The host-pathogen interactions of Chinook Salmon (*Oncorhynchus tshawytscha*) and the aquatic rhabdoviral pathogen infectious hematopoietic necrosis virus

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Abstract

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Infectious hematopoietic necrosis virus (IHNV) is an aquatic rhabdovirus that causes acute disease in Pacific salmon and trout populations in aquaculture facilities and freshwater conservation hatcheries across the northern hemisphere. While disease occurs primarily in juvenile fish, assessment of IHNV surveillance records across the US Pacific Northwest revealed a great majority of IHNV detections to be in adult fish at spawning. This investigation was premised on the high prevalence of IHNV infection in adult CRB Chinook salmon in the absence of disease in juvenile fish. Here, we evaluated field occurrence patterns of IHNV prevalence in Chinook salmon populations and conducted controlled virus exposures to characterize intraspecific variation in IHNV infection, disease and viral shedding in diverse Chinook salmon populations of the US Pacific Northwest. Our goal was to define the unique host-pathogen interactions of Columbia River Basin Chinook salmon and their interactions with the two genogroups (U and M) of IHN viruses prevalent in the CRB to better understand the role that Chinook salmon may have in the ecology and

epidemiology of IHNV across the Columbia River watershed. Throughout this work, L genogroup IHNV was included as a positive control virus known to cause disease and mortality in California Chinook salmon. In the first stage of this work, experimental exposures of two genetically distinct populations of Chinook salmon from Washington State to U, M and L genogroup strains of IHNV showed observable differences in host susceptibility to infection and mortality at 1g. While not statistically significant, infection prevalence was higher in the stream-type population with each of the U and M viruses when compared to the ocean-type population. Similarly, mortality was also higher in the stream-type population when compared to the ocean-type population. While differences in host susceptibility were initially attributed to differences in life history phenotype, these findings may instead be linked to more fundamental differences in the origin of each host population. In this initial set of controlled virus exposures, one host population was sourced from outside of the CRB and the other was sourced from within the CRB. Assessment of field surveillance data for the prevalence of IHNV infection in Chinook salmon populations from the CRB and coastal watersheds of Washington and Oregon showed a disproportionally low prevalence of IHNV detection in Chinook salmon populations outside of the CRB. While differences in host susceptibility to IHNV infection and mortality between the stream- and ocean-type populations tested were consistent with field occurrence patterns, additional experimentation with Chinook salmon populations of the CRB showed juvenile susceptibility to IHNV infection to be less likely linked to life history phenotype. To comprehensively characterize intraspecific variation in juvenile Chinook salmon susceptibility to IHNV infection and disease, four populations of CRB Chinook salmon were experimentally exposed to U, M and L genogroup strains of IHNV. These controlled laboratory studies showed little variation in

the overall susceptibility of CRB Chinook salmon to IHNV infection. Each host population became infected with the U and M genogroup viruses, at comparable levels observed with the positive control L genogroup virus. While infection prevalence and viral loads were comparably high among the four host populations, mortality was observably low following exposure to the U and M genogroup viruses relative to the L virus. Together, these experimental exposures empirically showed that juvenile Chinook salmon of the CRB can acquire U and M IHNV infections without the virulence observed with these virus types in juvenile sockeye salmon or rainbow and anadromous steelhead trout. These findings suggest that the absence of epizootic events in juvenile Chinook salmon of the CRB is not driven by the inability for U and M viruses to enter these host, but rather the ability of juvenile Chinook salmon to effectively control viral infections. In an effort to empirically test the hypothesis that subclinically infected Chinook salmon serve as vectors of IHNV, controlled laboratory exposures were conducted to characterized the shedding kinetics of U, M and L genogroup strains of IHNV in two diverse Chinook salmon populations of the CRB. A proportion of fish from each host population shed detectable quantities of U, M and L IHNV, where virus shedding peaked between 2-3 days post exposure and was no longer detected after day 5. While each host population shed comparable quantities of M and L IHN virus, a notable difference in the number of fish shedding U virus was observed. A disproportionately low number of lower CRB ocean-type (fall-run) Chinook salmon shed detectible U virus relative to the upper CRB stream-type (spring-run) population. Moreover, the stream-type population shed approximately 1 log more U virus consistently over the course of the infection, relative to the M and L IHN viruses. While our infectivity studies showed the U and M viruses to be equivalently infectious in four diverse populations of CRB Chinook salmon, results of our

shedding studies revealed intraspecific variation in the shedding of U virus in CRB Chinook salmon. Our investigational approach made it possible to assess results of our controlled laboratory exposures relative to field occurrence patterns of U and M IHNV infection in Chinook salmon with diverse life history phenotypes. Assessment of the spatial and temporal distributions of spring-run (stream-type) and fall-run (ocean-type) Chinook salmon were observed to coincide with the geographic distributions and prevalence patterns of U and M IHNV across Chinook salmon populations of the CRB. Together, results of this investigation suggest that CRB Chinook salmon populations of the spring-run (stream-type) life history phenotype may be differentially contributing to the successful maintenance of IHNV across the Columbia River watershed.

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DEDICATION

This dissertation is dedicated to my grandfathers, Jesus Gomez and Rafael Hernandez, who came to the United States from Mexico as “Braceros”, Mexican laborers allowed into the US as seasonal agricultural workers. To my grandmother, Maria Luisa Hernandez Aries, whose final words to me were “Todo se puede”. Anything is possible. To my grandmother, Concepcion Gomez Romero, who raised my mother to be the strong and determined individual that she is. To my mother, Concepcion Hernandez Gomez, the woman who not only brought me into this world but taught me how to be relentless and my own self advocate. To my father, Miguel Angel Hernandez. The man who, by day, was a truck driver, delivering freshly laundered uniforms and linens all throughout Los Angeles. By night, delivered pizzas. And, on Saturdays, went out in search of glass bottles and cans for their recycling redemption value. Obtaining a Doctor of Philosophy degree would not have been possible without the innumerable sacrifices made by each of these individuals. To Dr. Gael Kurath, without whom my development as a research scientist would not have been possible. Gael, without your dedicated training and advisorship, this dissertation would not be a reality. To Drs. Jacquelyn Bolman and Brian Bingham, my earliest mentors and biggest advocates. You each saw that small fire that burned inside of me which, with your guidance and mentorship, you stoked until it became the fire that helped carry me through my doctoral training. Finally, this dissertation is dedicated to Dr. George James Kenagy. Jim, I will forever attribute your friendship, mentorship and unwavering advocacy to the successful completion of my PhD.

Chapter 1. INTRODUCTION

1.1 INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS

Infectious hematopoietic necrosis virus (IHNV) is an enveloped, single-stranded, negative-sense RNA aquatic rhabdovirus that has a native host specificity for Pacific salmon and trout (genus *Oncorhynchus*). IHNV (species *Salmonid novirhabdovirus*) belongs to the taxonomic family *Rhabdoviridae* and is the type species of the genus *Novirhabdovirus*. Disease caused by IHNV infection occurs primarily in juvenile fish in aquaculture facilities and conservation hatcheries, where losses of up to 90% of fish have been reported (Bootland and Leong 2011; Kurath 2012). Originally endemic in western North America, IHNV has been spread by the movement of infected eggs and fish to Europe and Asia where it produces high losses in rainbow trout aquaculture facilities. As a result, IHNV has been placed on the list of "Reportable Aquatic Animal Pathogens" as defined by the World Organisation for Animal Health (OIE, 2018). While IHNV is a notable disease of aquaculture populations of rainbow trout (*Oncorhynchus mykiss*), it has also been important since the 1950s in conservation efforts to support naturally occurring populations of Pacific salmonids (Bootland and Leong 2011; Kurath 2012).

In the Pacific Northwest of the contiguous United States, Tribal, Federal and State agencies are tasked with the management of naturally occurring populations of Pacific salmonids for conservation purposes (i.e. Pacific salmon stocks listed under the Endangered Species Act) and capture by Native Americans (i.e. Federal Tribal Treaties) or the commercial and recreational fisheries. In an effort to supplement naturally occurring populations of Pacific salmonids, Tribal, Federal and State agencies maintain hatchery propagation programs for population enhancement.

Since the inception of hatchery programs, losses due to disease agents have been noted (Rucker et al. 1953; Parisot and Pelnar 1962). Among them is IHN virus (Amend et al. 1969).

Large mortality events of sockeye salmon (*O. nerka*), Chinook salmon (*O. tshawytscha*) and steelhead trout (*O. mykiss*) at hatchery facilities have been caused by IHNV since the 1950s and 1960s (Rucker et al. 1953; Parisot and Pelnar 1962). As more information has been gleaned about IHNV and IHNV related mortality events, screening and surveillance for this pathogen have been conducted since approximately the 1980s. Screening and surveillance of nearly all populations of Pacific salmonids in the Pacific Northwest for pathogens is now conducted by fish health laboratories of the Washington Department of Fish and Wildlife, Oregon Department of Fish and Wildlife, Idaho Department of Fish and Game, US Fish and Wildlife Service and the Northwest Indian Fisheries Commission. As a part of a larger research effort, the United States Geological Survey Western Fisheries Research Center developed a genetic typing system to genetically characterize IHNV isolates from across the virus' observed geographic range. The Molecular Epidemiology of Aquatic Pathogens Infectious Hematopoietic Necrosis Virus (MEAP-IHNV) database is comprised of genetic information (genotype) for over 3000 virus isolates and metadata pertaining to the origin of each isolate (phenotype) (http://gis.nacse.org/ihnv/). Together, the MEAP-IHNV Database has served as the foundation upon which this and other investigations assessing the ecology and epidemiology of IHNV have been built. The successful construction of this database is owed to the following key elements.

1.1.1 *Virus Isolate Genotype*

The IHNV genome encodes six genes including a nucleocapsid (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), non-virion (NV) protein and polymerase (L) protein

(Bootland and Leong 2011; Kurath 2012). Of these genes, the glycoprotein (G) gene was selected and is used for phylogenetic analysis of virus isolates. Genetic sequencing is conducted on a 303 nucleotide region within the glycoprotein gene (midG). Each unique midG genetic sequence is considered a "sequence type" and denoted by a universal designator (i.e. mG ###) (described in the background section of the MEAP-IHNV database). Many virus isolates have the same "sequence type" and thus the same universal designator. The development of this genetic typing system has allowed phylogenetic analysis to be performed on virus isolates.

Virus isolates can be obtained from whole-fish or fish tissue sampled (as dead or moribund fish) during an epidemic (mortality events), or from fish tissue attained as a part of a screening/surveillance event. For genetic typing of an isolate, viral RNA must be isolated and used to construct a complimentary DNA molecule on which conventional genetic sequencing is then performed.

1.1.2 *Virus Isolate Phenotype*

For each virus isolate, metadata such as location of isolation (e.g. State, Hatchery) including latitude and longitude, host species (e.g. Chinook salmon, steelhead trout), age class (e.g. juvenile, adult), host type/race (e.g. Spring-, Winter-, Fall-run) and host sex (e.g. male, female) is recorded.

1.1.3 *Genetic Typing of Virus Isolates Genotype*

IHN virus isolates from Tribal, Federal and State hatcheries from throughout western North America have been genetically typed at the USGS Western Fisheries Research Center for over 20 years. Collectively, the MEAP-IHNV database is currently comprised of 3013 IHNV isolates constituting 3309 sequences. This database, publically available online, contains all IHNV genetic

typing data produced at the USGS Western Fisheries Research Center and epidemiological metadata recorded for each virus isolate.

1.2 INTERSPECIFIC HOST VARIATION IN IHNV INFECTION AND DISEASE PREVALENCE

The development and use of the genetic typing system for IHNV isolates led to the identification of three phylogenetic subgroups of IHNV that were designated as genogroups U (upper), M (middle) and L (lower) based on their relative geographic occurrence in western North America (Garver et al. 2003; Kurath et al. 2003). Within each major genetic group of IHNV, genogroup-specific patterns of host specificity have been observed. U genogroup viruses are prevalent and highly virulent in sockeye salmon (Garver et al. 2006), whereas M genogroup viruses are prevalent and highly virulent in rainbow trout and steelhead trout (anadromous *O. mykiss*) (Garver et al. 2006). L genogroup viruses are prevalent and highly virulent in juvenile Chinook salmon of California and southern Oregon (Bendorf et al. 2007). In addition to the Pacific Northwest, IHNV is also endemic in salmonid fishes in Japan and has been isolated in several countries in Europe and Asia (OIE, 2018; Kurath 2012). Japanese IHNV isolates have been shown to group with U genogroup viruses of North America, consistent with the reported introduction of IHN virus via shipment of contaminated sockeye salmon eggs from Alaska in 1967 (Nishizawa et al. 2006). In Europe, IHNV first appeared in rainbow trout in 1987 (Enzmann et al. 2005; Bootland and Leong 2011). Since, disease outbreaks have been reported in rainbow trout aquaculture facilities in France, Italy, Germany and Switzerland, and linked to the trading of infected fish (Enzman et al. 2010). Genetic typing of European IHNV isolates group with the M genogroup viruses (Kurath et al. 2003), likely originating from an ancestral North American M genogroup

virus (Bootland and Leong 2011). Together, the anthropogenic movement and introduction of fish and eggs to new regions has the potential to influence the emergence and evolution of IHNV in aquaculture settings. While this investigation does not examine anthropogenic influences on the host-pathogen interactions of Chinook salmon and IHNV in conservation hatcheries across western North America, these studies provide insight to the evolution and adaptation of IHNV in response to the physical moment of infected fish. Here, we consider the natural movement and migrations of juvenile and adult Chinook salmon across the Columbia River watershed to evaluate field occurrence patterns of IHNV prevalence in distinct Chinook salmon populations.

1.3 Virology and Genetic Surveillance (VGS) Database for IHNV

Virological surveillance records for IHNV were combined with genetic surveillance data for IHNV in Pacific salmon and trout populations across freshwater ecosystems of the Pacific Northwest of North America. The IHNV Virology and Genetic Surveillance (VGS) database (Breyta et al. 2017) is comprised of virology testing records for a total of 15 salmonid species sampled between the years 2000-2012. While the VGS database includes a subset of genotyping data published in the MEAP-IHNV database, the VGS database differs from the MEAP-IHNV database as it includes IHNV positive and negative test results for those host populations tested. This data set was contributed to by all five resource management agencies that operate conservation hatcheries across the Columbia River watershed, as well as coastal rivers of Washington and Oregon (Breyta et al. 2017). While the VGS database includes records for salmonid populations from coastal watersheds of Washington and Oregon, this dataset does not contain IHNV surveillance and genotyping records for salmonid populations from the Salish Sea (Washington state coastal watersheds north of Cape Flattery). Together, the geospatial extent of

this dataset comprises the Columbia River watershed and coastal regions of Washington and Oregon (Breyta et al. 2017). While genotyping data is available for only a subset of the total 6766 VGS database records, all IHNV surveillance records for those salmonid populations tested between 2000-2012 across these watersheds are included in this database. This database was used to conduct ecological and epidemiological analyses of the prevalence of U and M genogroup IHNV in Chinook salmon populations of the CRB versus coastal watersheds, presented in Chapter 2 of this dissertation.

1.4 Intraspecific variation in IHNV infection and disease in Chinook salmon

This investigation aimed to characterize the intraspecific variation in IHNV infection and disease in diverse populations of Columbia River Chinook salmon, with the goal of understanding how specific life history attributes may influence differences in host susceptibility to IHNV infection. The basis for this investigation was the epidemiological variation in infection and pathology observed among Chinook salmon across the US Pacific coast. Chinook salmon in California and southern Oregon are well known to suffer high levels of infection and disease associated specifically with L genogroup IHNV (Bendorf et al. 2007). This contrasts with the epidemiological associations observed for IHN viruses in Chinook salmon across the Columbia River Basin (CRB). In a noteworthy exception to the general host tropism of U genogroup viruses to sockeye salmon and M genogroup viruses to rainbow trout and anadromous steelhead, both U and M group viruses are found frequently in adult Chinook salmon of the CRB with low disease impacts in juvenile fish (Breyta et al. 2016). As a key to understanding this observation, a comprehensive phylogenetic analysis of U genogroup IHNV isolates identified a newly recognized

subgroup, designated UC for its geographic range within the CRB (Black et al. 2016). Assessment of host and geographic structuring within the U genogroup virus phylogeny revealed UC genotype viruses are isolated primarily in Chinook salmon in the CRB, while the rest of the U genogroup viruses (now designated subgroup UP) were isolated primarily in sockeye salmon in coastal watersheds outside the CRB (Black et al. 2016). While it is unclear what epidemiological dynamics may be driving the adaptation of U genogroup virus to different hosts, this investigation sought to characterize the host-pathogen interactions unique to Columbia River Chinook salmon and the U and M strains of IHNV that co-occur across the watershed (specifically subgroups UC and MD). Moreover, this investigation aimed to characterize the intraspecific variation in IHNV infection, disease and viral shedding in diverse Chinook salmon populations of the CRB, to assess the transmission potential of IHN virus by subclinically infected Chinook salmon.

1.5 Chinook salmon of the Columbia River Basin

Chinook salmon are one of eight species of fish belonging to the genus *Onchorhynchus*. The extensive biological diversity observed across members of this genus reflects the successful evolutionary journey of this group throughout its divergence from other salmonids over the past ten million years (Behnke 1992). Across the Eastern Pacific Rim, Chinook salmon populations have experienced numerous extirpation events over geologic time. As a result, extant populations of Chinook salmon are derived from divergent ancestral lineages that have evolved in accordance to the selection pressures imposed by the biophysical landscapes they inhabit. In both the juvenile and adult life stages, various life history strategies have evolved across Chinook salmon populations to maximize their use of available spawning and rearing habitat (Quinn 2018).

The Columbia River Basin (CRB), which spans portions of the North American states of Washington, Oregon, Idaho, Montana, Wyoming, Utah and Nevada, and the province of British Columbia (Fig. 1.1), sustains numerous genetically diverse populations of Chinook salmon with expressed phenotypic differences in behavioral patterns, life histories, and geographic distributions (Waples et al. 2008; Quinn, 2018). Across Chinook salmon populations of the CRB, three adult migratory phenotypes, referred to as the spring-, summer-, or fall-run types, have evolved to coincide with arrival at one's spawning grounds in a physiological state that supports competition for mates and reproduction, after which individuals die (Fleming and Gross 1994). Generally, the energetic costs of upstream migration and reproduction have selected for migratory times that avoid high water temperatures, low dissolved oxygen levels, and low flows that inhibit upstream movement (Brannon et al. 2004). Chinook salmon populations that migrate to interior reaches of the CRB for spawning begin their upstream migrations earlier in a given year as less sexually developed adults (Brannon et al. 2004). These early migrating fish constitute the spring- and summer-run fish that are observed to spawn in smaller order, tributary streams of the upper Columbia River watershed (Myers et al. 1998) (appendix E). Fall-run Chinook salmon begin their upstream migrations later as more sexually developed adults (Hearsey et al. 2015) and spawn in larger order, river mainstems (Myers et al. 1998) (appendix E). Although largely distributed in coastal watersheds (Healey et al. 1991), many fall-run populations also migrate to the mid and upper Columbia River Basin (Brannon et al. 2004). Thermal regimes that favor embryonic development, in the gravel of natal streams, have selected for migration and spawn timing that coincides with incubation periods that optimize egg-to-fry survival and synchrony of fry emergence with the spring timing of aquatic insect emergence (Quinn 2018). Because these distinct life-history types have evolved in response to temperature and its direct influence on the

physiologies of adult and juvenile fish, adult migratory type is observed to co-vary with juvenile freshwater residence (Brannon et al. 2004).

Spring-run Chinook salmon offspring often reside in freshwater for a full year prior to seaward migration and are consider stream-type fish, whereas fall-run Chinook salmon offspring often migrate to the ocean within their first year of life and are considered ocean-type fish (Healey et al. 1991). Summer-run offspring from Snake River Chinook salmon populations generally exhibit a stream-type life history, whereas summer-run offspring from Columbia River Chinook salmon populations exhibit an ocean-type life history (Taylor 1990). Together, the present discussion of IHNV infection, disease and viral shedding in CRB Chinook salmon considered landscape features and host life history types as potential factors influencing the infection and pathology UC, MD, and L genogroup IHN virus strains in diverse Chinook salmon populations.

1.6 Research Objective

Our research objective was to define the extent of IHNV infection, disease and viral shedding in Columbia River Basin Chinook salmon following exposure to UC, MD, and L genogroup IHN virus strains, to understand how Chinook salmon may be contributing to the transmission and overall maintenance of IHNV across the Columbia River watershed. Building from established methods for studying the virulence and infectivity of select IHNV strains, we conducted controlled virus exposures to experimentally quantify IHNV infection and disease pathology in diverse Chinook salmon populations of the CRB. To characterize intraspecific variation in Columbia River Chinook salmon susceptibility to UC, MD and L IHNV infection and disease relative to host genetics and life history traits, multiple populations were included in our controlled laboratory studies. Using a comparative experimental approach, we tested the null hypotheses of equal

susceptibility of all Chinook salmon host populations (stream and ocean-type fish from upper and lower CRB populations) and equal susceptibility to all IHNV genotypes (UC, MD, and L). We analyzed IHNV Virology, Genotyping and Surveillance database records for Chinook salmon populations of the CRB versus coastal watersheds of Washington and Oregon based on host attributes such as age-class and life history type, to define the field occurrence patterns of U and M IHNV prevalence in juvenile versus adult Chinook salmon relative to their spatial and temporal distributions across their respective freshwater environments. These findings were used to interpret results of our controlled virus exposures, allowing us to:

- Characterize the prevalence of IHNV infection in spring-, summer- and fall-run Chinook salmon populations of the Columbia River Basin and coastal watersheds of Washington and Oregon.
- Define the geospatial distributions of U and M IHNV positive cohorts of spring-, summer- and fall-run Chinook salmon across the Columbia River Basin and coastal watersheds of Washington and Oregon.
- Characterize the susceptibility of stream- and ocean-type Chinook salmon to infection and disease following exposure to U, M and L genogroup isolates of IHNV.
- Characterize the infectivity of UC, MD and L IHNV strains in four Columbia River Basin Chinook salmon populations.
- Characterize the frequency, magnitude, and kinetics of L, UC and MD IHN virus shedding in juvenile stream- and ocean-type Chinook salmon of the Columbia River Basin.

Together, the analyses carried out as a part of this dissertation provide novel insights into the host-pathogen interactions of Columbia River Basin Chinook salmon and U and M IHNV.

1.7 FIGURES

Figure 1.1. The Columbia River Basin (CRB) located in western North America. Numerous watersheds, spanning the US states of Washington, Oregon, Montana, Idaho, Wyoming, and portions of the Canadian province of British Columbia, collectively comprise the CRB.

1.8 LITERATURE CITED

Amend, D. F., Yasutake, W. T., & Mead, R. W. (1969). A hematopoietic virus disease of rainbow trout and sockeye salmon. Transactions of the American Fisheries Society, 98 (4), 796-804.

Bendorf, C. M., Kelley, G. O., Yun, S. C., Kurath, G., Andree, K. B., & Hedrick, R. P. (2007). Genetic diversity of infectious hematopoietic necrosis virus from Feather River and Lake Oroville, California, and virulence of selected isolates for Chinook salmon and rainbow trout. Journal of aquatic animal health, 19(4), 254-269.

Behnke, R. J. (1992). Native trout of western North America. American Fisheries Society monograph (USA). no. 6.

Black, A., Breyta, R., Bedford, T., & Kurath, G. (2016). Geography and host species shape the evolutionary dynamics of U genogroup infectious hematopoietic necrosis virus. Virus evolution, 2(2), vew034.

Bootland, L. M., & Leong, J. C. (2011). Infectious haematopoietic necrosis virus. Fish diseases and disorders, 3, 66-109.

Brannon, E. L., Powell, M. S., Quinn, T. P., & Talbot, A. (2004). Population structure of Columbia River Basin Chinook salmon and steelhead trout. Reviews in Fisheries Science, 12(2-3), 99-232.

Breyta, R., Samson, C., Blair, M., Black, A., & Kurath, G. (2016). Successful mitigation of viral disease based on a delayed exposure rearing strategy at a large-scale steelhead trout conservation hatchery. Aquaculture, 450, 213-224.

Breyta, R., Brito, I., Kurath, G., & LaDeau, S. (2017). Infectious hematopoietic necrosis virus virological and genetic surveillance 2000–2012. Ecology, 98(1), 283-283.

Enzmann, P. J., Kurath, G., Fichtner, D., & Bergmann, S. M. (2005). Infectious hematopoietic necrosis virus: monophyletic origin of European isolates from North American genogroup M. Diseases of aquatic organisms, 66(3), 187-195.

Enzmann, P. J., Castric, J., Bovo, G., Thiery, R., Fichtner, D., Schütze, H., & Wahli, T. (2010). Evolution of infectious hematopoietic necrosis virus (IHNV), a fish rhabdovirus, in Europe over 20 years: implications for control. Diseases of aquatic organisms, 89(1), 9-15.

Fleming, I. A., & Gross, M. R. (1994). Breeding competition in a Pacific salmon (coho: Oncorhynchus kisutch): measures of natural and sexual selection. Evolution, 48(3), 637-657.

Garver, K. A., Troyer, R. M., & Kurath, G. (2003). Two distinct phylogenetic clades of infectious hematopoietic necrosis virus overlap within the Columbia River basin. Diseases of aquatic organisms, 55(3), 187-203.

Garver, K. A., Batts, W. N., & Kurath, G. (2006). Virulence comparisons of infectious hematopoietic necrosis virus U and M genogroups in sockeye salmon and rainbow trout. Journal of Aquatic Animal Health, 18(4), 232-243.

Healey, M. C. (1991). Life history of chinook salmon (*Oncorhynchus tshawytscha*). Pacific salmon life histories.

Hearsey, J. W., & Kinziger, A. P. (2015). Diversity in sympatric chinook salmon runs: timing, relative fat content and maturation. Environmental biology of fishes, 98(1), 413-423.

Kurath, G., Garver, K. A., Troyer, R. M., Emmenegger, E. J., Einer-Jensen, K., & Anderson, E. D. (2003). Phylogeography of infectious haematopoietic necrosis virus in North America. Journal of General Virology, 84(4), 803-814.

Kurath, G. (2012). Fish novirhabdoviruses. In: Dietzgen RG, Kuzman IV, eds. Rhabdoviruses: Molecular taxonomy, Evolution, Genomics, Ecology, Host-Vector interactions, Cytopathology, and Control. Caister Academic Press; pp.89-117.

Myers, J. M. (1998). Status review of chinook salmon from Washington, Idaho, Oregon, and California.

Nishizawa, T., Kinoshita, S., Kim, W. S., Higashi, S., & Yoshimizu, M. (2006). Nucleotide diversity of Japanese isolates of infectious hematopoietic necrosis virus (IHNV) based on the glycoprotein gene. Diseases of aquatic organisms, 71(3), 267-272.

Parisot, T. J., & Pelnar, J. (1962). An interim report on Sacramento River chinook disease: a viruslike disease of chinook salmon. The Progressive Fish-Culturist, 24(2), 51-55.

Quinn, T. P. (2018). The behavior and ecology of Pacific salmon and trout. University of Washington Press.

Rucker, R. R., Whipple, W. J., Parvin, J. R., & Evans, C. A. (1953). A contagious disease of salmon possibly of virus origin.

StreamNet GIS Data (2017). Columbia River Watershed Boundary spatial data set. Portland (OR): StreamNet, March 2019. Available at https://www.streamnet.org/data/interactive-maps-and-gis-data/

Taylor, E. B. (1990). Phenotypic correlates of life-history variation in juvenile chinook salmon, *Oncorhynchus tshawytscha*. The Journal of Animal Ecology, 455-468.

Waples, R. S., Teel, D. J., Myers, J. M., & Marshall, A. R. (2004). Life-history divergence in Chinook salmon: historic contingency and parallel evolution. Evolution, 58(2), 386-403.

Waples, R. S., Pess, G. R., & Beechie, T. (2008). Evolutionary history of Pacific salmon in dynamic environments. Evolutionary Applications, 1(2), 189-206.

World Organisation for Animal Health (2018). Aquatic Animal Health Code, Chapter 1.3, article 1.3.1, http://www.oie.int/index.php?id=171&L=0&htmfile=chapitre_diseases_listed.htm. World Organisation for Animal Health, Paris, France.

Chapter 2. THE PREVALENCE OF IHNV INFECTION IN CHINOOK SALMON POPULATIONS OF THE COLUMBIA RIVER BASIN AND COASTAL WATERSHEDS

2.1 INTRODUCTION

Central to the discussion of the persistence and spread of an infectious disease is a comprehensive understanding of the field occurrence patterns and unique host associations across its respective landscape. Numerous watersheds, spanning the US states of Washington, Oregon, Montana, Idaho, Wyoming, and portions of the Canadian province of British Columbia, collectively comprise the Columbia River Basin (CRB). Across the CRB, both U and M genogroup strains of IHNV are sympatric within the watershed (Garver et al. 2003), infecting numerous Pacific salmon and trout species (Breyta et al. 2016). In a noteworthy exception to the general host tropism of U genogroup viruses to sockeye salmon and M genogroup viruses to rainbow trout and anadromous steelhead, both U and M group viruses are found frequently in adult Chinook salmon of the CRB with low disease impacts in juvenile fish (Breyta et al. 2016). In an effort to better understand what landscape features and host attributes may be influencing the host-pathogen interactions of CRB Chinook salmon and IHNV, this investigation aimed to define the field occurrence patterns of U and M IHNV prevalence in Chinook salmon populations with expressed differences in behavior and life histories.

The biophysical environment differs markedly throughout the CRB, where differences in climate, topography and vegetation across this landscape have shaped the genetic diversity and population structure of extant Chinook salmon (*Oncorhynchus tshawytscha*) populations. Chinook salmon populations of the CRB have experienced numerous extirpation events over geologic time

(Waples et al. 2008). Extant populations of Chinook salmon have evolved to maximize their use of available spawning and rearing habitat throughout the CRB (Brannon et al. 2004). This is evident in the parallel evolution of adult run-timing across divergent lineages of Chinook salmon (Waples et al. 2004). Chinook salmon populations of the CRB are genetically diverse with expressed phenotypic differences in behavioral patterns, life histories, and geographic distributions (Waples et al. 2008). In the CRB, Chinook salmon populations are commonly classified by the timing of adult upstream migration for spawning (Brannon et al. 2004). Across Chinook salmon populations of the CRB, three adult migratory phenotypes, referred to as the spring-, summer-, or fall-run types, have evolved to coincide with arrival at one's spawning grounds in a physiological state allowing competition for mates and reproduction (Fleming and Gross 1994). The energetic costs of upstream migration and reproduction have selected for migratory times that avoid high water temperatures, low dissolved oxygen levels, and low flows that inhibit upstream movement (Brannon et al. 2004). Chinook salmon populations that migrate to interior reaches of the CRB for spawning begin their upstream migrations earlier in a given year as less sexually developed adults (Brannon et al. 2004). These early migrating fish constitute the spring- and summer-run fish that are observed to spawn in smaller order, tributary streams of the upper Columbia River Watershed (Fig. 2.1). Fall-run Chinook salmon begin their upstream migrations later as more sexually developed adults (Hearsey et al. 2015) and spawn in larger order, river mainstems (Myers et al. 1998). Thermal regimes that favor embryonic development, in the gravel of natal streams, have selected for migration and spawn timing that coincides with incubation periods that optimize egg-to-fry survival and synchrony of fry emergence with the spring timing of aquatic insect emergence (Quinn 2018). Because these distinct life-history types have evolved in response to temperature

and its direct influence on the physiologies of adult and juvenile fish, adult migratory type is observed to co-vary with juvenile freshwater residence (Brannon et al. 2004).

Spring-run Chinook salmon offspring often reside in freshwater for a full year prior to seaward migration and are consider stream-type fish, whereas fall-run Chinook salmon offspring often migrate to the ocean within their first year of life and are considered ocean-type fish (Healey et al. 1991). Summer-run offspring from Snake River Chinook salmon populations generally exhibit a stream-type life history, whereas summer-run offspring from Columbia River Chinook salmon populations exhibit an ocean-type life history (Taylor 1990). A recent assessment of the field occurrence patterns of IHNV detections in numerous Pacific salmonid fishes showed significant detection of IHNV in Chinook salmon in the CRB (Breyta et al. 2016). Moreover, this comprehensive study showed that high numbers of adult Chinook salmon returning to the CRB are virus positive at the time of spawning with low disease impacts in juvenile fish. In an effort to identify what landscape or host attributes may be influencing the host-pathogen interactions of CRB Chinook salmon and IHNV, this investigation first aimed to characterize the prevalence of IHNV infection in Chinook salmon populations of the Columbia River Basin versus coastal watersheds of Washington and Oregon. To examine the influence of host life history attributes on the field occurrence patterns of IHNV infection in diverse Chinook salmon populations, the prevalence of U and M IHNV in spring-, summer- and fall-run fish was determined. Because Chinook salmon differ substantially in spatial and temporal distribution across a watershed relative to age and life history (Fig.2.2), defining field occurrence patterns of U and M IHNV prevalence in Chinook salmon populations based on geographic distribution, behavior and life history in addition to age-class may bring greater resolution to the potential roles that specific populations

may have in the ecology and epidemiology of IHNV in the CRB and the relative risk posed by IHNV to hosts of the various life history types.

2.2 Materials and Methods

2.2.1 *Surveillance of IHNV in the Columbia River Basin vs. Coastal Watersheds*

Surveillance records for IHNV across the freshwater ecosystems of the Columbia River watershed and coastal rivers of Washington and Oregon were assessed to characterize the field occurrence patterns of IHNV infection in Chinook salmon. Here, coastal watersheds are those that drain into the Pacific Ocean between the mouth of the Columbia River and Cape Flattery, and do not include watersheds of the Salish Sea. The IHNV Virology, Genotyping and Surveillance (VGS) database (Breyta et al. 2017) consists of 6766 records, representing 1146 sample sites and 15 different fish hosts, and is comprised of surveillance records collected between the years 2000–2012 by all five resource management agencies that operate conservation hatcheries across the US Pacific Northwest (Breyta et al. 2017). This investigation first sought to address the question: *Do Chinook salmon populations of the Columbia River Basin and coastal watersheds of Washington and Oregon differ in the prevalence of IHNV infection?* To do so, the VGS data set was imported into ArcMap, version 10.6.1 (Esri, 2018) where database records for Chinook salmon were subsetted using the select by attributes command. Initial subsetting of the VGS data set by our target species resulted in 1826 records for Chinook salmon across the CRB and coastal watersheds. Each Chinook salmon VGS database record was differentiated as a coastal river or CRB population tested, based on geospatial distribution (Latitude, Longitude) as projected in the NAD 1983 Lambert Conformal Conic Coordinate System (Esri, 2018). Spatial subsetting of these data were performed using the Columbia River Watershed Boundary (StreamNet GIS Data, 2017) and the

Clip command (Esri, 2018). Of the 1826 IHNV surveillance records available for Chinook salmon between the years 2000–2012, 404 records were reported for coastal watersheds of Washington and Oregon whereas 1422 records were reported for the CRB. To examine the influence of host life history on the field occurrence patterns of IHNV infection in diverse Chinook salmon populations, boolean operators were combined to further subset these data by adult migratory run timing. Together, positive and negative surveillance records and available virus isolate genotype data were used to conduct univariate analysis on the prevalence of U and M IHNV infection in Chinook salmon populations of the CRB versus coastal watersheds.

2.2.2 *Geostatistical analysis of the distributions of IHNV positive Chinook salmon cohorts*

IHNV VGS database records available for Chinook salmon between the years 2000-2012 each constitute a cohort of fish tested. Briefly, a cohort represents fish of the same species, age (adult or juvenile), collection site location (e.g. Hatchery), and year of testing (Breyta et al. 2017). For the majority of Chinook salmon cohorts tested for IHNV, metadata pertaining to adult migratory timing was available. To assess differences in the geospatial distributions of IHNV positive Chinook salmon cohorts, two geostatistical metrics were compared. The mean center of concentration was used to identify the geographic center for a select set of points (Esri, 2018). Measures of mean center were calculated in ArcMap, version 10.6.1 as the mean x-coordinate value and the mean y-coordinate value for a select set of points. Further, for each select set of points, the directional distribution (Standard Deviational Ellipse) was calculated as the standard deviation of x-coordinates and y-coordinates from their mean center (Esri, 2018). Together, these two geostatistical measures allowed comparison of the spatial distributions between two select sets of points.

2.2.3 *Prevalence of IHNV in Chinook salmon of the Columbia River Basin*

Chinook salmon IHNV VGS database records were subsetted for the CRB, as described above, to address the questions: *Do spring-, summer- and fall-run Chinook salmon populations of the CRB differ in prevalence of IHNV infection?* and *Do spring-, summer- and fall-run Chinook salmon populations of the CRB differ in prevalence of U and M IHNV infection?*. The 1422 records available for CRB Chinook salmon collectively represented a total of 128 sample sites across the Columbia River watershed. Of these 1422 CRB Chinook salmon cohorts tested, 315 were IHNV positive. Adult migratory timing was available for 1011 of the CRB Chinook salmon cohorts tested, of which 260 were virus positive. Of the 315 total IHNV positive Chinook salmon cohorts, genotyping data was available for 186 records. Overall, IHNV was detected in 22% of CRB Chinook salmon tested, for which 59% had viral genotype data available. Together, these data were used to characterize the prevalence of U and M IHNV infection in spring-, summer- and fall-run Chinook salmon of the CRB.

2.2.4 *Abundance of spring-, summer- and fall-run Chinook salmon across the CRB*

The relative abundance of spring-, summer- and fall-run Chinook salmon across the CRB was estimated for each life history phenotype based on the average number of adult fish migrating past the Bonneville Dam between the years 2000–2012 (Columbia River DART Database, cbr.washington. edu/dart/). Host abundance was integrated with IHNV field surveillance data to characterize possible intraspecific patterns of IHNV infection across the diverse life history phenotypes of CRB Chinook salmon.

2.3 RESULTS

2.3.1 *Prevalence of IHNV infection in Chinook salmon of the CRB vs. Coastal Watersheds*

Assessment of the prevalence of IHNV infection across Chinook salmon populations of the CRB and coastal watersheds of Washington and Oregon showed greater proportions of CRB Chinook salmon to be infected with IHNV (22%) relative to populations of the coastal watersheds (3%) (Table 2.1). While more Chinook salmon populations of the CRB are tested for IHNV in comparison to coastal populations, differences in the prevalence of IHNV infection did not appear to be driven by overall differences in sampling pressure. Between the years 2000-2012, 404 Chinook salmon cohorts across the coastal watersheds of Washington and Oregon were tested for IHNV. Of these 404 cohorts tested, only 14 were virus positive. Together, differences in the prevalence of IHNV infection across Chinook salmon populations of these differing geographic regions appear to be driven by a relatively low level of IHNV infection in Chinook salmon of the coastal streams.

2.3.2 *Prevalence of U and M IHNV infection in Chinook salmon of the CRB vs. Coastal Watersheds*

The prevalence of U and M IHNV infection across Chinook salmon populations of the CRB and coastal watersheds of Washington and Oregon was reported as the proportion of U and M IHNV positive cohorts relative to the total number of IHNV positive cohorts for which genotyping data was available (Table 2.1). Across Chinook salmon populations of the CRB, prevalence of IHNV infection was higher with U genogroup viruses (83%) than with M viruses (17%). In contrast, prevalence of IHNV infection in Chinook salmon of the coastal watersheds was observed to be higher with M virus (64%) relative to the U virus (36%). It is important to note that due to

the overall low prevalence of IHNV in coastal populations of Chinook salmon, there were only 11 cohorts genotyped, so the relative abundance of U and M virus is based on very small numbers. Differences in the prevalence of U and M IHNV infection in Chinook salmon cohorts of the CRB and coastal watersheds were consistent with the spatial distributions of each virus genogroup across the two geographic regions. U and M viruses are broadly distributed across the Columbia River watershed, with the mean center of M virus detections in Chinook salmon occurring lower in the CRB whereas for U virus it is observed eastward toward the interior of the CRB (Fig. 2.3). Across the coastal watersheds of Washington and Oregon, M virus detections in Chinook salmon are very narrowly distributed occurring in a small number of hatchery facilities (Fig. 2.3). Far less U virus was detected than M viruses in Chinook salmon across the coastal watersheds, occurring only at a single hatchery facility between 2000-2012.

2.3.3 *Prevalence of IHNV infection relative to Chinook salmon life history phenotype*

The prevalence of IHNV infection in spring-, summer- and fall-run Chinook salmon populations of the CRB and coastal watersheds was determined for each respective life history phenotype as the proportion of those IHNV positive cohorts relative to their total number of cohorts tested (Table 2.2). Across the CRB, infection prevalence did not appear to differ between the spring- and fall-run life history phenotypes, 27% and 25%, respectively. However, infection prevalence was lower in CRB summer-run populations (13%). Across the coastal watersheds of Washington and Oregon, the prevalence of IHNV infection was equivalently low in spring-, summer- and fall-run Chinook salmon with no observable differences in infection prevalence among the differing host types.

The specific prevalence of U and M IHNV infection in spring-, summer- and fall-run Chinook salmon was determined for populations of the CRB and coastal watersheds. For each life history phenotype, the prevalence of U and M IHNV infection was reported as the proportion of U and M virus positive cohorts relative to the total number of IHNV positive cohorts for which genotyping data was available (Table 2.3). In both spring- and fall-run Chinook salmon of the CRB, infection prevalence was equivalently higher with U genogroup viruses (82% and 88%, respectively) and lower with M viruses (18% and 12%, respectively). In spring- and fall-run Chinook salmon of the coastal watersheds, however, the converse was observed. Across the coastal watersheds of Washington and Oregon, M genogroup viruses comprised 75% and 100% of IHNV detections in spring- and fall-run Chinook salmon, respectively. While far fewer Chinook salmon cohorts were virus positive across the coastal watersheds, a notable pattern emerged. Whereas U genogroup viruses constitute 83% of IHNV detected in CRB Chinook salmon (Table 2.1), U viruses comprise 25% of IHNV detections in coastal spring-run Chinook salmon and 0% in fall-run populations (Table 2.3). Across the three life history types, fall-run Chinook salmon were the host type most abundant (appendices E and F) and most tested for IHNV in coastal watersheds. Together, the absence of U virus detection in fall-run fish of the coastal watersheds between the years 2000-2012 is in stark contrast to the high prevalence of U virus in both fall- and spring-run Chinook salmon of the CRB.

2.3.4 *Geospatial distributions of IHNV positive cohorts of Chinook salmon across the CRB*

The mean center of all M IHNV positive Chinook salmon cohorts was located westward toward the lower CRB, whereas the mean center of all U IHNV positive cohorts was located eastward toward the interior CRB (Fig. 2.3b). Spring-run Chinook salmon infected with M IHN

virus had a narrow distribution that was centered over the mainstem of the Columbia River, whereas cohorts infected with U IHN virus were more broadly distributed across the interior CRB (Fig. 2.4). Too few summer-run Chinook salmon cohorts were infected with U and M virus, not allowing calculation of mean centers and directional distributions for each virus type. Notably, both IHNV positive cohorts of summer-run Chinook salmon were located in hatchery facilities in the state of Idaho. The mean center of U IHNV positive fall-run Chinook salmon cohorts was located westward toward the lower CRB, with a narrow distribution centered over the lower mainstem of the Columbia River. Too few fall-run Chinook salmon cohorts were infected with M virus across the CRB, not allowing calculation of a mean center and directional distribution. Notably, all fall-run Chinook salmon cohorts infected with M virus were located in one lower Columbia River hatchery facility in Washington state. Together, greater numbers of spring-run Chinook salmon cohorts were infected with U and M IHNV strains than cohorts of the summer- and fall-run life history types. Spring-run cohorts infected with U and M IHNV strains were broadly distributed across the CRB. While U and M virus infected spring-run cohorts overlap spatially in tributary streams of the Columbia River mainstem, two significant patterns in the distributions of U and M virus infections emerged. M virus infections in spring-run Chinook salmon were centered westward toward the lower CRB, whereas U virus infections were centered eastward toward the interior CRB. Geostatistical analysis of the mean center and directional distributions of all U and M IHNV positive hosts in the CRB revealed the same spatial pattern (Fig. 2.2). M virus infections in all host types were centered westward toward the lower CRB, whereas U virus infections were centered eastward toward the interior CRB. Further, the standard deviational ellipse calculated for all M IHN virus positive hosts was centered over the lower CRB, with an overall northwest to southwest orientation. For all U IHN virus positive hosts, the

calculated standard deviational ellipse was centered eastward toward the interior CRB with a more west to east orientation.

Of the three migratory run-types counted annually for numbers of returning adult Chinook salmon to the CRB, fall-run fish are most abundant (60%), followed by spring-run fish (27%), and summer-run fish comprising a small minority (13%) (Columbia River DART Database, cbr.washington.edu/dart/). It is unclear how differences in the relative abundance of each Chinook salmon host type may be influencing the reported field occurrence patterns of IHNV in CRB Chinook salmon. However, detections of IHNV in spring-run Chinook salmon are more broadly distributed and have a more interior distribution within the CRB. Further, detections of IHNV in fall-run Chinook salmon are more narrowly distributed along the mainstem Columbia River. While individual assessment of the prevalence of U and M IHNV suggest equal infection across diverse populations of CRB Chinook salmon, comparing the two virus genogroups revealed a disproportionately higher prevalence of U virus in CRB Chinook salmon relative to M virus. Together, these findings precipitated the following discussion.

2.4 DISCUSSION

This investigation examined the prevalence of IHNV infection in Chinook salmon populations in a large subregion of the US Pacific Northwest, with the added resolution of life history to characterize intraspecific variation in the prevalence of U and M IHNV infection. Comparison of field occurrence patterns of IHNV prevalence in Chinook salmon populations across two distinct geographic regions provided novel information about the associations of IHNV and Chinook salmon from within and outside of the CRB. Across our two study regions, far fewer cohorts of Chinook salmon were observed to be infected with IHNV across coastal watersheds of Washington

and Oregon relative to the CRB. The disproportionately low prevalence of IHNV in Chinook salmon populations of the coastal watersheds is a newly defined pattern which brought novel insights to a well-known epidemiological pattern. As a part of this investigation, the prevalence of IHNV infection in Chinook salmon populations of the CRB was assessed relative to age class. Consistent with previously reported field occurrence patterns of IHNV infection in Chinook salmon across the US Pacific Northwest (Breyta et al. 2017), infection prevalence was higher in Chinook salmon adult fish (36%) than in juvenile fish (9%) across the CRB (Table 2.4). While disease associated with IHNV infection is observed principally in juvenile fish (Breyta et al. 2016), the high prevalence of IHNV infection in adult fish prompted additional assessment of the influence of both age class and life history phenotype on the prevalence of IHNV infection. When assessed by both age class and life history phenotype, infection prevalence in spring- and fall-run Chinook salmon of the CRB appeared to be less comparable between the two life history types. Infection prevalence in adult spring-run fish was 43%, whereas in adult fall-run fish infection prevalence was 32% (Table 2.5). While a difference of 11% may not appear significant or biologically relevant, one must consider the relative abundance of each host type across the CRB. Fall-run fish constitute 60% of adult Chinook salmon returning to the CRB, whereas spring-run fish comprise only 27% of the average annual returns of Chinook salmon (Table 2.7). While these data alone do not give a complete picture of the association of CRB Chinook salmon and IHNV, they do suggest that adult spring-run Chinook salmon maybe more closely linked to the ecology of IHNV in the CRB. This observation is of interest when considering the disproportionately low prevalence of IHNV in Chinook salmon populations of the coastal watersheds relative to the CRB. Across coastal streams of Washington and Oregon, adult spring-run fish comprise a small proportion of the Chinook salmon that spawn in these watersheds (Healey 1991). Coastal streams

are more commonly inhabited by fall-run Chinook salmon, potentially contributing to the overall low prevalence IHNV infection in Chinook salmon populations of the coastal watersheds. Empirical testing is needed to characterize intraspecific differences in host susceptibility to IHNV infection (presented in subsequent chapters).

Evaluation of the prevalence of U and M IHNV in juvenile and adult spring-, summer-, and fall-run Chinook salmon of the CRB provided additional insight into the unique association of IHNV within the CRB. Initial assessment of the prevalence of IHNV in juvenile spring-, summer-, and fall-run Chinook salmon showed equivalently low prevalence (8%, 4%, and 12%) between the host types (Table 2.5). When assessed based on age class and life history type, two potentially interesting differences in the prevalence of U and M viral infections in CRB Chinook salmon emerged. While greater numbers of juvenile spring-run Chinook salmon cohorts were tested, IHNV prevalence was slightly higher in juvenile fall-run fish (Table 2.6). Infection prevalence was observed to be 8% in juvenile spring-run fish, whereas in fall-run juvenile fish infection prevalence was 12%. Most notable, was the lack of M IHNV detection in juvenile fall-run Chinook salmon, although only 5 cohorts were genotyped. All IHN virus detected in juvenile fall-run fish was U genogroup virus, whereas in juvenile spring-run fish both U and M viruses were detected. While caution must be taken when making inferences from such few records (Table 2.6), the following observations are worth noting. Whereas U genogroup viruses constitute 83% of IHNV detected in CRB Chinook salmon (Table 2.1), U viruses comprise 25% of IHNV detections in Coastal spring-run Chinook salmon and 0% in fall-run populations (Table 2.3). While the absence of U virus detection in fall-run fish of the coastal watersheds is in stark contrast to the high prevalence of U virus in both fall- and spring-run Chinook salmon of the CRB, the converse is observed when examining juvenile fall-run Chinook salmon in the CRB. While numerous

factors may be contributing to the observed differences in the prevalence of U and M IHNV infection across Chinook salmon populations of these two geographic regions, these findings suggest that infection prevalence in Chinook salmon populations may be more closely linked to biophysical attributes corresponding to the expanse of a watershed (i.e. stream order, diversity of host assemblages, rain-dominated vs. snow-dominated streams) rather than simply adult run phenotypes. While the spatial and temporal distributions of spring-run (stream-type) and fall-run (ocean-type) Chinook salmon coincide with the geographic distributions of U and M IHN viruses across the CRB, experimental studies are needed to characterize how U and M virus strains infect diverse Chinook salmon populations. While it is unclear how IHNV is maintained over time across the CRB, this investigation suggests that diverse host assemblages across a landscape as well as host age and life history type may differentially contribute to the successful maintenance of IHNV across a watershed.

2.5 TABLES

Table 2.1. Prevalence of IHNV infection in Chinook salmon population of the Columbia River Basin (CRB) and coastal watersheds of Washington and Oregon. Infection prevalence is based on IHNV Virology, Genotyping and Surveillance (VGS) data[a] available for Chinook salmon cohorts[b] for the years 2000-2012. The prevalence of U and M IHNV infection across Chinook salmon populations of the CRB and coastal watersheds is reported as the proportion of U and M IHNV positive Chinook salmon cohorts relative to the total number of IHNV positive cohorts for which genotyping data was available.

Chinook salmon populations	Total cohorts tested	Pos. cohorts tested	Percent pos.	Pos. cohorts genotyped	U pos. cohorts	Percent U pos.	M pos. cohorts	Percent M pos.
Columbia River Basin	1422	315	22%	186	154	83%	32	17%
Coastal WA,OR	404	14	3%	11	4	36%	7	64%

[a]IHNV Virology, Genotyping and Surveillance (VGS) database records for CRB Chinook salmon 2000-2012 (Breyta et al. 2017).
[b]A cohort represents fish of the same species, age (adult or juvenile), collection site location (e.g. Hatchery), and year of testing (Breyta et al. 2017).

Table 2.2. Prevalence of IHNV infection in spring-run (stream-type), summer-run (stream/ocean-type) and fall-run (ocean-type) Chinook salmon populations of the Columbia River Basin (CRB) and Coastal Watersheds of Washington and Oregon. Infection prevalence is based on IHNV Virology, Genotyping and Surveillance (VGS) data[a] available for Chinook salmon cohorts[b] of the CRB and Coastal Watersheds for the years 2000-2012. Infection prevalence is reported as the proportion of IHNV positive Chinook salmon cohorts relative to the total number of IHNV positive cohorts of each life history type.

Chinook salmon populations Adult migration timing (juvenile life history)	Total cohorts tested	Pos. cohorts tested	Percent pos.
Columbia River Basin			
Spring-run (stream-type)	777	209	27%
Summer-run (stream/ocean-type)	62	8	13%
Fall-run (ocean-type)	172	43	25%
Unknown migration timing	411	55	13%
Coastal WA,OR			
Spring-run (stream-type)	65	5	8%
Summer-run (stream/ocean-type)	30	2	7%
Fall-run (ocean-type)	173	4	2%
Unknown migration timing	136	3	2%

[a]IHNV Virology, Genotyping and Surveillance (VGS) database records for CRB Chinook salmon 2000-2012 (Breyta et al. 2017).
[b]A cohort represents fish of the same species, age (adult or juvenile), collection site location (e.g. Hatchery), and year of testing (Breyta et al. 2017).

Table 2.3. Prevalence of U and M IHNV infection in spring-run (stream-type), summer-run (stream/ocean-type) and fall-run (ocean-type) Chinook salmon of the Columbia River Basin (CRB) and coastal watersheds of Washington and Oregon. Prevalence of U and M viral infection is based on IHNV Virology, Genotyping and Surveillance (VGS) data[a] available for Chinook salmon cohorts[b] of the CRB and coastal watersheds for the years 2000-2012. Prevalence of U and M IHNV infection is reported as the proportion of Chinook salmon cohorts infected with U or M viruses relative to the total number of IHNV positive cohorts for which genotype data is available.

Chinook salmon populations Adult migration timing (juvenile life history)	Total cohorts tested	Pos. cohorts tested	Percent pos.	Pos. cohorts genotyped	U pos. cohorts	Percent U pos.	M pos. cohorts	Percent M pos.
Columbia River Basin								
Spring-run (stream-type)	777	209	27%	126	103	82%	23	18%
Summer-run (stream/ocean-type)	62	8	13%	2	1	50%	1	50%
Fall-run (ocean-type)	172	43	25%	26	23	88%	3	12%
Unknown migration timing	411	55	13%	32	27	84%	5	16%
Coastal WA,OR								
Spring-run (stream-type)	65	5	8%	4	1	25%	3	75%
Summer-run (stream/ocean-type)	30	2	7%	2	2	100%	0	0%
Fall-run (ocean-type)	173	4	2%	4	0	0%	4	100%
Unknown migration timing	136	3	2%	1	1	100%	0	0%

[a]IHNV Virology, Genotyping and Surveillance (VGS) database records for CRB Chinook salmon 2000-2012 (Breyta et al. 2017).
[b]A cohort represents fish of the same species, age (adult or juvenile), collection site location (e.g. Hatchery), and year of testing (Breyta et al. 2017).

Table 2.4. Prevalence of IHNV infection in adult and juvenile Chinook salmon of the Columbia River Basin (CRB)[a]. The prevalence of IHNV infection in adult and juvenile Chinook salmon is report as the proportion of virus positive cohorts[b] relative to the total number of cohorts tested.

Chinook salmon Age class	Total cohorts tested	Pos. cohorts tested	Percent Pos.
Adult	713	259	36%
Juvenile	487	44	9%
Unknown age	222	12	5%
Total	1422	315	22%

[a]IHNV Virology, Genotyping and Surveillance (VGS) database records for CRB Chinook salmon 2000-2012 (Breyta et al. 2017).
[b]A cohort represents fish of the same species, age (adult or juvenile), collection site location (e.g. Hatchery), and year of testing (Breyta et al. 2017).

Table 2.5. Prevalence of IHNV infection in spring-run (stream-type), summer-run (stream/ocean), and fall-run (ocean-type) Chinook salmon populations of the Columbia River Basin (CRB)[a]. Infection prevalence is reported for each life history phenotype as the proportion of IHNV positive adult and juvenile Chinook salmon cohorts[b] relative to the total number of adult and juvenile cohorts tested[c].

Chinook salmon Age class	Spring-run cohorts tested	Spring-run cohorts pos.	Spring % pos.	Summer-run cohorts tested	Summer-run cohorts pos.	Summer % pos.	Fall-run cohorts tested	Fall-run cohorts pos.	Fall % pos.	Unknown run cohorts tested	Unknown run cohorts pos.	Unknown % pos.
Adult	421	181	43%	35	7	20%	112	36	32%	144	35	24%
Juvenile	356	28	8%	27	1	4%	60	7	12%	42	8	19%
Total	777	209	27%	62	8	13%	172	43	25%	186	43	23%

[a]IHNV Virology, Genotyping and Surveillance (VGS) database records for CRB Chinook salmon 2000-2012 (Breyta et al. 2017).
[b]A cohort represents fish of the same species, age (adult or juvenile), collection site location (e.g. Hatchery), and year of testing (Breyta et al. 2017).
[c] CRB Chinook salmon VGS database records for which age class was reported but migratory timing (life history) was unknown were included in this analysis.

Table 2.6. Prevalence of U and M IHNV infection in spring-run (stream-type), summer-run (stream/ocean-type) and fall-run (ocean-type) Chinook salmon of the Columbia River Basin (CRB). Prevalence of U and M viral infection is based on IHNV Virology, Genotyping and Surveillance (VGS) data[a] available for Chinook salmon cohorts[b] of the CRB for the years 2000-2012. Prevalence of U and M IHNV infection is reported as the proportion of adult and juvenile Chinook salmon cohorts infected with U or M viruses relative to the total number of IHNV positive cohorts for which genotyping data was available.

Chinook salmon populations Adult migration timing (juvenile life history) Age class	Total cohorts tested	Pos. cohorts tested	Percent pos.	Pos. cohorts genotyped	U pos. cohorts	Percent U pos.	M pos. cohorts	Percent M pos.
Spring-run (stream-type)	777	209	27%	126	103	82%	23	18%
Adult	421	181	43%	110	89	81%	21	19%
Juvenile	356	28	8%	16	14	88%	2	13%
Summer-run (stream/ocean-type)	62	8	13%	2	1	50%	1	50%
Adult	35	7	20%	2	1	50%	1	50%
Juvenile	27	1	4%	0	0	-	0	-
Fall-run (ocean-type)	172	43	25%	26	23	88%	3	12%
Adult	112	36	32%	21	18	86%	3	14%
Juvenile	60	7	12%	5	5	100%	0	-
Unknown migration timing[c]	186	55	30%	32	27	84%	5	16%
Adult	144	35	24%	25	20	80%	5	20%
Juvenile	42	8	19%	7	7	100%	0	-

[a]IHNV Virology, Genotyping and Surveillance (VGS) database records for CRB Chinook salmon 2000-2012 (Breyta et al. 2017).
[b]A cohort represents fish of the same species, age (adult or juvenile), collection site location (e.g. Hatchery), and year of testing (Breyta et al. 2017).
[c]Two hundred and twenty five additional CRB Chinook salmon cohorts were tested for IHNV, of which twelve were IHNV positive. Age class was not reported for these records and excluded from this analysis.

Table 2.7. Relative abundance[a] of spring-run (stream-type), summer-run (stream/ocean-type) and fall-run (ocean-type) Chinook salmon in the CRB.

Adult migration timing (juvenile life history)	Avg. annual returns (2000-2012)	Percent of avg. adult returns
Spring-run (stream-type)	172,876	27%
Summer-run (stream/ocean-type)	85,655	13%
Fall-run (ocean-type)	381,195	60%
Total	639,726	100%

[a]Average annual returns of adult Chinook salmon to the CRB, for the years 2000-2012, are based on passage numbers above Bonneville Dam (Columbia River DART database) and do not include abundance of Chinook salmon in the lower reaches of the Columbia River Watershed. Calculated average annual returns do not include Chinook salmon Jacks.

2.6 FIGURES

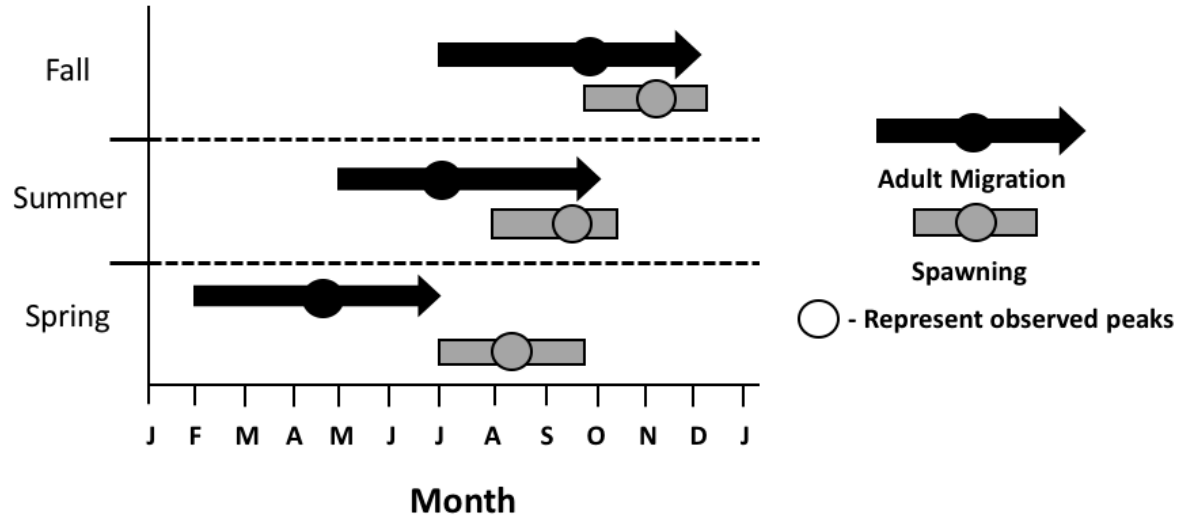

Figure 2.1. The upstream migration and spawn timing of spring-, summer- and fall-run Chinook salmon of the Columbia River Basin (CRB). Black arrows represent the generalized upstream migratory times of spring-, summer- and fall-run Chinook salmon, with circles denoting the time of year when peak numbers of migrating fish are observed. Grey boxes represent the generalized timing and observed peaks in adult spawning, over the course of a year. Time is reported along the *x*-axis as month of year (abbreviated by a single letter), between January 1st and January 31st. Figure prepared using data from Fulton (1968), Howell et al. (1985), Hymer et al. (1992), Marshall et al. (1995) and Brannon et al. (2004).

Figure 2.2. The geographic distributions of spring- (top), summer- (middle) and fall-run (bottom) Chinook salmon across the Columbia River Basin (CRB). Figure prepared in ESRI ArcMap (10.6) using fish distribution data for the Pacific Northwest and the Columbia River Watershed Boundary published by StreamNet (2017).

a)

b)

Figure 2.3. The geographic distributions of U (light grey) and M (dark grey) IHNV positive cohorts of Chinook salmon in the (a) Coastal Watersheds of Washington and Oregon and (b) the Columbia River Basin. Mean center (large dots) and directional distributions (standard deviational ellipses) were calculated for U and M IHNV positive cohorts Chinook salmon positive between the years 2000-2012.

Figure 2.4. The geographic distributions of U (light grey) and M (dark grey) IHNV positive cohorts of spring-run (top), summer-run (middle), and fall-run (bottom) Chinook salmon in the CRB and their relative geographic distributions across the watershed. Mean center (large dots) and directional distributions (standard deviational ellipses) were calculated for U and M IHNV positive cohorts of spring-, summer-, and fall-run Chinook salmon.

2.7 LITERATURE CITED

ArcMap, E. S. R. I. Version 10.6.1. (2018). Redlands, California: ESRI.
Brannon, E. L., Powell, M. S., Quinn, T. P., & Talbot, A. (2004). Population structure of Columbia River Basin Chinook salmon and steelhead trout. Reviews in Fisheries Science, 12(2-3), 99-232.

Breyta, R., Samson, C., Blair, M., Black, A., & Kurath, G. (2016). Successful mitigation of viral disease based on a delayed exposure rearing strategy at a large-scale steelhead trout conservation hatchery. Aquaculture, 450, 213-224.

Breyta, R., Brito, I., Kurath, G., & LaDeau, S. (2017). Infectious hematopoietic necrosis virus virological and genetic surveillance 2000–2012. Ecology, 98(1), 283-283.

Columbia River DART Database (Data Access in Real Time), Columbia Basin Research. University of Washington, School of Aquatic & Fishery Sciences. Seattle (WA). Available at http://www.cbr.washington.edu/dart

Fleming, I. A., & Gross, M. R. (1994). Breeding competition in a Pacific salmon (coho: Oncorhynchus kisutch): measures of natural and sexual selection. Evolution, 48(3), 637-657.

Healey, M. C. (1991). Life history of chinook salmon (*Oncorhynchus tshawytscha*). Pacific salmon life histories.

Hearsey, J. W., & Kinziger, A. P. (2015). Diversity in sympatric chinook salmon runs: timing, relative fat content and maturation. Environmental biology of fishes, 98(1), 413-423.
Myers, J. M. (1998). Status review of chinook salmon from Washington, Idaho, Oregon, and California.

Quinn, T. P. (2018). The behavior and ecology of Pacific salmon and trout. University of Washington Press.

StreamNet GIS Data (2017). Columbia River Watershed Boundary spatial data set. Portland (OR): StreamNet, March 2019. Available at https://www.streamnet.org/data/interactive-maps-and-gis-data/

Taylor, E. B. (1990). Phenotypic correlates of life-history variation in juvenile chinook salmon, *Oncorhynchus tshawytscha*. The Journal of Animal Ecology, 455-468.

Waples, R. S., Teel, D. J., Myers, J. M., & Marshall, A. R. (2004). Life-history divergence in Chinook salmon: historic contingency and parallel evolution. Evolution, 58(2), 386-403.

Waples, R. S., Pess, G. R., & Beechie, T. (2008). Evolutionary history of Pacific salmon in dynamic environments. Evolutionary Applications, 1(2), 189-206.

Chapter 3. SUSCEPTIBILITY OF OCEAN- AND STREAM-TYPE CHINOOK SALMON TO ISOLATES OF THE L, U, AND M GENOGROUPS OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS (IHNV)

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3.1 ABSTRACT

This study examined the susceptibility of Chinook salmon *Oncorhynchus tshawytscha* to viral strains from the L, U, and M genogroups of infectious hematopoietic necrosis virus (IHNV) present in western North America. The goal of this investigation was to establish a baseline understanding of the susceptibility of ocean- and stream-type Chinook salmon to infection and mortality caused by exposure to commonly detected strains of L, U, and M IHNV. The L IHNV strain tested here was highly infectious and virulent in both Chinook salmon populations, following patterns previously reported for Chinook salmon. Furthermore, ocean- and stream-type Chinook salmon fry at 1 g can also become subclinically infected with U and M strains of IHNV without experiencing significant mortality. The stream-type life history phenotype was generally more susceptible to infection and suffered greater mortality than the ocean-type phenotype. Between the U and M genogroup strains tested, the U group strains were generally more infectious than the M group strains in both Chinook salmon types. Substantial viral clearance occurred by 30

d post exposure, but persistent viral infection was observed with L, U, and M strains in both host populations. While mortality decreased with increased host size in stream-type Chinook salmon, infection prevalence was not lower for all strains at a greater size. These results suggest that Chinook salmon may serve as reservoirs and/or vectors of U and M genogroup IHNV.

3.2 INTRODUCTION

Infectious hematopoietic necrosis virus (IHNV) is an enveloped, single-stranded, negative-sense RNA virus that causes acute, systemic disease in salmonids. Genetic sequencing of a 303 nucleotide region within the glycoprotein gene (mid-G) from hundreds of virus isolates has identified 3 genetic subgroups of IHNV designated U (upper), M (middle), and L (lower) for their relative geographic occurrence in western North America (Garver et al. 2003, Kurath et al. 2003). Each genogroup of IHNV contains many individual isolates, and genogroup-specific patterns of host specificity have been observed. Isolates in the U genogroup of IHNV are primarily virulent for sockeye salmon *Oncorhynchus nerka* (Garver et al. 2006) and occur as 2 phylogenetic subgroups designated U-P (Pacific) and U-C (Columbia River Basin) (Black 2015). Isolates in the M genogroup are primarily virulent for rainbow trout *O. mykiss* and steelhead trout (anadromous *O. mykiss*) (Garver et al. 2006) and can be separated into 6 subgroups, viz. M-A through M-F (Troyer & Kurath 2003). Isolates in the L genogroup are virulent in juvenile Chinook salmon *O. tshawytscha* of northern California and southern Oregon and form 2 subgroups designated L-I and L-II (Bendorf et al. 2007, Kelley et al. 2007). While IHNV is detected frequently in up-migrating adult salmonids, the majority of disease is expressed in juvenile fish at propagation facilities. Epidemics of IHNV can result in losses of up to 90% (Bootland & Leong 1999), making IHNV the most threatening viral pathogen to cultured Pacific salmonids.

Chinook salmon populations of the US Pacific Northwest are genetically diverse with expressed phenotypic differences in behavioral patterns, life history, and geographical distribution (Quinn 2005). Two juvenile life history patterns have been defined (Gilbert 1913, Healey 1991). Stream-type Chinook salmon reside in rivers for a full year prior to seaward migration, whereas ocean-type ('sea-type' in Gilbert 1913) migrate to the ocean within their first year of life. These juvenile life history types are linked to a suite of environmental conditions affecting growth rate, and in many cases, they co-vary with adult return migration timing and the spatial distribution of spawning (Taylor 1990a,b, Brannon et al. 2004). Because ocean- and stream-type Chinook salmon are sympatric with other salmonid species throughout many streams of the Pacific Northwest, defining their interactions with virus strains of the dominant IHNV genogroups is critical in understanding their potential role in the ecology and epidemiology of IHNV.

The Columbia River Basin (CRB), which spans portions of the US states of Washington, Oregon, Idaho, Montana, Wyoming, Utah, and Nevada, and the Canadian province of British Columbia, is host to a number of Pacific salmon, trout, and charr species (Fulton 1970, Wydoski & Whitney 2003) that are potential hosts for IHNV. In the CRB, strains of IHNV from both the U and M genogroups are found throughout much of the system (Garver et al. 2003), where disease outbreaks that occur in sockeye salmon are typically caused by U group IHNV, and disease in steelhead/rainbow trout is typically caused by M group IHNV (Garver et al. 2003, Breyta et al. 2013, 2016). Chinook salmon are the dominant salmonid species propagated and present in the CRB (Columbia River DART Database, cbr.washington.edu/dart/). While juvenile Chinook salmon in the CRB experience little disease due to IHNV, the virus is frequently detected in adults at spawning. Consistent presence of IHNV in adult Chinook salmon has led us to hypothesize that

this species may function as a reservoir and/or vector of IHNV in the CRB and perhaps facilitate transfer of the virus to other susceptible salmonid species.

Prior to this investigation, information regarding the interaction of Chinook salmon with IHNV was based mostly on observed disease epidemics caused by IHNV isolates from the L genogroup in juvenile Chinook salmon of California hatcheries. The virulence of various IHNV isolates has also been explored in laboratory experiments using juvenile Chinook salmon of southern Oregon and northern California (LaPatra et al. 1993, Bendorf 2010). The investigation presented here used controlled laboratory challenge experiments to define the survival, infection prevalence, viral load, and viral persistence of L, U, and M genogroup representative strains of IHNV in ocean- and stream-type Chinook salmon of the Pacific Northwest. Chinook salmon fry were exposed to selected strains of IHNV by immersion, and infection prevalence and viral load were quantified at 7 and 30 d post exposure (dpe) using both a plaque assay and reverse transcriptase quantitative PCR (RT-qPCR). Further, stream-type Chinook salmon were tested for survival, infection, and viral persistence at a larger size. The goal of this investigation was to establish a baseline understanding of the susceptibility of ocean- and stream-type Chinook salmon to L, U, and M IHNV strains.

3.3 MATERIALS AND METHODS

3.3.1 *Fish*

All fish rearing and experiments were conducted at the USGS Western Fisheries Research Center (WFRC) wet lab in Seattle, WA, using single-pass, flow-through, sand-filtered, and UV-treated fresh water from Lake Washington. Two Chinook salmon populations, representative of the ocean and stream life history phenotypes, were selected for viral challenges. Ocean-type Chinook salmon, returning to the Soos Creek Salmon Hatchery (Puget Sound), were artificially

spawned at the WFRC from male and female gametes of 20 mating pairs. Soos Creek is a tributary of the Green River in central Puget Sound, and this Chinook salmon population is representative of the ocean life history type in this region. For the stream-type Chinook salmon, approximately 2000 fertilized eggs, from a total of 84 adult mating pairs, were obtained from the Little White Salmon National Fish Hatchery in the CRB. All eggs were obtained from females that had been screened and found negative for *Renibacterium salmoninarum*, a vertically transmitted bacterial pathogen that is common in Chinook salmon (Munson et al. 2010). Ocean- and stream-type Chinook salmon eggs were incubated and hatched at the WFRC, where they were reared to approximately 1 g (referred to hereafter as 'fry') at a constant temperature of 10°C and fed daily with a semi-moist pellet diet (BioOregon) at a rate of 1.0 to 2.0% body weight. Stream-type Chinook salmon were subsequently reared to an average size of 28 g for testing at a larger size.

3.3.2 *Virus exposures*

All viral challenges were conducted at a constant water temperature of 10°C to mimic general conditions of the CRB and allow comparison of results with previous challenges in rainbow trout (Garver et al. 2006), steelhead trout (Breyta et al. 2014), and sockeye salmon (Garver et al. 2006). Three controlled laboratory challenges were performed on Chinook salmon.

In Expt 1, ocean-type Chinook salmon at an average weight of 0.98 g were exposed to each of 5 viral strains (Table 3.1) or a mock control treatment (virus- free media), creating 6 treatment groups. Triplicate groups of 20 fry were challenged by static immersion for 1 h in 1 l of laboratory water containing 2×10^5 plaque-forming units (PFU) ml^{-1} of a designated virus strain, or mock control, as previously described by Garver et al. (2006). After static immersion, independent flow-through water was resumed to each tank at a final volume of 5 l. These triplicate groups were monitored daily over the course of 30 d for mortality, morbidity, and clinical signs of infection.

All deceased fish were removed and stored at −80°C, and a minimum of 20%, selected to include fish from all treatments over time, were later tested by plaque assay (Batts & Winton 1989) for the presence and quantity of IHNV to confirm virus as the likely cause of mortality. Cumulative percent survival (CPS) for each treatment was calculated as the average CPS among triplicate groups of 20 fish exposed to L, U, and M strains of IHNV, and virulence was compared by survival analyses as described below. For each treatment group, a fourth replicate group of 20 fish was challenged, as described above, and 10 fish were sampled at 7 dpe to assay for subclinical infection. Fish of these sampled groups were euthanized with 240 mg l^{-1} of buffered tricaine methanesulfonate (Western Chemical) and individually stored at −80°C until processing. Thirty dpe, a total of 10 surviving fish per treatment, selected randomly from the 3 replicate tanks, were also sampled for assay of persistent infection.

In Expt 2, stream-type Chinook salmon at an average weight of 1.23 g were challenged using the same methods described above.

In Expt 3, stream-type Chinook salmon at an average weight of 28.1 g were exposed to 1 of 3 viral treatments by batch immersion. Treatment groups in this experiment were viral strains FR0031 (L genogroup), RB1 (U genogroup), DW09 (M genogroup), and the mock control. There were 3 replicate tanks $treatment^{-1}$, with 25 fish $tank^{-1}$. The virus treatment concentration was 2×10^5 PFU ml^{-1} in a total of 10 l to accommodate increased fish size. After a 1 h static immersion, single flow-through laboratory water was resumed, and tanks held a total volume of 38 l. Fish were monitored daily, and virulence was assessed as described above. Kidney and spleen tissues from deceased fish were excised, pooled for each fish, and stored at −80°C. As above, a minimum of 20% were later tested by plaque assay to confirm virus-related mortality. Seven dpe, 5 fish were sampled from each tank, totaling 15 fish $treatment^{-1}$. Kidney and spleen tissues were aseptically

excised from each sampled fish and stored at −80°C for later processing. Thirty dpe, 5 surviving fish were sampled from each replicate, for a total of 15 fish treatment^{-1}, for assay of persistent infection.

3.3.3 *Virus quantification assays*

Viral plaque assay and RT-qPCR measure different indicators of viral presence in a host. Plaque assay quantifies infectious viral titer in PFU g^{-1} of fish (Batts & Winton 1989), while RT-qPCR quantifies viral RNA copy number g^{-1} of fish (viral load) as a proxy for IHNV infection (Purcell et al. 2006). In order to compare each metric of virus infection in 1 g Chinook salmon exposed to IHNV, both assays were carried out in each fish sampled from Expts 1 and 2. Viral titers of fish sampled 7 and 30 dpe were determined using plaque assay of whole fish homogenates as previously described by Batts & Winton (1989), with the exception that fish were diluted 1:4 (weight:volume) in MEM (with no serum) for homogenization. RNA was also extracted from fish homogenates by diluting 500 µl of the homogenate 1:2 in a guanidine-based denaturing solution (Wargo et al. 2010) within 3 min of homogenization. RNA was then extracted as out- lined by Wargo et al. (2010). Viral load was determined using the IHNV glycoprotein (G) gene RT-qPCR assay described by Purcell et al. (2006). All RT- qPCR assays were run in duplicate, and absolute quantification standards based on transcript RNA were included on each qPCR plate as previously de- scribed by Wargo et al. (2010). For the purpose of our analysis, samples were considered positive if both replicate wells reached the RT-qPCR threshold by 40 cycles (Ct). Samples for which both replicate wells did not reach the threshold by 40 Ct were considered negative by the test. If 1 replicate well reached 40 Ct while the other did not, the test result was considered suspect, but interpreted as a negative test result for statistical analyses. Given this criterion, 38 samples yielded suspect test results but were assigned a negative test result. The calculated average

detection limit of the G IHNV RT-qPCR assay in whole fry homogenates was 7272 copies g^{-1} of fish (log 3.86 copies) based on our ability to detect a minimum of 2 viral RNA copies $well^{-1}$ in the transcript RNA standard curves. The detection limit for the plaque assay was 100 PFU g^{-1} of fish, calculated as the virus concentration that would result in 1 plaque formed in 1 of the 2 duplicate wells at the lowest dilution plated.

In the combined kidney and spleen tissues excised from individual 28 g stream-type Chinook salmon exposed to IHNV, only the RT-qPCR assay was carried out. Tissues were weighed and homogenized in a guanidine-based denaturing solution at a dilution of 1:8 as outlined by Wargo et al. (2010), with the exception that homogenization was done in Whirl- Pak® bags using a roller rather than a stomacher. While the majority of tissue samples weighed ≥1 g, some samples collected 30 dpe were too small for processing as individual fish, so tissues from 2 fish were pooled in 13 instances. For the purpose of statistical analysis, fish tissues processed individually or as a pool from the tissues of 2 fish represented a single data point. For kidney and spleen samples, the detection limit of the G IHNV RT-qPCR assay was 4545 copies g^{-1} of fish tissue (log 3.65 copies), which differed from the detection limit in whole fry due to differences in dilutions during sample processing.

3.3.4 *Statistical Analyses*

Survival curves for triplicate groups of 20 fry in each of the 5 viral treatments or mock control were estimated using the Kaplan-Meier method (Sigma- Plot, version 13). No significant differences were found for replicate tanks within treatments except for the FR0031 treatment in 1 g stream-type Chinook salmon. In this treatment, 1 of the 3 replicate tanks differed significantly in survival ($p = 0.023$; see Table 3.2), but repeated analysis using individual replicates did not change the statistical outcome of the data set. Subsequent analyses were therefore conducted using

data pooled from the replicate tanks within each treatment. Survival curves were com- pared within and between experiments with a log- rank test where, if the log-rank test for the survival curves was greater than would be expected by chance ($p < 0.001$), there was a significant difference between survival curves. To identify the viral treatment group that differed, multiple pairwise comparisons using the Holm-Sidak method were carried out with an overall significance level of $\alpha = 0.05$ (SigmaPlot, version 13). Significant differences in infection prevalence were determined using Fisher's exact test with Bonferroni corrected p-values to account for multiple pairwise comparisons. In Expts 1 and 2, a p-value ≤ 0.0125 was considered significant, while in Expt 3, $p \leq 0.025$ was considered significant. Analysis of variance (ANOVA) and Tukey's HSD test were used to assess differences in mean log viral load in positively infected fish from distinct viral treatment groups. Pearson's product moment correlation analysis was used to measure the linear correlation of viral titer (by viral plaque assay) and viral load (by RT-qPCR) for each juvenile fish that was positive by 1 or both techniques, using combined data from fish sampled at 7 and 30 dpe.

3.4 RESULTS

3.4.1 *Survival of ocean- and stream-type Chinook salmon fry exposed to L, U, and M strains of IHNV*

Ocean-type Chinook salmon fry in the positive control L genogroup FR0031 treatment showed an average CPS of 63%, with kinetics illustrated in Fig. 3.1a. The onset of mortality occurred at 7 dpe with a steep decline in survival between Days 7 and 11, followed by more gradual losses through the end of the 30 d monitoring period. Clinical signs of disease including exophthalmia, skin darkening, and hemorrhaging were observed in approximately one-third of the fish prior to death (Table 3.2). Mortality occurred in the RB1 treatment at 17 and 22 dpe, resulting in an average CPS of 97% for this treatment. Although there was no mortality (average 100% CPS)

in the other 3 treatments, clinical signs similar to those described above were observed in a small number of fish in the BLK94, RB1, and DW09 treatments (approximately 1 to 5 fish treatment^{-1}; Table 3.2). The mock-exposed fish also had an average CPS of 100%. The log rank statistic for the survival curve of fish in the FR0031 virus treatment differed significantly ($p < 0.001$) from those of fish in all other virus treatments and the mock control (Fig. 3.1a). There were no significant differences among treatment groups other than FR0031. A subset of 7 of 24 fish that died during challenge were tested, and all were found positive for virus by plaque assay with an average titer of 5.13×10^6 PFU g^{-1} (SEM = 0.64), indicating virus infection as the cause of the observed mortality.

In stream-type Chinook salmon fry, the L genogroup FR0031 treatment caused slightly higher, but not significantly different, mortality than in ocean- type fry, resulting in an average CPS of 51%, with kinetics shown in Fig. 3.1b. Mortality began at 6 dpe and continued through 16 dpe, plateauing at 22 dpe. Clinical signs of disease, as described above, were observed in approximately two-thirds of the fish (Table 3.2). The other 4 viral treatments each had low mortality. The U genogroup treatments BLK94 and RB1 each had an average CPS of 93%, and the M genogroup treatments 220-90 and DW09 each had an average CPS of 98%. Clinical signs, as described above, were observed in a small number of fish in each of the U and M viral treatments (at most 2 to 11 fish treatment^{-1}; Table 3.2). There was no mortality in mock-exposed fish. As observed in ocean-type Chinook fry, only the survival curve for the FR0031 treatment differed significantly (Fig. 3.1b) when compared to all other treatment groups. Differences in survival among the U and M viral treatments were not statistically significant. A subset of 9 of 41 fish that died were tested, and all were found positive for virus by plaque assay with an average titer of 4.1 $\times 10^6$ PFU g^{-1} of fish (SEM = 0.54).

Virulence comparisons between the 2 Chinook salmon populations were performed using survival data from each virus treatment. The log rank statistic for each pair of survival curves indicated that only the BLK94 virus treatment differed significantly ($p = 0.043$) between ocean- and stream-type Chinook salmon fry (Fig. 3.1).

3.4.2 *Infection of ocean- and stream-type Chinook salmon fry at 7 dpe*

Among ocean-type fry in the positive control L genogroup FR0031 treatment, 100% of the fish sampled were infected at 7 dpe when assessed by viral plaque assay (Fig. 3.2a). Using this method, infection occurred in 10 to 30% of fish in the U genogroup BLK94 and RB1 treatments, and in 0 to 20% of fish in the M genogroup 220-90 and DW09 treatments. When assayed by RT-qPCR, infection was detected in 70% of ocean-type fry in the FR0031 treatment, 0 to 50% of fish in the BLK94 and RB1 treatments, and 0 to 20% of fish in the 220-90 and DW09 treatments. When compared to the positive control FR0031 treatment, infection prevalence in ocean-type fry was significantly lower ($p < 0.0125$) for all other viral treatments groups tested at 7 dpe by viral plaque assay. By RT-qPCR, infection prevalence was significantly lower ($p < 0.0125$) only in the BLK94 and DW09 treatments where no infection was detected.

In stream-type Chinook salmon, 90% of the fish in the FR0031 treatment were infected at 7 dpe when assayed by viral plaque assay (Fig. 3.2b). Infection prevalence ranged from 60 to 90% in the BLK94 and RB1 treatments, and 20 to 30% in the 220-90 and DW09 treatments. When assayed by RT-qPCR at 7 dpe, infection prevalence in the FR0031, 220-90, and DW09 virus treatments was the same as determined by plaque assay, and prevalence in the BLK94 and RB1 treatments was slightly lower, at 50%. At 7 dpe, infection prevalence differed significantly from the FR0031treatment only in the 220-90 treatment group ($p = 0.0055$).

3.4.3 *Persistence of infection in ocean- and stream-type Chinook salmon fry at 30 dpe*

At 30 dpe, infection prevalence in ocean-type Chinook fry in the FR0031 L genogroup treatment was 20% when assayed by viral plaque assay (Fig. 3.2c). By this assay, infection prevalence in the DW09 (M) treatment was 10%, and no infection was detected in the BLK94, RB1, or 220-90 viral treatments. When assayed by RT-qPCR, 40% of ocean-type fry in the FR0031 treatment were infected at 30 dpe, and infection prevalence ranged between 0 and 20% in the U and M viral treatments. In stream-type Chinook salmon fry in the FR0031 treatment, infection prevalence was 37.5% at 30 dpe, and prevalence ranged between 0 and 10% in the U and M viral treatments when assayed by viral plaque assay (Fig. 3.2d). When assayed by RT-qPCR, infection prevalence in the FR0031 treatment was 37.5%, as with viral plaque assay. By this assay, infection pre- valence in the RB1 (U) treatment was 20%, and no infection was detected in the BLK94, 220-90, and DW09 viral treatments at 30 dpe. Pairwise comparisons using Fisher's exact test did not find variation in infection prevalence between viral treatments to be significant within or among Chinook salmon hosts tested at 30 dpe.

3.4.4 *Quantity of virus in ocean- and stream-type Chinook salmon fry at 7 and 30 dpe*

Ocean- and stream-type Chinook salmon fry positively infected with L, U, or M IHNV strains had viral titers ranging between 10^3 and 10^7 PFU g^{-1} of fish as determined by plaque assay and between 10^4 and 10^6 viral RNA copies g^{-1} of fish by RT-qPCR (Fig. 3.3). Among ocean-type fry positive at 7 dpe with L, U, and M virus strains, little variation was noted among mean log viral titers or mean log viral loads (Fig. 3.3a). In stream-type fry, however, viral titers and viral loads were highest in fish with the FR0031 strain and lowest in fish with the M strains, although the differences were not significant (Fig. 3.3b). Overall, fewer fish were infected at 30 dpe (Fig. 3.3c,d); however, viral titers and viral loads were not significantly lower than those observed at 7

dpe. ANOVA of positively infected fish indicated no significant differences in viral titer or viral load among viral treatments, within or between Chinook salmon hosts at both 7 and 30 dpe.

3.4.5 *Survival of stream-type Chinook salmon exposed at a greater size to L, U, and M strains of IHNV*

In 28 g stream-type Chinook salmon, average CPS in the FR0031 treatment was 78% (Fig. 3.4). The onset of mortality occurred at 11 dpe, with a decline in survival between Days 11 and 18, followed by gradual losses through the end of the 30 d monitoring period. Clinical signs of disease including exophthalmia, skin darkening, and some hemorrhaging were observed prior to death in the majority of fish that died (Table 3.2). There was no mortality in the RB1 (U) and DW09 (M) viral treatments or the mock control group. The survival curve of the FR0031 treatment differed significantly ($p < 0.001$) from those of the U and M viral treatments and mock control (Fig. 3.4). All fish that died were tested and found positive for virus by plaque assay with an average titer of 1.82×10^4 PFU g^{-1} of combined kidney and spleen tissues (SEM = 1.17).

3.4.6 *Infection of stream-type Chinook salmon of a greater size at 7 and 30 dpe*

At 7 dpe, 93% of the 28 g stream-type Chinook salmon in the FR0031 (L) treatment were infected when assayed by RT-qPCR (Fig. 3.5a). Infection prevalence was 26% in the RB1 (U) treatment and 20% in the DW09 (M) treatment. When compared to fish in the positive control FR0031 viral treatment, infection prevalence was significantly less ($p < 0.025$) in both the U and M treatment groups at 7 dpe, and prevalence did not differ significantly between the U and M treatment groups. At 30 dpe, infection prevalence of 28 g stream-type Chinook salmon in the FR0031 treatment was 46% (Fig. 3.5b). There was no detectable infection in the RB1 treatment,

while infection prevalence in the DW09 strain was 15%. There were no significant differences in infection prevalence between any of the treatment groups at 30 dpe.

Mean log viral loads at 7 dpe were highest in fish infected with the FR0031 strain; however, little variation was observed in mean log viral loads of fish infected with the U or M strains of IHNV (Fig. 3.6a). While viral loads were higher at 7 dpe than at 30 dpe (Fig. 3.6), there were no significant differences in mean log viral loads among viral treatment groups between the 2 time points.

3.4.7 *Correlation of plaque assay titers and RT-qPCR viral load*

Among the total of 223 fry from Expts 1 and 2 that were tested by both plaque assay and RT-qPCR, 41 were positive by both assays, and 8 were positive by RT-qPCR but negative by plaque assay. Another 11 samples were positive by plaque assay but negative by RT-qPCR; however, 9 of these 11 samples were classified as suspect by RT-qPCR (1 replicate well tested positive and the other tested negative, see 'Materials and methods'). Viral titer and viral load estimates from individual fish that were positive by both viral plaque assay and RT-qPCR indicated a significant positive correlation coefficient ($r = 0.644$, $p < 0.005$). This was only slightly different from the value calculated when samples that were positive by only 1 of the techniques were also included ($r = 0.648$, $p < 0.005$; Fig. 3.7).

3.5 DISCUSSION

Variation in the virulence of IHNV strains was previously reported in a foundational study using isolates originally differentiated by electropherotype (now known to include U, M, and L strains) in controlled challenges of juvenile Chinook salmon and steelhead trout (LaPatra et al. 1993). Subsequent investigations have described the host-specific virulence and the infectivity of

U and M genogroup strains of IHNV in rainbow trout (Garver et al. 2006, Peñaranda et al. 2009, Wargo et al. 2010), steelhead trout (Breyta et al. 2014), and sockeye salmon (Garver et al. 2006, Purcell et al. 2009, Wargo et al. 2010). More recently, virulence of virus strains representing the L genogroup has been tested in California Chinook salmon (Bendorf 2010). Altogether, these studies have broadened our understanding of the host specificity of IHNV in western North America. The data presented here expand on this knowledge by defining the survival, infection prevalence, viral load, and viral persistence of L, U, and M genogroup representative strains of IHNV in ocean- and stream-type Chinook salmon.

In rainbow trout, viral exposures with M genogroup isolates of IHNV resulted in high mortality, whereas exposures with U genogroup isolates resulted in low mortality (Garver et al. 2006). In sockeye salmon, the converse was observed, where exposure to U isolates of IHNV resulted in high mortality and M isolates caused low mortality (Garver et al. 2006). While both U and M type viruses were able to enter and infect both sockeye salmon and rainbow trout, in each host the more virulent isolate replicated faster, to higher levels, in a higher proportion of the fish, and persisted longer, than the less virulent virus (Peñaranda et al. 2009, Purcell et al. 2009).

In our investigation, the higher virulence of the L genogroup strain was consistent with the losses observed in the field during epizootic events in juvenile Chinook salmon of California hatcheries, and with previously reported L genogroup virulence challenges (Bendorf 2010). Among the U and M strains tested here, the observation of low or no mortality also mimicked general field patterns where Chinook salmon generally show little disease when infected with U or M group IHNV (Garver et al. 2003, Kurath & Breyta 2013). In stream-type Chinook fry, the onset of mortality in the U strain treatments was earlier than in the M strain treatments. While not

statistically significant, the U strains also resulted in measurably higher mortality than the M strains.

Infection prevalence at 7 dpe, as determined by both viral plaque and RT-qPCR assays, indicated greatest susceptibility of both ocean- and stream- type Chinook fry to the L genogroup strain of IHNV. Both infection assays indicated that ocean- and stream-type Chinook fry could also become infected with U and M strains of IHNV. Although not statistically significant, a trend of higher susceptibility to infection with U genogroup strains than M strains was noted in both Chinook salmon types when sampled at 7 dpe. This pattern was consistent among stream-type Chinook salmon exposed both at 1 and 28 g.

When infected, quantities of virus in ocean-type Chinook fry were generally comparable among viral treatments at 7 dpe. However, in stream-type Chinook fry, a trend was observed in which the highest virus levels were found in the L virus treatment group, with moderate levels in the U virus treatments, and lower levels in the M virus treatments. As with infection prevalence, this pattern was consistent among stream-type Chinook salmon exposed both at 1 and 28 g. Although no significant differences were noted in viral quantities among IHNV strains tested at 7 dpe, it is possible that differences in kinetics may have occurred at earlier times in infection. While the pattern of higher infectivity and virulence with U strains than with M strains was not statistically significant in Chinook salmon as it was in sockeye salmon (Purcell et al. 2009), the observed trend may be biologically relevant and should be tested with larger sample numbers in the future.

In both Chinook salmon populations, infection prevalence was lower at 30 dpe than at 7 dpe. While not completely concordant, both virus quantification assays detected persistence of L, U, and M strains of IHNV in small numbers of both ocean- and stream- type Chinook salmon.

Notably, persistent infections at 30 dpe showed virus quantities similar to those in fish positive at 7 dpe. While infection with an L genogroup strain of IHNV may persist in ocean-type Chinook salmon for as long as 216 dpe (Bendorf 2010), here infections with U and M strains of IHNV are shown to persist in the 2 dominant Chinook salmon life history phenotypes. Altogether, the isolation of infectious L, U, and M IHNV strains from both ocean- and stream-type Chinook salmon at 30 dpe supports previously published evidence for the possibility of a carrier state for IHNV in Chinook salmon (St-Hilaire et al. 2001).

Size-dependent susceptibility to IHNV has been previously observed in stream-type Chinook salmon (LaPatra 1989) and other salmonid hosts (LaPatra et al. 1990), where increased host size and/or age was associated with decreased mortality upon virus exposure. Consistent with these previously published reports, 28 g stream-type Chinook salmon in the L IHNV treatment had increased survival relative to 1 g fish, although this difference was not statistically significant. While infection prevalence at 7 dpe was lower in 28 g fish in the U and M viral treatments when compared to 1 g stream-type Chinook salmon, infection of fish in the L virus treatment was equal in the 2 size classes. While infection prevalence decreased over the course of 30 d, viral loads were not significantly less than reported at 7 dpe. Thus, results from Expts 2 and 3 support previous reports of size- dependent susceptibility of Chinook salmon to IHNV-related mortality, but we did not observe a size-dependent decrease in susceptibility to infection for the L virus. At the 28 g size chosen to represent out-migrating juveniles, stream-type Chinook salmon can become subclinically infected with L, U, and M strains of IHNV.

One of the goals of our experimental design was to compare viral plaque assay and RT-qPCR as indicators of viral infection. Previously published correlation analyses between viable IHNV quantities in infected fish tissues measured by plaque assay and viral load by RT-qPCR showed a

strong correlation (r = 0.94) and an average ratio of 8.3×10^3 genome copies per PFU in infected fish tissue (Purcell et al. 2006). In our investigation, we observed a significant but moderate correlation (r = 0.648), and our average ratio of viral RNA copies to PFU was 74:1. Although the 2 studies are not strictly comparable, it is notable that our ratio was approximately 100-fold lower than the previously published relationship. This difference in ratio may be due to differences in fish species, experimental design, RNA extraction, or RT-qPCR methods. Here, whole fish were homogenized in MEM to preserve the ability to detect viable virus prior to a dilution in a guanidine-based denaturing solution for RNA extraction, while Purcell et al. (2006) homogenized tissues directly in a commercial RNA extraction kit. In general, the RT-qPCR assay has been reported to have increased sensitivity relative to viral plaque assay. Consistent with this, in our study, we found that 8 of the 60 samples tested by both methods were positive by RT-qPCR but not by plaque assay. However, we also had 11 instances where samples were positive by plaque assay but negative by RT- qPCR. Nine of these 11 samples actually had 'suspect' results in the RT-qPCR (1 positive and 1 negative in the duplicate wells), suggesting that the viral copy number in these samples was near the detection limit of the assay. Altogether, the data suggest an overall low sensitivity of our RT-qPCR assay relative to that of Purcell et al. (2006). It is possible that homogenization in MEM prior to the guanidine-based extraction may have resulted in a reduced RNA yield due to RNA degradation. Nevertheless, the ability to re-isolate culturable virus from Chinook salmon exposed to IHNV demonstrated that the fish were infected with viable virus of all 3 genogroups. The positive correlation observed between viable viral titers and viral loads supports the use of the G gene IHNV RT-qPCR assay as a higher throughput technique for determining IHNV prevalence and infection levels in fish.

The observations outlined in this investigation confirm that Chinook salmon fry and larger juveniles can be infected with U and M strains of IHNV, maintain infections for at least 30 d, and not experience high levels of mortality. Such observations mirror field patterns of IHNV detection in Chinook salmon (Garver et al. 2003, Kurath & Breyta 2013). Altogether, the data presented here support the hypothesis that Chinook salmon may be reservoirs and/or vectors of U and M genogroup IHNV. Such a reservoir role would not be unique to IHNV. In the ecology of viral hemorrhagic septicemia virus (VHSV) in the US Great Lakes, the round goby *Neogobius melanostomus* and yellow perch *Perca flavescens* have been identified as species that may be subclinical carriers of VHSV transmissible to many other fish species (Bain et al. 2010). Although IHN disease in juvenile CRB Chinook salmon is relatively infrequent, it has been observed occasionally and can cause substantial losses (Kurath & Breyta 2013), such as during a 1995 epidemic in a federally threatened stream-type Chinook population (W. Groberg pers. comm.). While further studies are needed to better understand the role that Chinook salmon may play in the ecology and epidemiology of IHNV in western North America, this study is the first to characterize the susceptibility of juvenile Chinook salmon to U and M IHNV in a controlled laboratory setting.

3.6 ACKNOWLEDGEMENTS

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3.7 TABLES

Table 3.8. Infectious hematopoietic necrosis virus (IHNV) strains used in experimental challenges

Virus	Genogroup-subgenogroup	Host	Isolation site	Year of isolation
FR0031	L-II	Chinook salmon	Feather River Hatchery, CA	2000
BLK94	U-P	Sockeye salmon	Baker Lake, WA	1994
RB1	U-C	Steelhead trout	Round Butte Hatchery, OR	1975
220-90	M-B	Rainbow trout	Hagerman Valley, ID	1990
DW09	M-D	Steelhead trout	Dworshak Hatchery, ID	2009

Table 3.9. Summary of results from 3 experiments in which Chinook salmon *Oncorhynchus tshawytscha* were exposed to various strains of IHNV and then monitored for both survival (cumulative percent survival, CPS) and infection, measured as viral load by RT-qPCR and viral titer by plaque assay (PA). The number of observations of clinical signs during daily monitoring of each replicate tank in each treatment is shown. Note that this is not necessarily equivalent to the actual number of fish that developed signs because the same fish may have been observed on sequential days. dpe: days post exposure; na: not applicable, as the PA was not conducted for samples from 28 g fish.

Host	CPS			Clinical	No. positive/total tested			
Virus (genogroup)	Individual tank	Mean	SEM	signs	7 dpe		30 dpe	
					RT-qPCR	PA	RT-qPCR	PA
Ocean-type Chinook, 1g								
FR0031 (L)	55, 60, 75	63	6.01	5, 6, 8	7/10	10/10	2/5	1/5
BLK94 (U)	100, 100, 100	100	0.00	0, 1, 2	0/10	1/10	1/10	0/10
RB1 (U)	100, 100, 90	97	3.33	0, 0, 1	5/10	3/10	0/10	0/10
220-90 (M)	100, 100, 100	100	0.00	0, 0, 0	2/10	2/10	1/10	0/10
DW09 (M)	100, 100, 100	100	0.00	0, 2, 3	0/10	0/10	2/10	1/10
Mock	100, 100, 100	100	0.00	0, 0, 0	0/10	0/10	0/5	0/5
Stream-type Chinook, 1g								
FR0031 (L)	32, 47, 74	51	12.29	11, 16, 19	9/10	9/10	3/8	3/8
BLK94 (U)	90, 95, 95	93	1.67	2, 2, 5	5/10	6/10	0/10	0/10
RB1 (U)	90, 95, 95	93	1.67	1, 2, 2	5/10	9/10	2/10	1/10
220-90 (M)	95, 95, 100	97	1.67	0, 0, 2	2/10	2/10	0/10	1/10
DW09 (M)	95, 100, 100	98	1.67	0, 2, 2	3/10	3/10	0/10	0/10
Mock	100, 100, 100	100	0.00	0, 0, 0	0/10	0/10	0/5	0/5
Stream-type Chinook, 28g								
FR0031 (L)	67, 80, 87	78	5.85	2, 2, 5	14/15	na	6/13[a]	na
RB1 (U)	100, 100, 100	100	0.00	0, 0, 0	4/15	na	0/9[a]	na
DW09 (M)	100, 100, 100	100	0.00	0, 0, 0	3/15	na	2/13[a]	na
Mock	100, 100, 100	100	0.00	0, 0, 0	0/15	na	0/11[a]	na

[a]Some samples from 28 g fish were tested as pools of tissue from 2 fish (see 'Materials and methods')

3.8 FIGURES

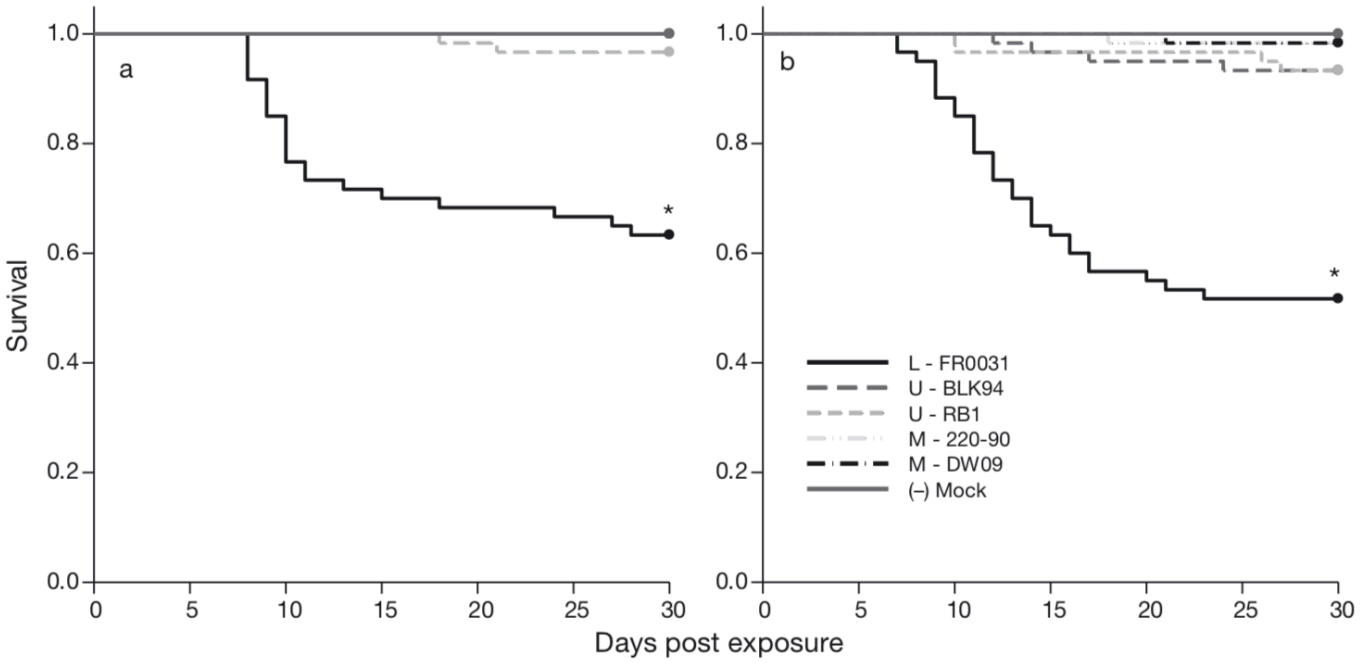


Figure 3.1. Daily cumulative proportion survival of 1 g (a) ocean- and (b) stream-type Chinook salmon *Oncorhynchus tshawytscha* exposed by immersion to 5 strains of infectious hematopoietic necrosis virus (IHNV; strain names and genogroups are given in the key). Data are pooled from triplicate groups of 20 fish treatment^{-1}. Data overlap on the 100% survival line for several virus treatments. Asterisks indicate that fish in the positive control L genogroup (FR0031) treatment had significantly lower survival than fish in the other treatments ($p < 0.001$). No other significant differences were found among virus treatments.

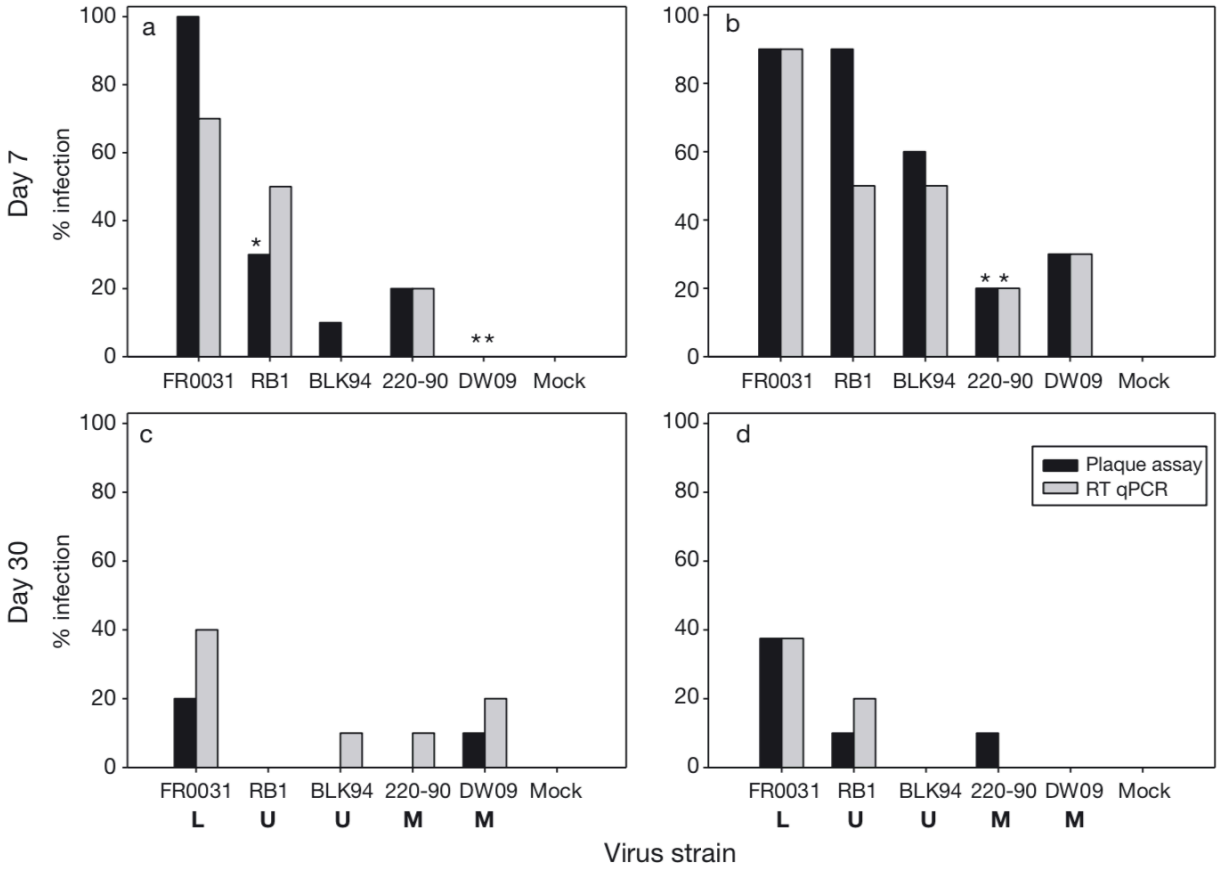


Figure 3.2. Frequency of infection of 1 g (a,c) ocean- and (b,d) stream-type Chinook salmon *Oncorhynchus tshawytscha* sampled 7 d (a,b) and 30 d (c,d) post exposure to 5 L, U, and M IHNV isolates. Ten fish per treatment group were sampled and processed by plaque assay and RT-qPCR, with the exception of the FR0031 treatment groups at 30 d, where 5 ocean- and 8 stream-type fish were processed. Asterisks denote significant differences ($p < 0.0125$) from the positive control FR0031 treatment tested by the relevant assay.

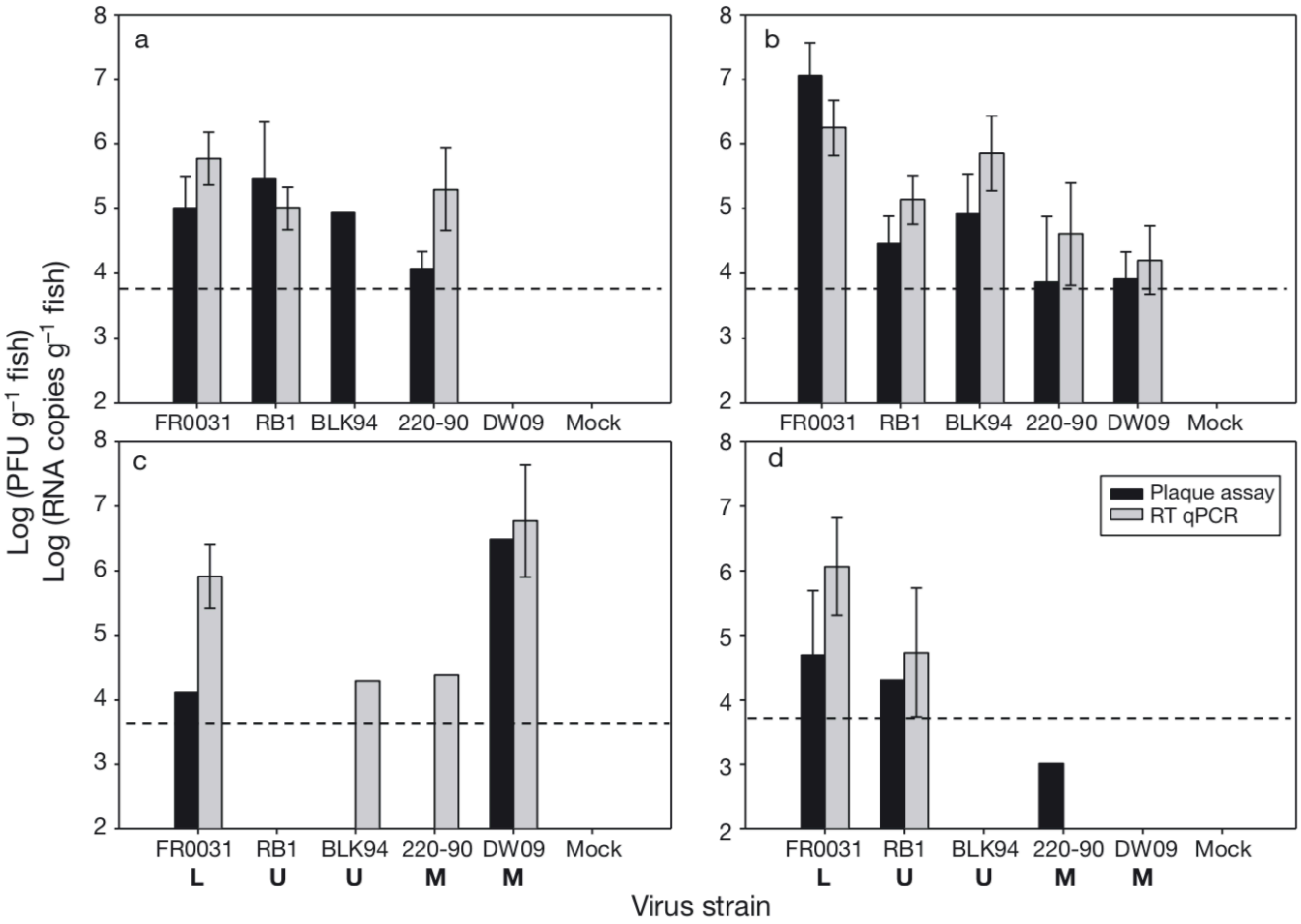


Figure 3.3. Mean (± SE) viral load of 1 g (a,c) ocean- and (b,d) stream-type Chinook salmon *Oncorhynchus tshawytscha* infected 7 d (a,b) and 30 d (c,d) post exposure to 5 L, U, and M IHNV isolates. Viral quantities were determined by plaque assay (plaque-forming units, PFU g^{-1}) and RT-qPCR (viral RNA copies g^{-1}). Only virus-positive fish were used to determine aver- ages. Where there are no error bars, data represent a single fish as detailed in Table 3.2. The detection limit for each assay is denoted by the dashed horizontal lines.

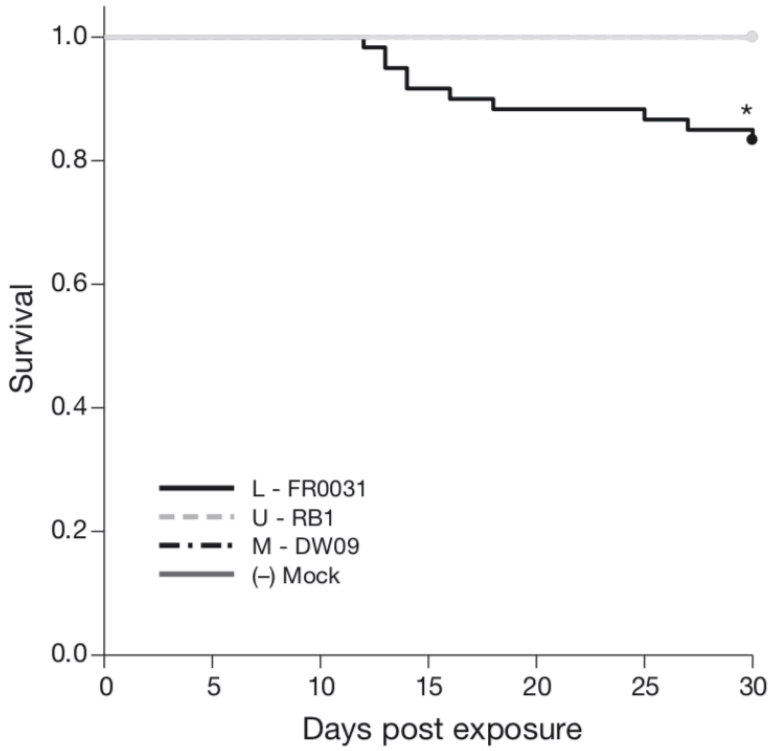


Figure 3.4. Daily cumulative proportion survival of 28 g stream- type Chinook salmon *Oncorhynchus tshawytscha* exposed by immersion to 3 strains of IHNV. Data shown are pooled from triplicate groups of 20 fish treatment^{-1}. There was 100% survival in fish in the RB1 (U) and DW09 (M) virus treatments (lines overlap at 100%). Fish in the positive control FR0031 L genogroup treatment had significantly lower survival than fish in the other treatments (*$p < 0.001$).

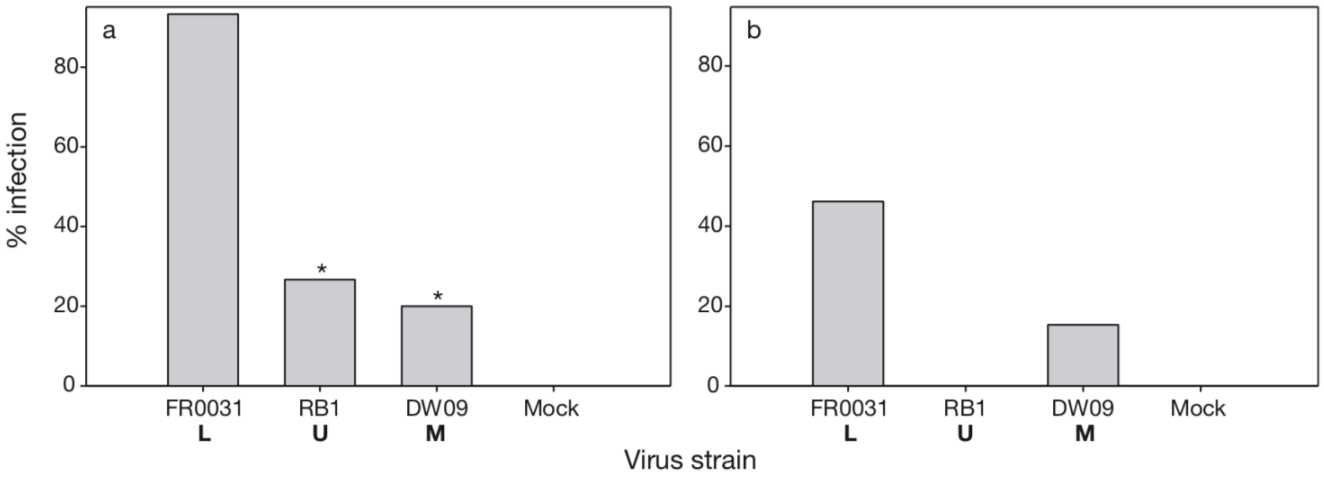


Figure 3.5. Frequency of infection of 28 g stream-type Chinook salmon *Oncorhynchus tshawytscha* sampled (a) 7 d and (b) 30 d post exposure to L, U, and M strains of IHNV by immersion. Samples consisted of pooled kidney and spleen tissues processed by RT-qPCR. Day 7 data represent groups of 15 fish processed individually, and Day 30 data are groups of 9 to 13 fish processed individually or as pools of 2 fish. Asterisks denote significant differences ($p < 0.025$) from the positive control FR0031 treatment.

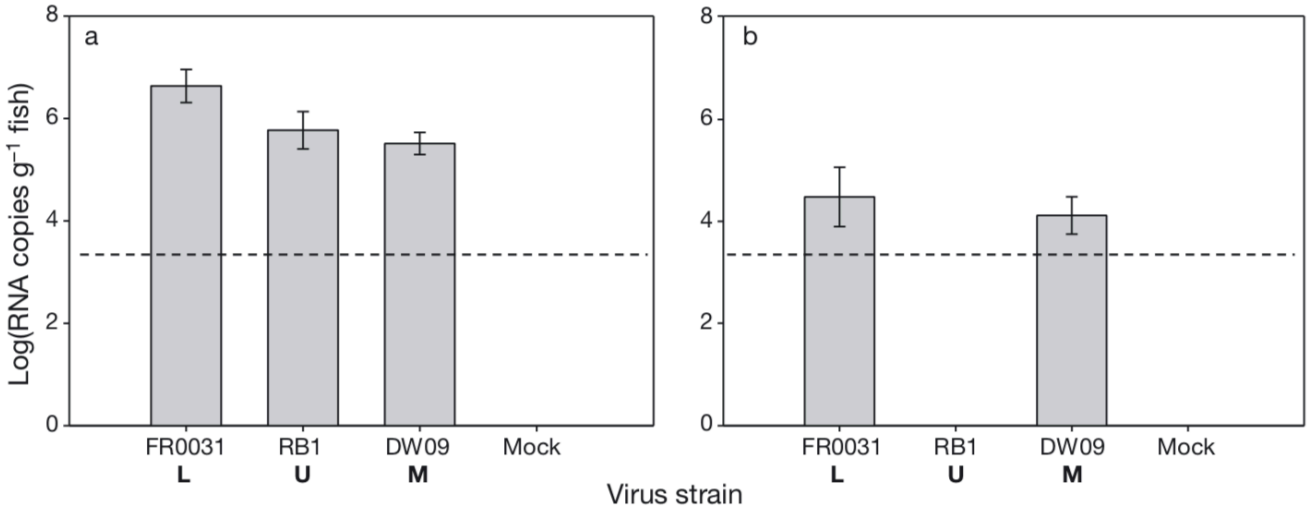


Figure 3.6. Mean (± SE) viral load of 28 g stream-type Chinook salmon *Oncorhynchus tshawytscha* infected (a) 7 d and (b) 30 d post exposure to L, U, and M IHNV isolates. Viral load was determined by RT-qPCR in viral RNA copies g^{-1} of fish tissue. Only virus-positive fish were used to determine averages. The detection limit for the RT-qPCR assay is denoted by the dashed horizontal lines.

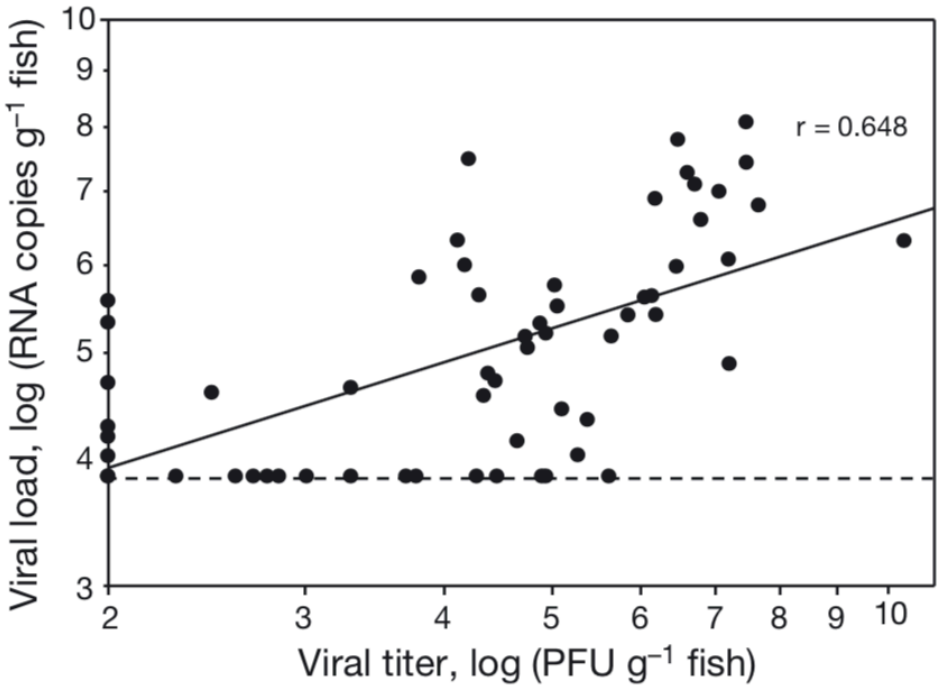


Figure 3.7. Correlation between viral quantities determined by plaque assay and by RT-qPCR (r = 0.648). Data shown are for individual ocean- and stream-type Chinook salmon *Oncorhynchus tshawytscha* exposed to IHNV at 1 g and assayed for virus by both methods at 7 or 30 d post exposure (see Fig. 3.2). Log plaque-forming units (PFU) g^{-1} of fish was determined by plaque assay and log viral RNA copies g^{-1} of fish was determined using the IHNV RT-qPCR assay. The lower limits of detection were 100 PFU g^{-1} of fish (log 2, x-axis) and 7272 RNA copies g^{-1} of fish (log 3.86, y-axis; dashed line). Eight samples that were positive by RT-qPCR and not by plaque assay are displayed on the y-axis. Eleven samples that were positive by plaque assay but not by RT-qPCR, as well as 5 samples that were positive by RT-qPCR, but below the calculated average for the detection limit, are displayed on the dashed line.

3.9 LITERATURE CITED

Bain MB, Cornwell ER, Hope KM, Eckerlin GE and others (2010) Distribution of an invasive aquatic pathogen (viral hemorrhagic septicemia virus) in the Great Lakes and its relationship to shipping. PLoS ONE 5:e10156

Batts W, Winton J (1989) Enhanced detection of infectious hematopoietic necrosis virus and other fish viruses by pretreatment of cell monolayers with polyethylene glycol. J Aquat Anim Health 1:284−290

Bendorf CM (2010) Phylogenetic analysis and virulence characteristics for Chinook salmon, rainbow trout, and steelhead of L genogroup infectious hematopoietic necrosis virus (IHNV) from the Feather River, California. PhD thesis, University of California Davis, CA

Bendorf CM, Kelley GO, Yun SC, Kurath G, Andree KB, Hedrick RP (2007) Genetic diversity of infectious hematopoietic necrosis virus from Feather River and Lake Oroville, California, and virulence of selected isolates for chinook salmon and rainbow trout. J Aquat Anim Health 19:254−269

Black A (2015) Geographic and host factors shape the evolution of a newly recognized subgroup within the U genogroup of the fish rhabdovirus IHNV. MSc thesis, University of Washington, Seattle, WA

Bootland L, Leong J (1999) Infectious haematopoietic necrosis virus. In: Woo PTK, Bruno DW (eds) Fish diseases and disorders, Vol 3. CAB International, New York, NY, p 57–121

Brannon EL, Powell MS, Quinn TP, Talbot A (2004) Population structure of Columbia River basin Chinook salmon and steelhead trout. Rev Fish Sci 12:99−232

Breyta R, Jones A, Stewart B, Brunson R and others (2013) Emergence of MD type infectious hematopoietic necrosis virus in Washington State coastal steelhead trout. Dis Aquat Org 104:179−195

Breyta R, Jones A, Kurath G (2014) Differential susceptibility in steelhead trout populations to an emergent MD strain of infectious hematopoietic necrosis virus. Dis Aquat Org 112:17−28

Breyta R, Samson C, Blair M, Black A, Kurath G (2016) Successful mitigation of viral disease based on a delayed exposure rearing strategy at a large-scale steelhead trout conservation hatchery. Aquaculture 450:213−224

Emmenegger EJ, Kurath G (2002) Genetic characterization of infectious hematopoietic necrosis virus of coastal salmonid stocks in Washington State. J Aquat Anim Health 14:25−34

Fulton LA (1970) Spawning areas and abundance of steelhead trout and coho, sockeye, and chum salmon in the Columbia River Basin — past and present. US Department of Commerce,

National Oceanic and Atmospheric Administration, National Marine Fisheries Service, Washington, DC

Garver KA, Troyer RM, Kurath G (2003) Two distinct phylogenetic clades of infectious hematopoietic necrosis virus overlap within the Columbia River basin. Dis Aquat Org 55:187−203

Garver KA, Batts WN, Kurath G (2006) Virulence comparisons of infectious hematopoietic necrosis virus U and M genogroups in sockeye salmon and rainbow trout. J Aquat Anim Health 18:232−243

Gilbert CH (1913) Age at maturity of the Pacific coast salmon of the genus Oncorhynchus. Bull Bur Fish 32: 1−22

Healey M (1991) Life history of chinook salmon (*Oncorhynchus tshawytscha*). In: Groot C, Margolis L (eds) Pacific salmon life histories. University of British Columbia Press, Vancouver, BC, p 311−394

Kell AM, Wargo AR, Kurath G (2014) Viral fitness does not correlate with three genotype displacement events involving infectious hematopoietic necrosis virus. Virology 464-465:146−155

Kelley GO, Bendorf CM, Yun SC, Kurath G, Hedrick RP (2007) Genotypes and phylogeographical relationships of infectious hematopoietic necrosis virus in California, USA. Dis Aquat Org 77:29−40

Kurath G, Breyta R (2013) Molecular epidemiology of aquatic pathogens — infectious hematopoietic necrosis virus (MEAP—IHNV) database. US Geological Survey. Available at gis.nacse.org/ihnv

Kurath G, Garver KA, Troyer RM, Emmenegger EJ, Einer- Jensen K, Anderson ED (2003) Phylogeography of infectious haematopoietic necrosis virus in North America. J Gen Virol 84:803−814

LaPatra SE (1989) Strain differentiation and detection of infectious hematopoietic necrosis virus. PhD dissertation, Oregon State University, Corvallis, OR

LaPatra S, Groberg W, Rohovec J, Fryer J (1990) Size- related susceptibility of salmonids to two strains of infectious hematopoietic necrosis virus. Trans Am Fish Soc 119:25−30

LaPatra SE, Fryer JL, Rohovec JS (1993) Virulence comparison of different electropherotypes of infectious hematopoietic necrosis virus. Dis Aquat Org 16: 115−120

LaPatra SE, Lauda KA, Jones GR (1994) Antigenic variants of infectious hematopoietic necrosis virus and implications for vaccine development. Dis Aquat Org 20: 119−126

Munson AD, Elliott DG, Johnson K (2010) Management of bacterial kidney disease in Chinook salmon hatcheries based on broodstock testing by enzyme-linked immunosorbent assay: a multiyear study. N Am J Fish Manag 30: 940−955

Peñaranda MMD, Purcell MK, Kurath G (2009) Differential virulence mechanisms of infectious hematopoietic necrosis virus in rainbow trout (Oncorhynchus mykiss) include host entry and virus replication kinetics. J Gen Virol 90: 2172−2182

Purcell MK, Hart SA, Kurath G, Winton JR (2006) Strand- specific, real-time RT-PCR assays for quantification of genomic and positive-sense RNAs of the fish rhabdovirus, Infectious hematopoietic necrosis virus. J Virol Methods 132:18−24

Purcell M, Garver K, Conway C, Elliott D, Kurath G (2009) Infectious haematopoietic necrosis virus genogroup- specific virulence mechanisms in sockeye salmon, Oncorhynchus nerka (Walbaum), from Redfish Lake, Idaho. J Fish Dis 32:619−631

Quinn TP (2005) The behavior and ecology of Pacific salmon and trout. University of British Columbia Press, Vancouver

St-Hilaire S, Ribble C, Traxler G, Davies T, Kent ML (2001) Evidence for a carrier state of infectious hematopoietic necrosis virus in chinook salmon *Oncorhynchus tshawytscha*. Dis Aquat Org 46:173−179

Taylor EB (1990a) Environmental correlates of life-history variation in juvenile chinook salmon, *Oncorhynchus tshawytscha* (Walbaum). J Fish Biol 37:1−17

Taylor EB (1990b) Variability in agonistic behaviour and salinity tolerance between and within two populations of juvenile chinook salmon, *Oncorhynchus tshawytscha*, with contrasting life histories. Can J Fish Aquat Sci 47:2172−2180

Troyer RM, Kurath G (2003) Molecular epidemiology of infectious hematopoietic necrosis virus reveals complex virus traffic and evolution within southern Idaho aquaculture Dis Aquat Org 55:175-185

Wargo AR, Garver KA, Kurath G (2010) Virulence correlates with fitness in vivo for two M group genotypes of Infectious hematopoietic necrosis virus (IHNV). Virology 404:51−58

Wydoski RS, Whitney RR (2003) Inland fishes of Washington. American Fisheries Society, Bethesda, MD

Chapter 4. VIRULENCE AND INFECTIVITY OF UC, MD AND L STRAINS OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS (IHNV) IN FOUR POPULATIONS OF COLUMBIA RIVER BASIN CHINOOK SALMON

4.1 ABSTRACT

This investigation sought to characterize the virulence and infectivity of UC, MD and L strains of IHNV in genetically diverse populations of Columbia River Basin Chinook salmon (*Oncorhynchus tshawytscha*) by examining the susceptibility of host populations to mortality and infection following exposure to each viral strain by static immersion. Kinetics of daily cumulative percent survival were used to construct survival curves for each Chinook salmon population following exposure to UC, MD and L strains of IHNV for one hour. The influence of viral treatment on host survival was assessed using survival analysis, whereas the influence of viral treatment and experimental host population on the frequency of infection was assessed using generalized linear models. Exposure of juvenile Chinook salmon to a range of virus concentrations allowed calculation of the dosages needed to infect 50 percent of an experimental host population (ID_{50}) with a select virus strain. These ID_{50} estimates served as a metric of virus infectivity and were compared between IHNV strains. Controlled laboratory challenges showed the Chinook salmon specialist L virus to be significantly higher in virulence than the UC and MD viral strains in all host populations, causing between 17-45 percent reductions in survival. Virulence was observably low with the UC and MD virus strains tested, with reductions in host survival ranging between 3-8 percent. However, infectivity of the UC and MD strains of IHNV were equivalent to the Chinook salmon specialist L virus strain. Results of this investigation suggest that the virulence of UC, MD and L strains of IHNV in CRB Chinook salmon is not driven by the ability of these

viruses to gain entry into *O. tshawytscha*, but rather the ability of juvenile fish to control viral infections. While Chinook salmon populations of the CRB are effective in controlling IHNV infections with commonly detected UC and MD virus strains, genetic variants of the UC and MD virus types have the potential to evolve with higher virulence to juvenile *O. tshawytscha.*

4.2 INTRODUCTION

Landscape features can greatly contribute to the population structure and life history diversification of organisms in both aquatic and terrestrial habitats, where physiological, morphological and behavioral attributes evolve in response to environmental selection pressures. In stable environments, natural selection predictably favors organismal specialism. In highly variable environments, however, organismal plasticity is highly advantages. The extensive biological diversity that is expressed within the genus *Oncorhynchus* illustrates the broad adaptive potential of Pacific salmon and trout to highly variable environments. As obligate intracellular parasites, for viruses the "environment" is defined largely by their hosts. Heterogeneous multi-host landscapes provide an opportunity for the evolution of both specialist viruses that typically infect a single host type, and generalists that infect two or more host types. For the aquatic rhabdoviral pathogen infectious hematopoietic necrosis virus (IHNV), diverse assemblages of Pacific salmonid fishes have served as the landscape upon which IHNV has evolved.

IHNV is an enveloped, single-stranded, negative-sense RNA virus that causes acute, systemic disease in Pacific salmon and trout populations (genus *Oncorhynchus*). IHNV (species *Salmonid novirhabdovirus*) belongs to the taxonomic family *Rhabdoviridae* and is the type species of the genus *Novirhabdovirus*. IHNV has a broad geographic distribution in the northern hemisphere (Bootland and Leong 2011; Kurath 2012) and is the most economically significant viral pathogen

affecting salmonid conservation hatchery programs and rainbow trout aquaculture (Bootland and Leong 2011).

IHNV is observed to infect both juvenile and adult Oncorhynchids, however epizootic events occur predominately in juvenile fish (Bootland and Leong, 2011; Breyta et al. 2016; Breyta et al. 2017). Genetic typing and phylogenetic analysis of thousands of virus isolates has identified three major genogroups of IHNV, designated U (upper), M (middle), and L (lower) for their relative geographic distributions across western North America (Garver et al. 2003; Kurath et al. 2003). Within each IHNV genogroup, distinct patterns of host specific virulence have been observed. In both field observations and controlled laboratory studies U genogroup strains of IHNV are highly virulent in juvenile sockeye salmon (*O. nerka*), M genogroup strains are highly virulent in juvenile rainbow trout and anadromous steelhead (two life history forms of *O. mykiss*), and L genogroup strains are highly virulent in juvenile Chinook salmon (*O. tshawytscha*). Viruses in each genogroup are detected in other salmonid host species, but at relatively low frequencies, and with lower virulence. Thus, the U, M, and L genogroups of IHNV appear to function largely as specialists adapted to a single host species, but this specialism is not absolute. This specialist pattern of U, M, and L host specificities is found throughout most of the geographic range of IHNV, but a major exception occurs in the large, complex Columbia River watershed.

The Columbia River Basin (CRB), which spans portions of the US states of Washington, Oregon, Idaho, Montana, Wyoming, Utah, and Nevada, and the Canadian province of British Columbia, is host to a number of Pacific salmon, trout, and charr species (Fulton 1970; Wydoski & Whitney 2003). The CRB supports all three major IHNV host species, with Chinook salmon most abundant, followed by steelhead trout, and smaller populations of sockeye salmon (Columbia River DART Database, cbr.washington. edu/dart/). IHNV prevalence in these three hosts ranges

from 26-32% in adult fish, and 8-25% in juvenile fish of the CRB (Breyta et al. 2017). Genetic typing of IHNV in the CRB has shown that viruses from both the U and M genogroups are sympatric throughout portions of the watershed (Garver et al. 2003; Breyta et al. 2016). In the CRB, M viruses continue to occur as specialists in steelhead and rainbow trout, with only a low frequency of detections in other host species (Breyta et al. 2016). M viruses in the CRB are nearly all in the MD subgroup of the M genogroup, and they periodically cause epidemic disease in steelhead trout (Breyta et al. 2016). In contrast, U genogroup viruses in the CRB are detected frequently in both Chinook salmon and steelhead trout, as well as in the small numbers of sockeye salmon (Breyta et al. 2016). This is a unique host association pattern for U genogroup viruses, which occur as sockeye specialists outside the CRB, occurring only rarely in sympatric populations of Chinook salmon or steelhead trout. A detailed phylogenetic study of U genogroup viruses revealed that a novel U subgroup has evolved in the CRB, designated UC, which is associated with this more generalist host pattern infecting Chinook salmon, steelhead trout, and sockeye salmon (Black et al. 2016). The high prevalence and unique host specificity patterns of IHNV in the CRB warrant investigations of the novel host-pathogen interactions. As the most abundant salmonid species in the CRB, we focus here on the interactions of CRB Chinook salmon with UC and MD IHNV strains that represent the great majority of virus detected in the CRB.

Chinook salmon populations of the CRB are genetically diverse with expressed phenotypic differences in behavioral patterns, life histories, and geographic distributions (Waples et al. 2008). In the CRB, Chinook salmon populations are commonly classified by the timing of adult upstream migration for spawning (Brannon et al. 2004). Across Chinook salmon populations of the CRB, three adult migratory phenotypes, referred to as the spring-, summer-, or fall-run types, have evolved to coincide with arrival at one's spawning grounds in a physiological state allowing

competition for mates and reproduction (Fleming and Gross 1994). The energetic costs of upstream migration and reproduction have selected for migratory times that avoid high water temperatures, low dissolved oxygen levels, and low flows that inhibit upstream movement (Brannon et al. 2004). Chinook salmon populations that migrate to interior reaches of the CRB for spawning begin their upstream migrations earlier in a given year as less sexually developed adults (Myers et al. 1998). These early migrating fish constitute spring- and summer-run Chinook salmon populations that are observed to spawn in smaller order, tributary streams of the interior Columbia River watershed (Myers et al. 1998). Fall-run Chinook salmon begin their upstream migrations later as more sexually developed adults (Hearsey et al. 2015) and spawn in larger order, river mainstems (Myers et al. 1998). Because these distinct life-history phenotypes have evolved in response to temperature and its direct influence on the physiologies of adult and juvenile fish, adult migration timing is observed to co-vary with juvenile freshwater residence (Brannon et al. 2004). Spring-run Chinook salmon offspring often reside in freshwater for a full year prior to seaward migration and are considered stream-type fish, whereas fall-run Chinook salmon offspring often migrate to the ocean within their first year of life and are considered ocean-type fish (Healey et al. 1991). Summer-run offspring from interior Chinook salmon populations exhibit a stream-type life history, whereas summer-run offspring from lower Columbia River Chinook salmon populations exhibit an ocean-type life history (Taylor 1990). Of these three adult migratory run-types, fall-run fish are most abundant (60%), followed by spring-run fish (27%), and summer-run fish comprise a small minority (13%) in the CRB (Columbia River DART Database, cbr.washington. edu/dart/). Here we focus on spring- and fall-run Chinook salmon, which differ substantially in spatial and temporal distribution across the CRB (Fig. 4.1), potentially differing in how they interact with IHNV. Because spring- (stream-type) and fall-run (ocean-type) Chinook salmon populations are

sympatric with other IHNV-susceptible salmonid species throughout the CRB, defining the interactions of differing Chinook salmon populations with the UC and MD IHN virus types commonly detected across the watershed is critical in understanding their potential role in the ecology and epidemiology of IHNV.

This investigation aimed to characterize the host-pathogen interactions between CRB Chinook salmon and virus strains of the dominant IHNV genogroups present in the Columbia River watershed. Assessment of IHNV surveillance data enabled us to identify field occurrence patterns of UC and MD IHNV infection in CRB Chinook salmon. Field occurrence patterns were used to formulate and test hypotheses specific to host and virus using controlled laboratory studies. To characterize intraspecific variation in the susceptibility of CRB Chinook salmon to UC and MD IHNV infection and disease, controlled laboratory exposures were used to compare infection prevalence and prevalence of mortality between experimental host populations. The same experimental data was also used to characterize differences in the infectivity and virulence between IHNV strains.

Juvenile fish from each of four CRB Chinook salmon population were exposed to a range of virus concentrations by batch immersion. Infection and mortality outcomes were assessed from the host and virus perspectives. From the host perspective, infection status was used to test the null hypothesis of equal susceptibility to IHNV infection among host populations. Similarly, mortality outcomes were used to test the null hypothesis of equal susceptibility to mortality following exposure to IHNV. From the virus perspective, mortality outcomes were used to test the null hypothesis of equal virulence between IHNV strains. The dosages needed to infect 50 percent of an experimental host population (ID_{50}) with a select virus strain were determined as a metric of virus infectivity and were compared between virus strains and host populations to test the null

hypothesis of equal infectivity between IHNV strains. The influence of host population, viral strain and exposure dose on the outcomes of infection (infection status and viral load) was evaluated using logistic regression and generalized linear models.

To date, no study has comprehensively examined the susceptibility of Chinook salmon to IHNV infection and mortality with U and M viral strains. Of specific relevance to the evolution of a novel generalist virus subgroup in the CRB, there are no previous studies of UC and MD IHNV in CRB chinook salmon. In addition, the possibility of intra-specific variation in susceptibility to IHNV among highly diverse populations of Chinook salmon has not been explored. This unique investigation combined field surveillance data and controlled laboratory studies to examine attributes of both the host and pathogen leading to the intra-species-specific patterns observed for CRB Chinook salmon and the UC and MD IHN virus types.

4.3 METHODS

4.3.1 *Ethics statement*

All animal experiments were approved by the University of Washington Institutional Animal Care and Use Committee (UW IACUC 3042-12).

4.3.2 *Analysis of IHNV field surveillance data*

Surveillance records for IHNV in the freshwater ecosystems of the Columbia River watershed and coastal rivers in Washington and Oregon were assessed to characterize the field occurrence patterns of U and M IHNV infection in CRB Chinook salmon. The IHNV Virology, Genotyping and Surveillance (VGS) database consists of 6766 records, representing 1146 sample sites, and 15 different fish hosts, and is comprised of surveillance records collected between the years 2000–2012 by all five resource management agencies that operate conservation hatcheries across the US

Pacific Northwest (Breyta et al. 2017). To curate this data set to Chinook salmon of the CRB, the VGS data set was imported into ArcMap, version 10.6.1 (Esri, 2018) where a spatial subset of the database was extracted based on the Columbia River Watershed Boundary (StreamNet GIS Data, 2017) using the Clip command. Further subsetting of these data by host species resulted in a total of 1422 records for CRB Chinook salmon, representing 128 total sample sites. Migratory run data was available for 1011 records, comprising the VGS database subset analyzed for CRB Chinook salmon. The virus was found in an average of 25.7% of records, and of these 59.2% had viral genotype data available. Together, these data were used to conduct univariate analysis of the prevalence of U and M IHNV infection in spring-, summer- and fall-run Chinook salmon of the CRB.

The relative abundance of spring-, summer- and fall-run Chinook salmon across the CRB was estimated for each life history type based on the average number of adult fish migrating passed the Bonneville Dam between the years 2000-2012 (Columbia River DART Database, cbr.washington.edu/dart/). Host abundance was integrated with IHNV field surveillance data to characterize possible intra-specific patterns of IHNV infection across the diverse Chinook salmon life history phenotypes.

4.3.3 *Experimental host populations*

Chinook salmon of the Winthrop National Fish Hatchery (United States Fish and Wildlife Service) and North Santiam Hatchery (Oregon Department of Fish and Wildlife) were selected as upper and lower CRB stream-type populations (Table 4.1), respectively (Narum et al. 2010). Chinook salmon of the Priest Rapids Hatchery and Cowlitz Hatchery (Washington Department of Fish and Wildlife) were selected as upper and lower CRB ocean-type populations, respectively (Narum et al. 2010). A total of 2000 eyed eggs (developing embryo), sourced from a minimum of

12 parental spawning pairs, were obtained from each of the four populations. All Chinook salmon eggs were incubated and hatched at the USGS Western Fisheries Research Center (WFRC) laboratory in Seattle, WA where they were reared to approximately 1 g at a constant temperature of 10°C. Juvenile Chinook salmon were fed a semi-moist pellet diet (BioOregon) at a rate of 1.0 - 2.0% body weight per day. All fish rearing and experimental exposures were conducted at the USGS WFRC using single-pass, flow-through, sand-filtered and UV-treated freshwater from Lake Washington.

4.3.4 *Virus exposures*

All viral challenges were conducted at a constant water temperature of 10°C to mimic general conditions observed throughout the CRB (Brannon et al. 2004). Controlled laboratory challenges were performed on juvenile Chinook salmon at an average weight of 1 g. Each host population was exposed to L, UC and MD genogroups strains of IHNV (Table 4.2) or a virus-free media treatment (negative control). To assess the virulence, triplicate groups of twenty fish were exposed to each viral strain by static immersion for 1 hr in 1 L of water containing virus at two doses, 2 x 10^3 plaque forming units (PFU) ml-1 (referred to as moderate dose) and 2 x 10^5 PFU ml^{-1} (referred to as high dose). After immersion challenge, water flow was resumed and each tank filled to a final volume of 5 L. These triplicate groups were monitored daily over the course of 30 days for mortality. All deceased fish were removed and individually stored at -80°C for later testing by viral plaque assay (Batts and Winton 1989). Cumulative percent survival (CPS) was calculated as the average CPS among triplicate groups of twenty fish exposed to L, UC and MD strains of IHNV. Kinetics of daily CPS were used to construct survival curves for each experimental host population following exposure to L, UC and MD strains of IHNV as described below. At the end of the 30-day challenge observation period all surviving fish were euthanized and 8 fish from each high dose

exposure group were saved for a preliminary analysis of viral persistence. These fish were stored at -80°C for later RNA extraction and virus detection and quantification.

To characterize the infectivity of L, UC and MD virus types in diverse populations of CRB Chinook salmon, twenty additional fish from each host population were exposed to each virus strain at of four virus concentrations: 2×10^2, 2×10^3, 2×10^4, and 2×10^5 PFU ml^{-1}. Viral challenges were conducted for 1 hr as described above. Post immersion challenge, flow-through water was resumed and fish were rinsed in total volumes of 5 L for 1 hr. Following this rinse, 8 of the 20 juvenile fish in each viral treatment were placed into individual 1 L beakers containing 400 ml of static water maintained at a constant temperature of 10°C by circulating temperature-controlled water around the beakers. The remaining 12 fish from each virus treatment were euthanized using buffered Tricaine Methanesulfonate (Western Chemical, Inc.) at a concentration of 240 mg L-1. Three days post exposure (dpe), each set of 8 fish per treatment group were euthanized and individually stored at -80°C for later RNA extraction and virus detection and quantification.

4.3.5 *RNA extraction and cDNA synthesis*

Total RNA was extracted from whole fish as previously described (Wargo et al. 2010). Briefly, 4 ml g^{-1} of fish of a guanidinium thiocyanate-based denaturation solution was added to each fish individually stored in a Whirl-Pak® bag (Nasco). Each fish was homogenized using a Stomacher® 80 Biomaster (Seward, Ltd.), after which the homogenate was centrifuged for 30 minutes (2,200 rcf). RNA was extracted from 1 ml of the homogenate with phenol-chloroform, precipitated and resuspended in 50 µl of RNase-free water. RNA was assessed for quality and concentration by spectrophotometry before complementary DNA (cDNA) was synthesized using M-MLV reverse transcriptase with random hexamer primers (Wargo et al. 2010). A standard

volume of 5 µl of RNA was used in each cDNA reaction and the final 20 µl of cDNA was diluted 1:5 by adding 80 µl of RNase-free water. Newly synthesized cDNA was stored at -80°C for polymerase chain reaction (PCR) analysis.

4.3.6 *Viral RNA quantification via reverse transcriptase real-time PCR*

Viral RNA was quantified using the universal IHNV N gene reverse transcriptase real-time PCR (RT-rPCR) assay as previously described (Purcell et al. 2013). Briefly, 5 µl of each diluted cDNA sample was combined with forward and reverse primers, TaqMan® FAM-labeled probe for the IHNV N gene, VIC® - labeled probe for the artificial positive control (APC) and amplified on an Applied Biosystems ViiA7™ real-time PCR machine. APC plasmid DNA was linearized and used to construct a standard curve (5×10^7 to 5 DNA copies) with which to quantify the absolute copy number of viral RNA (Purcell et al. 2013). Each sample was run in duplicate wells and interpreted as positive only when amplification was detected in both replicates within 40 cycles. The analytical sensitivity of the IHNV RT-rPCR assay was determined based on the PCR efficiencies observed for the APC plasmid DNA standard curves for all assays included in this analysis. The reaction efficiencies varied from −3.4 to −3.5 and the y-intercept values varied from 39.8 to 40.9, indicating similar limits of detection across the IHNV RT-rPCR assays. The detection limit of the IHNV N gene RT-rPCR assay was 2512.9 viral RNA copies per gram of fish tissue (3.40 $\log_{10}$ RNA copies g^{-1}). This assay detects both genomic and messenger RNA, and this combined quantity will be referred to hereafter as viral load.

4.3.7 *Statistical analyses*

Survival of experimental host populations

To assess the influence of viral treatment on host survival, survival analysis was conducted using the Kaplan-Meyer method as previously described (Hernandez et al. 2016). Briefly, survival curves were constructed for each set of 20 Chinook salmon fry exposed to a virus or virus-free treatment. Comparison of survival curves between experimental replicates demonstrated no significant differences. Therefore, data were pooled from triplicate groups of 20 fish per treatment to construct a single survival curve. Survival curves were compared within and across Chinook salmon populations using a log-rank test ($\alpha = 0.001$). To identify statistically significant differences between survival curves, multiple pairwise comparisons were conducted using the Holm-Sidak method ($\alpha = 0.05$).

Infection of experimental host populations

To characterize differences in host susceptibility to infection with L, UC and MD genogroup viruses, univariate analysis was conducted on binary infection data and viral load data. For each host population, infection prevalence at 3 dpe is reported as an indicator of virus infectivity. For virus positive fish, the quantities of IHN virus detected at 3 dpe are reported as the mean log10 viral load (log10 RNA copies g-1), indicating early virus replication capability once inside the host. Exposure of juvenile Chinook salmon to a range of virus concentrations allowed estimation of the dosages needed to infect 50 percent of each host population (ID50) with each IHNV strain. Briefly, a logistic regression model was fit to exposure dose to estimate the level at which 50 percent of a host population is expected to become infected. These regression analyses were conducted in the statistical program R, version 3.4.4 (R Core Team, 2018). For ten of the twelve total models (three virus strains in four host populations), the binomial error family was used for the logistic regression. For the remaining two models, upper and lower CRB stream-type Chinook salmon populations exposed to the L virus, a quasibinomial error family was favored because of

apparent overdispersion (Crawley 2013). Estimates of ID50 were determined using the dose.p function of the Mass package in R (Venables and Ripley 2002), and were used as a metric of virus infectivity. Significant differences between ID50 values were assessed using the Welch-Satterthwaite 2-tailed t-test (Breyta et al. 2014). To account for multiple pairwise comparisons, a Bonferroni correction of the 0.05 significance level was performed by dividing it by the number of comparisons in a given analysis. Significant differences between ID50 estimates were evaluated between viral strains within host populations (three comparisons, Bonferroni $\alpha = 0.0167$), or between host populations within viral strains (six comparisons, Bonferroni $\alpha = 0.0083$).

The influence of exposure dose, viral strain and host population on infection frequency

While comparisons of ID50 estimates have precedence in evaluating differences in the infectivity of IHNV strains (Breyta et al. 2014; McKenney et al. 2016), a pooled logistic regression approach was taken to evaluate the influence of exposure dose, viral strain and host population on the frequency of IHNV infection. The full regression model evaluated infection status at 3 dpe relative to exposure dose, viral strain, and host population. Reduced models omitted either viral strain, host population or both. Model comparison and selection was based on the minimization of Akaike's information criterion (AIC), which decreases as the model's out-of-sample predictive accuracy improves.

The inclusion of categorical variables, such as host population, in regression models requires them to be dummy coded first. Briefly, a dummy variable is created for all but one level of the categorical variable. The level that is held out serves as the reference level. The influence of this reference level on the outcome of infection is represented by a model's y-intercept, whereas the influences of the alternative levels is captured by regression coefficients that represent offsets from

the y-intercept. A logistic regression models the log odds ratio as a linear effect of the model's independent variables, which in the present case is formalized as follows:

$$\log(OR_i) = \beta_0 + \log_{10}(dose_i) \times \beta_1 + \sum_{h=2}^{H} host_{h,i} \times \alpha_h + \sum_{s=2}^{S} strain_{s,i} \times \gamma_s \quad [4.1]$$

where

- $\log(OR_i)$ is the log odds ratio associated with the *i*th fish;
- β_0 is the model's y-intercept, which also captures the reference level's influence on the log odds;
- $\log_{10}(dose_i)$ is the $\log_{10}$ of the exposure dose for the *i*th fish;
- β_1 is the effect size of exposure dose on the log odds;
- $host_{h,i}$ is the dummy variable for the *h*th host population for the *i*th fish;
- α_h is the offset from the y- intercept associated with the *h*th host population;
- $strain_{s,i}$ is the dummy variable for the *s*th viral strain for the *i*th fish;
- γ_s is the offset from the y- intercept associated with the *s*th viral strain.

To identify significant differences between all pairs of levels, refitting the same model multiple times was necessary to reassign the reference level. Because multiple pairs were evaluated, Bonferroni correction was once again applied.

The influence of exposure dose, viral strain and host population on viral load

The influence of exposure dose, viral strain and host population on viral load was evaluated using a generalized linear model (GLM) in R. The form of the full model closely resembles Eq.4.1, with the exception that the dependent variable was $\log_{10}(viral\ load_i)$. Reduced models once again omitted either viral strain, host population or both. Model comparison and selection was also based on the minimization of the AIC. As before, refitting the same model multiple times to reassign the reference level was necessary to identify significant differences between all pairs of levels.

4.4 RESULTS

4.4.1 *Field occurrence patterns of U and M IHNV infection in Chinook salmon*

Assessment of IHNV Virology, Genotyping and Surveillance (VGS) database records available for CRB Chinook salmon between the years 2000-2012 (Breyta et al. 2017) showed infection prevalence to be equivalent between the two dominant life history phenotypes of CRB Chinook salmon (Table 4.3). Across spring-run Chinook salmon populations tested, infection prevalence was 27%, whereas for fall-run Chinook salmon infection prevalence was 25%. Infection prevalence was lower in summer-run Chinook salmon (13%), with only 8 virus-positive cohorts reported. Due to their overall low abundance and small number of virus detections, summer-run fish were not included in our subsequent analyses. Overall, surveillance pressure was far higher for spring-run fish (277 cohorts) than for fall-run fish (172 cohorts). However, for each host type 60% of the virus-positive tests had virus genotype data available, allowing us to compare relative frequencies of U and M viral infections. Prevalence of U and M IHNV infection in spring- and fall-run Chinook salmon were equivalent between the two life history phenotypes. In CRB spring-run Chinook salmon, U viral strains comprised 82% of IHNV positive cohorts genotyped, whereas M viral strains comprised only 18%. Similarly, in fall-run Chinook salmon, U viral strains comprised 88% of IHNV positive cohorts genotyped, whereas M viral strains comprised only 12%. From the host perspective, these findings suggest spring- and fall-run Chinook salmon of the CRB are equally susceptible to IHNV infection. From the virus perspective, these findings suggest that UC and MD IHNV strains differ dramatically, with UC viruses potentially more infectious than MD viruses.

4.4.2 *Chinook salmon populations and virus strains for controlled laboratory studies*

Four Chinook salmon populations, representative of the genetic and life history diversity of *O. tshawytscha* in the CRB, were selected for experimental virus exposures. In Waples et al. (2004), four major genetic lineages were described for Chinook salmon of the Columbia River Watershed: interior Columbia River spring-run populations, interior Columbia summer- and fall-run populations, Willamette River populations and lower Columbia River spring- and fall run populations (referred to here as genetic lineages 1-4). Experimental host populations of CRB Chinook salmon were selected to represent each of these four genetic lineages (Table 4.1). Geographically, two of these Chinook salmon populations were obtained from the interior Columbia River (east of the Cascade Mountain Range) and two from the lower Columbia River (west of the Cascade Mountain Range) (Waples et al. 2008). Within each geographic region, populations were included to represent the distinct stream and ocean life history phenotypes observed in juvenile freshwater rearing (Brannon et al. 2004; Narum et al. 2010), corresponding to spring-run and fall-run adult phenotypes, respectively.

Three viral strains were included in this investigation (Table 4.2). Strain RB1, representing the UC subgroup, is virulent in sockeye salmon (Garver et al. 2006), and has the most common UC sequence type detected in the CRB (type mG001U) (Garver et al. 2003; Breyta et al. 2016). Strain QTS07, representing the MD subgroup, has high virulence in steelhead trout (Breyta et al. 2014) and was selected because it has the most common MD IHNV sequence type (type mG110M) detected throughout the CRB (Breyta et al. 2016). Although L genogroup IHNV does not occur in the CRB, the California L genogroup strain FR0031 was included as a positive control known to have high virulence and infectivity in juvenile Chinook salmon (Bendorf 2010; Hernandez et al. 2016).

4.4.3 *Survival of experimental populations of Columbia River Basin Chinook salmon*

For each Chinook salmon population included this investigation, kinetics of daily cumulative percent survival (CPS) are illustrated in Fig. 4.2. Of the four populations exposed to the positive control L genogroup virus at a high concentration of 2 x 10^5 PFU ml^{-1}, the upper CRB stream-type population had the lowest survival (Fig. 4.2a) with an average CPS of 55%. In contrast, the lower CRB stream-type population had the highest survival (Fig. 4.2c) with an average CPS of 83%. The upper and lower CRB ocean-type populations had average CPS of 57% and 60%, respectively. Statistically significant reductions in survival were observed in each host population of Chinook salmon exposed to the positive control L genogroup virus at a high concentration of 2 x 10^5 PFU ml^{-1} ($P < 0.001$), when compared to the virus-free media treatment (Fig. 4.2). Among the four host populations, survival analysis showed that three of the populations were equally susceptible to mortality following exposure to a high concentration of L virus, while the lower CRB stream-type population was statistically less susceptible ($P < 0.001$) (Fig. 4.2c).

Cumulative percent survival was higher overall in host populations exposed to the L genogroup virus at a moderate concentration of 2 x 10^3 PFU ml^{-1} (Fig. 4.2). Among the four Chinook salmon populations, the upper CRB ocean-type population had the lowest survival (Fig. 4. 2f) with an average CPS of 75%. The lower CRB ocean-type population had the second lowest survival (Fig. 4.2h) with an average CPS of 82%, and higher survival was observed in the upper and lower CRB stream-type population with CPS averages of 97% and 93%, respectively. In comparisons of survival curves the stream-type populations were not significantly different than the mock treatments. At this moderate exposure dose of the L virus both upper and lower CRB stream-type host populations were significantly less susceptible to mortality than the upper CRB ocean-type population ($P < 0.001$).

Across experimental host populations exposed to moderate or high concentrations of the UC virus, average CPS ranged from 92% to 98%. Across host populations exposed to MD virus at moderate or high concentrations, average CPS ranged from 92% to 100% and 93% to 100%, respectively. Multiple pairwise comparisons of survival curves within and across Chinook salmon populations showed no significant differences in survival following exposure with the UC and MD viral treatments, and neither differed from the mock-exposed control treatment. Survival was significantly higher ($P < 0.001$) in populations exposed to UC and MD strains of IHNV relative to the L virus. Altogether, the positive control L virus strain was significantly higher in virulence than the UC and MD virus strains. Between the UC and MD virus strains tested, virulence was low and not observed to differ within or across host populations.

4.4.4 *Infection of experimental populations of Columbia River Basin Chinook salmon*

Infection prevalence and viral load ($\log_{10}$ RNA copies g^{-1} of fish) were used to evaluate differences in the infectivity and early in-host replication of the three strains of IHNV. Three days following exposure, the proportion of fish from each experimental host population infected with each of the virus strains was determined (Figure 4.3). Exposure dosages ranged from 2×10^2 PFU ml^{-1} to 2×10^5 PFU ml^{-1}, for which an increasing dose response was consistently observed (Figure 4.3). Of the four Chinook salmon populations included in this investigation, the upper CRB stream-type population had the highest number of virus positive fish overall, totaling 48 individuals (Table 4.4). The lower CRB stream-type population had the lowest number of virus positive fish, totaling 30 individuals. Between the upper and lower CRB ocean-type Chinook salmon populations, equivalent numbers of virus positive fish were observed with 38 and 39 total individuals, respectively.

As an indication of the ability to replicate early in infection, mean log10 viral loads (± SEM) are reported for virus-positive fish within each viral treatment at 3 dpe (Table 4.5), with individual fish data illustrated in Fig. 4.4. There was little evidence of a dose response or of differences in mean log10 viral loads among IHNV strains within each host population (Figure 4.4). Therefore, viral load data were analyzed as total RNA copies produced for each fish host population over all exposure doses (Table 4.5). On a whole population basis, greater overall quantities of virus were produced by Chinook salmon of the lower CRB (8.61-8.92 $\log_{10}$ viral RNA copies) relative to those of the upper CRB (8.15-8.27 $\log_{10}$ viral RNA copies).

Of the three virus strains tested, the positive control L virus strain infected more fish than the UC and MD virus strains (Table 4.6). However, the highest total quantity of virus replication (viral load) was observed with the UC virus strain (Table 4.6). Most notably, the total quantity of UC virus was greater than the total quantity of virus observed for the positive control L strain.

4.4.5 *Viral dosages needed to infect 50 percent of a host population (ID50)*

Following exposure to a range of virus concentrations, infection frequencies both below and above 50 percent were attained in all four experimental host populations of Chinook salmon with each viral strain (Figure 4.3). Estimates of the viral dosages at which 50 percent of a population would become infected (ID_{50}) with each virus type were generated using binary infection outcomes for fish in each host population at 3 dpe. The ID_{50} values for each host population are reported in $\log_{10}$ PFU ml^{-1}, where lower ID_{50} estimates represent higher infectivity because a lower concentration is sufficient to infect 50 percent of a population. ID_{50} estimates were first compared within each host population to evaluate differences in the infectivity of L, UC and MD virus types (Figure 4.5). To account for multiple pairwise comparisons, the 0.05 significance level was Bonferroni-corrected to $\alpha = 0.0166$. For all virus strains in all host populations, ID_{50} estimates

varied within a range of approximately 1.5 logs, from 3.46 to 4.96 $\log_{10}$ PFU ml^{-1}. ID_{50} estimates were overall lower in the upper CRB stream-type population (Figure 4.5a) and higher for the lower CRB stream-type Chinook salmon population (Figure 4.5c). While not statistically significant, these differences suggest a trend of higher susceptibility to IHNV infection in upper CRB stream-type Chinook salmon relative to the lower CRB stream-type population. In the ocean-type host populations, greater variation between ID_{50} estimates for the L, UC and MD virus types was observed. Multiple pairwise comparisons of ID_{50} estimates within each experimental host population revealed only one statistically significant difference between two ID_{50} values. In the lower CRB ocean-type Chinook salmon population, the L virus was significantly higher in infectivity ($P = 0.008$) than the UC virus strain (Figure 4.5d). While not statistically significant, a similar pattern can be observed in the upper CRB ocean-type Chinook salmon population (Figure 4.5b) suggesting higher infectivity with the L virus in the ocean-type host populations.

For each virus strain, comparison of the same ID50 estimates across host populations is shown in Fig. 4.6, again demonstrating only one statistically significant difference between two ID_{50} values. The MD virus was significantly higher in infectivity in the upper CRB stream-type population ($P = 0.0123$) when compared to the lower CRB stream-type population (Figure 4.6b). Multiple pairwise comparisons showed infectivity of the L, UC and MD virus types to be statistically equivalent across experimental host populations. Altogether, with two exceptions, little variation was observed among ID_{50} estimates, either within or across host populations.

4.4.6 *The influence of virus strain & exposure dose on infection status and viral load*

The influence of virus strain and exposure dose on the infection status and viral load was assessed using logistic regression (Table 4.7) and generalized linear models (Table 4.8), respectively. Each of the two models was refit three times to allow comparisons between all pairs

of levels. To account for multiple pairwise comparisons, the 0.05 significance level was Bonferroni-corrected to $\alpha = 0.0166$. Differences between all pair levels are reported as *P* values in Table 4.9. The models each indicated a statistically significant influence of exposure dose on infection status ($P < 0.001$) but not viral load (Table 4.9). Consistent with these analyses, an increasing dose response can be observed in the frequency of virus-positive fish at 3 dpe (Figure 4.3). Virus strain was not observed to significantly influence infection status or viral load (Table 4.9). However, the logistic regression and generalized linear models reported *P* values that were above the Bonferroni-corrected $\alpha = 0.0166$ but less than 0.05 for comparisons between L and UC virus strains (Table 4.9). Assessment of the total numbers of fish infected at 3 dpe revealed the greatest number of fish infected with the L strain of IHNV and the fewest with the UC strain (Table 4.6). In contrast, examination of the total quantities of each virus by viral loads revealed more UC virus than the positive control L strain of IHNV (Table 4.6). While not statistically significant, the *P* values reported by the logistic regression and generalized linear models are indicative of potentially biologically relevant effects of L virus on infection status ($P = 0.030$) and UC virus on viral load ($P = 0.042$).

4.4.7 *The influence of host population on infection status and viral load*

The influence of host population on infection status and viral load was assessed using logistic regression (Table 4.7) and generalized linear models (Table 4.8), respectively. Here each of the two models was refit six times, to allow comparisons between all pairs of levels. To account for multiple pairwise comparisons, the 0.05 significance level was Bonferroni-corrected to $\alpha = 0.0083$. Differences between all pair levels are reported as P values in Table 4.10. The logistic regression models indicated no statistically significant influence of host population on infection status, with the exception of one pair contrasting upper versus lower CRB stream-type Chinook salmon ($P <$

0.001) (Table 4.10). Consistent with this exception, stream-type Chinook salmon of the upper CRB were observed to have greater numbers of virus-positive fish than the lower CRB stream-type population tested (Table 4.4).

The generalized linear models evaluating viral load indicated no statistically significant influence of host population on viral load, with the exception of one pair contrasting stream-type Chinook salmon of the upper versus lower CRB ($P < 0.005$) (Table 4.10). While higher numbers of upper CRB stream-type Chinook salmon were infected with IHNV at 3 dpe, comparison of the total quantities of virus detected within each of the stream-type populations revealed greater quantities of virus detected in the lower CRB stream-type Chinook salmon (Table 4.5). Each of the models reported *P* values that were above the Bonferroni-corrected $\alpha = 0.0083$ but less than 0.05 for three additional pairs of levels (Table 4.10). While not statistically significant, these *P* values support the notion that experimental host population may influence infection status (Table 4.4) and viral load (Table 4.5) at 3 dpe.

4.4.8 *Persistence of IHNV infection in Columbia River Basin Chinook salmon*

To provide a preliminary indication of any variation in viral persistence infection prevalence and mean $\log_{10}$ viral loads ($\log_{10}$ RNA copies g^{-1} of fish) were determined for a subset of fish that survived the high dose viral challenges. Thirty days post exposure, L, UC and MD IHNV infections were observed to persist in a proportion of fish from each host population (Table 4.11). Although statistical analyses were not conducted due to the small numbers of survivors (n=8) analyzed in each group, several interesting trends were observed. Infection prevalence was highest among fish exposed to the positive control L virus, relative to the UC and MD viral treatments. Similarly, mean $\log_{10}$ viral loads were generally highest among fish infected with L IHNV strain at 30 dpe. The total quantity of L IHN virus detected at 30 dpe in all host populations combined was

approximately two logs higher than the total quantities of the UC and MD viruses. While fewer fish were observed to be infected with the UC and MD IHNV strains, the mean $\log_{10}$ viral loads of fish infected at 30 dpe with the UC and MD viral strains were not negligible. Among Chinook salmon persistently infected with the UC IHNV strain, mean $\log_{10}$ viral loads ranged between 4.38 – 5.83 $\log_{10}$ RNA copies g^{-1} of fish. Among fish persistently infected with the MD IHNV strain, mean $\log_{10}$ viral loads ranged between 5.18 – 5.87 $\log_{10}$ RNA copies g^{-1} of fish. Overall higher numbers of virus positive fish were observed with the UC IHNV strain relative to the MD strain. Further, higher total virus quantities were observed with the UC IHNV strain relative to the MD viral strain.

4.5 DISCUSSION

The interaction of Chinook salmon with IHNV has been well characterized with regard to disease epidemics caused by L genogroup IHNV in juvenile Chinook salmon of southern Oregon and northern California hatcheries. Experiments by Bendorf (2010) on the virulence of LI and and LII genogroup strains of IHNV in Chinook salmon populations of northern California showed no significant differences in virulence between the two virus types. Across three California Chinook salmon populations tested, the LI and LII viral strains were moderately virulent with cumulative percent mortality ranging between (47– 87 %) (Bendorf 2010). In experimental exposures of California Chinook salmon to IHNV isolates from an epizootic event in sockeye salmon from the Auke Creek Hatchery in Alaska (presumably a U genogroup IHNV strain) and an epizootic event in Chinook salmon of the Coleman National Fish Hatchery in California (presumably a L genogroup IHNV strain), low levels of mortality were observed in juvenile Chinook salmon exposed to the sockeye salmon isolate whereas significant mortality was reported with the Chinook salmon isolate (Wertheimer and Winton 1982). Later studies exploring the virulence of IHNV

isolates, representing different virus electropherotypes, in juvenile Chinook salmon showed similar results (LaPatra et al. 1993). At a mean weight of 0.6 g, LaPatra (1989) reported high levels of mortality (93%) in juvenile Chinook salmon exposed to high dosages of the ER (Elk River Hatchery) and TR (Trinity River Hatchery) IHNV isolates. In contrast, juvenile Chinook salmon exposed to the RBH (Round Butte Hatchery) IHNV isolate showed significantly lower mortality (40%) (LaPatra et al. 1993). Further, even lower levels of mortality (17%-20%) were observed following exposure to the COW (Cowlitz River Hatchery), HAG (Hagerman Valley, Idaho, International Aquaculture Research Center) and DW (Dworshak National Fish Hatchery) IHNV isolates. Later genetic analysis showed the ER and TR IHNV isolates to belong to the L genogroup of IHNV, the RBH isolate to belong to the U genogroup of IHNV, and the COW, HAG and DW isolates to belong to the M genogroup of IHNV (G. Kurath pers. comm.). While these and later studies examining the genogroup-specific virulence of IHNV support the notion that U, M, and L genogroup strains of IHNV function largely as specialists adapted to a single host species, this specialism is not absolute. In all laboratory studies examining the virulence of U, M and L IHNV strains in non-adapted hosts, low levels of mortality have been measured (Garver et al. 2006; Penaranda et al. 2009; Bendorf 2010).

The extensive biological diversity that is expressed in the life histories of Pacific salmon and trout populations of the CRB constitute a heterogeneous multi-host landscape upon which IHNV is actively evolving. The recently identified UC subgroup of the U genogroup of IHNV in the CRB is associated with more of a generalist host pattern infecting Chinook salmon, steelhead trout, and sockeye salmon (Black et al. 2016). The evolution of a novel generalist virus subgroup in the CRB warrant investigations of the interactions between the dominant host and viral types present across the watershed. In this investigation, four populations of CRB Chinook salmon were exposed to

UC, MD and L strains of IHNV. Although L genogroup IHNV does not occur in the CRB, the California L genogroup strain FR0031 was included as a positive control known to have high virulence and infectivity in juvenile Chinook salmon (Bendorf 2010; Hernandez et al. 2016). Following exposure to a high concentration of the L genogroup virus, significant reductions in survival were observed in each Chinook salmon population. At moderate and high viral concentrations, the positive control L genogroup virus was measurably more virulent than the UC and MD viral strains in all four Chinook salmon populations. Irrespective of exposure dose, reductions in survival did not surpass eight percent in groups of fish exposed to the UC and MD viruses. Reductions in survival were equivalent between fish exposed to the UC and MD viral strains and were not observed to differ from fish in the negative control (virus-free) treatment. Overall, the L genogroup virus was significantly higher in virulence than the UC and MD viruses in all Chinook salmon populations tested. The UC and MD virus strains had equivalently low virulence in all four host populations. Together, distinct Chinook salmon populations of CRB did not differ in susceptibility to mortality following exposure to UC and MD IHNV strains.

The UC, MD and L genogroup strains of IHNV were each capable of infecting a proportion of each host population tested. While exposure dose was observed to influence the frequency of infection, virus strains did not differ in their influence on infection frequency or viral load at 3 dpe. Assessment of ID_{50} values showed infectivity of the L virus to be equivalent across all four host populations. Similarly, infectivity of the UC virus was also equivalent across all Chinook salmon host populations. Infectivity of the MD virus was significantly higher in upper CRB stream-type than in lower CRB stream-type Chinook salmon, however, no additional significant differences were observed. Overall, infectivity with the UC, MD and L IHNV strains was comparable within and across experimental host populations. Consistent with results of controlled laboratory

exposures, field occurrence patterns of IHNV showed infection prevalence to be equivalent between spring-run (stream-type) and fall-run (ocean-type) Chinook salmon of the CRB. Prevalence of U and M IHNV infection in spring- and fall-run Chinook salmon was also equivalent between the two life history types. However, the overall field prevalence of U and M were distinctively different, with much more U virus (82-88%) than M viruses (12-18%) detected.

While previous studies have characterized intra-specific variation in sockeye salmon, rainbow trout and anadromous steelhead susceptibility to mortality following exposure to U, M and L strains of IHNV (Garver et al. 2006; Breyta et al. 2014), few studies have examined intra-specific variation in Chinook salmon (Bendorf et al. 2010), and none have tested U, M and L strains of IHNV. This investigation is the first to comprehensively examine intra-specific variation in CRB Chinook salmon susceptibility to infection and mortality with U, M and L strains of IHNV.

4.6 Conclusion

The host-pathogen interactions of CRB Chinook salmon with UC, MD and L strains of IHNV have not been previously characterized with regard to viral infectivity and virulence. The observations outlined in this investigation confirm that UC and MD viral strains, commonly detected across the CRB, have the ability to infect genetically diverse populations of Chinook salmon. Moreover, infectivity of the UC and MD strains of IHNV is equivalent to the L genogroup Chinook salmon specialist virus. Results of this investigation suggest that virulence of L, UC and MD strains of IHNV in juvenile Chinook salmon is not driven by the ability of these viruses to gain entry into the host, but rather the ability of juvenile fish to control viral infections. While Chinook salmon populations of the CRB are currently effective in controlling IHNV infections with commonly detected UC and MD viruses, genetic variants of these virus types have the potential to evolve with higher virulence to juvenile Chinook salmon. Although infrequent,

occasional epidemics due to UC IHNV have occurred in juvenile upper CRB Chinook salmon (G. Kurath, R. Breyta pers. comm., MEAP-IHNV database). Because innate and adaptive antiviral immune response systems in teleost fishes are controlled by highly polymorphic genetic systems (Workenhe et al. 2010), newly evolved more virulent strains of IHNV could pose a bigger threat to CRB Chinook salmon populations that have experienced severe (or repeated) bottlenecks in population size. Such Chinook salmon populations include but are not limited to upper CRB stream-type fish derived from interior spring-run adult fish, for which lower genetic diversity has been reported (Narum et al. 2010; Waples et al. 2004; Winans 1989). Here, upper CRB stream-type Chinook salmon were observed to have the highest numbers of virus positive fish at 3 and 30 dpe, and the lowest overall ID_{50} values. While these findings suggest that upper CRB stream-type Chinook salmon may be more susceptible to IHNV infection relative to other CRB Chinook salmon populations, results of our controlled laboratory studies showed Chinook salmon populations of the CRB, exhibiting different life history phenotypes, become infected with IHNV at equivalent proportions. Further, UC and MD IHNV strains are equivalent in infectivity and virulence in CRB Chinook salmon. Of ecological and epidemiological significance is the lack of concordance between results of controlled laboratory exposures and field occurrence patterns of U and M IHNV in CRB Chinook salmon. Whereas controlled laboratory exposures suggest equivalent infectivity of U and M viruses across diverse CRB populations of Chinook salmon, surveillance records for IHNV in CRB Chinook salmon between the years 2000-2012 reveal a disproportionately higher prevalence of U IHN virus (85%) detected than M IHN virus detected (15%) in CRB Chinook salmon. While it is unclear what specific Chinook salmon attributes may be driving differences in the overall prevalence of U and M IHNV in CRB Chinook salmon, host age class appears to be closely associated with infection status at the time of testing.

4.7 TABLES

Table 4.1. Columbia River Basin (CRB) Chinook salmon (*Oncorhynchus tshawytscha*) populations obtained for experimental IHNV exposures.

Genetic lineage[a]	Columbia River Basin Geographic region[b]	Juvenile life history (freshwater residence)	Adult migration timing	Chinook salmon Hatchery population[c]	Endangered Species Act Federal Listing Status
1	Upper	Stream–type (yearling)	Spring	Winthrop National Fish Hatchery	Endangered
2	Upper	Ocean–type (subyearling)	Fall	Priest Rapids Hatchery	Threatened
3	Lower	Stream–type (yearling)	Spring	North Santiam Hatchery	Threatened
4	Lower	Ocean–type (subyearling)	Fall	Cowlitz Hatchery	Threatened

[a]Waples et al. (2004) describes four major genetic lineages for Chinook salmon populations of the CRB. In this investigation, these major lineages are designated as genetic lineages 1-4.
[b]Populations sourced east of the Cascade Mountain Range represent upper CRB fish and those obtained west of the Cascade Range constitute lower CRB fish.
[c]Chinook salmon populations, from specific hatchery programs, were selected based on the analysis described in Narum et al. (2010).

Table 4.2. Infectious hematopoietic necrosis virus (IHNV) strains used in controlled laboratory exposures.

Genogroup–subgroup	Virus strain	midG type[a]	Host	Isolation site	Year of Isolation
LII	FR0031	mG011L	Chinook salmon	Feather River Hatchery, California	2000
MD	QTS07	mG110M	Steelhead trout	Salmon River Hatchery, Washington	2007
UC	RB1	mG001U	Steelhead trout	Round Butte Hatchery, Oregon	1975

[a]Genotyping data based on 303 nt of the IHNV glycoprotein gene, referred to as the variable midG region (Kurath et al. 2003).

Table 4.3. Prevalence of U and M IHNV infection in spring-run (stream-type), summer-run (stream/ocean-type) and fall-run (ocean-type) Chinook salmon of the CRB.

Adult migration timing (juvenile life history)	Avg. annual returns[a] (2000-2012)	Percent of avg. adult returns	Total cohorts tested[b,c]	Pos. cohorts tested	Percent pos.	Pos. cohorts genotyped	U pos. cohorts	Percent U pos.	M pos. cohorts	Percent M pos.
Spring-Run (stream-type)	172,876	27%	777	209	27%	126	103	82%	23	18%
Summer-run (stream/ocean-type)[d]	85,655	13%	62	8	13%	2	1	50%	1	50%
Fall-run (ocean-type)	381,195	60%	172	43	25%	26	23	88%	3	12%

[a]Average annual returns of adult Chinook salmon to the CRB, for the years 2000-2012, are based on passage numbers above Bonneville Dam (Columbia River DART database) and do not include abundance of Chinook salmon in the lower reaches of the Columbia River Watershed. Calculated average annual returns do not include Chinook salmon Jacks.
[b]IHNV Virology, Genotyping and Surveillance (VGS) database records for CRB Chinook salmon 2000-2012 (Breyta et al. 2017).
[c]A cohort represents fish of the same species, age (adult or juvenile), collection site location (e.g. Hatchery), and year of testing (Breyta et al. 2017).
[d]Summer-run offspring from Chinook salmon populations of the Snake River exhibit a stream-type juvenile life history, whereas Columbia River populations exhibit an ocean-type life history (Taylor 1990).

Table 4.4. Infection frequency in experimental host populations of juvenile Chinook salmon at 3 days post exposure to L, UC and MD strains of IHNV. Exposure dosages are reported in plaque forming units (PFU) ml^{-1}. For each experimental treatment, virus positive fish are reported relative to the number of fish tested.

		L (FR0031)	UC (RB1)	MD (QTS07)		
Host population (Genetic lineage)	Dose	Virus pos. fish/total tested			Total fish pos.[a]	Total fish neg.[a]
Upper CRB Stream–type (Lineage 1)	$2x10^2$	0/8	0/8	2/8	48	48
	$2x10^3$	1/8	3/8	4/8		
	$2x10^4$	7/8	5/8	5/8		
	$2x10^5$	7/8	7/8	7/8		
	Mock	0/8	0/8	0/8		
Upper CRB Ocean–type (Lineage 2)	$2x10^2$	0/8	0/8	0/8	38	58
	$2x10^3$	1/8	0/8	1/8		
	$2x10^4$	6/8	3/8	6/8		
	$2x10^5$	8/8	6/8	7/8		
	Mock	0/8	0/8	0/8		
Lower CRB Stream–type (Lineage 3)	$2x10^2$	0/8	0/8	0/8	30	66
	$2x10^3$	1/8	1/8	1/8		
	$2x10^4$	1/8	4/8	2/8		
	$2x10^5$	8/8	7/8	5/8		
	Mock	0/8	0/8	0/8		
Lower CRB Ocean–type (Lineage 4)	$2x10^2$	1/8	0/8	0/8	39	57
	$2x10^3$	4/8	1/8	2/8		
	$2x10^4$	5/8	3/8	5/8		
	$2x10^5$	8/8	5/8	5/8		
	Mock	0/8	0/8	0/8		

[a]Totals are for combined results of all exposure for each host population.

Table 4.5. Quantities of IHN virus detected in 1 g stream- and ocean-type Chinook salmon of the upper and lower CRB at 3 days post exposure to L, UC or MD strains of IHNV.

		L (FR0031)		UC (RB1)		MD (QTS07)		
Host population (Genetic lineage)	Dose[a]	Mean log viral load[b]	SEM[c]	Mean log viral load[b]	SEM[c]	Mean log viral load[b]	SEM[c]	Log of total virus[d] (RNA copies)
Upper CRB Stream–type	$2x10^2$	-	-	-	-	5.61	0.365	8.15
(Lineage 1)	$2x10^3$	4.58	-	5.00	0.613	5.45	0.268	
	$2x10^4$	5.38	0.316	4.56	0.628	5.68	0.374	
	$2x10^5$	5.90	0.364	4.44	0.38	5.72	0.482	
	Mock	-	-	-	-	-	-	
Upper CRB Ocean–type	$2x10^2$	-	-	-	-	-	-	8.27
(Lineage 2)	$2x10^3$	5.44	-	-	-	5.41	-	
	$2x10^4$	5.72	0.429	5.47	0.61	5.02	0.18	
	$2x10^5$	6.29	0.426	5.71	0.513	6.08	0.30	
	Mock	-	-	-	-	-	-	
Lower CRB Stream–type	$2x10^2$	-	-	-	-	-	-	8.61
(Lineage 3)	$2x10^3$	4.67	-	7.78	-	5.12	-	
	$2x10^4$	6.98	-	5.79	0.662	6.41	0.9	
	$2x10^5$	6.61	0.315	5.91	0.434	6.77	0.6	
	Mock	-	-	-	-	-	-	
						-		
Lower CRB Ocean–type	$2x10^2$	6.68	-	-	-	-	-	8.92
(Lineage 4)	$2x10^3$	6.63	0.592	8.43	-	5.68	0.505	
	$2x10^4$	6.02	0.228	4.62	0.76	6.11	0.527	
	$2x10^5$	6.69	0.269	6.04	0.734	6.10	0.234	
	Mock	-	-	-	-	-	-	

[a]Exposure dosages are reported in plaque forming units (PFU) ml^{-1}.
[b]Mean log_{10} viral load (log_{10} RNA copies g^{-1} of fish) is reported for virus positive fish within each treatment group.
[c]Standard error of the mean (SEM) is reported for virus positive fish within each treatment group.
[d]For each experimental host population, the sum total of virus detected is reported as the log_{10} of total viral RNA copies detected at 3 dpe.

Table 4.6. Summary of juvenile Chinook salmon infection frequency and log_{10} viral loads 3 days after exposure to L, UC and MD strains of IHNV. Data for each virus strain are combined totals[a] from all four host populations, for all virus exposure doses.

IHNV genogroup-subgroup (experimental virus strain)	Total fish pos.	Total fish neg.	Log_{10} of total virus (viral RNA copies)
LII (FR0031)	58	70	8.65
UC (RB1)	45	83	8.91
MD (QTS07)	52	76	8.49
Mock	0	30	-

[a]Virus-specific totals are combined results from data presented in Table 5.

Table 4.7. Parameter estimates, with standard errors (SE), for evaluation of infection status using the full or reduced logistic regression models. The full regression model evaluated infection status against viral strain, exposure dose and host population (model 1). Reduced models omitted either host population (model 2), viral strain (model 3), or both (model 4). The full regression model was favored, as indicated by the lowest observable Akaike's information criterion (AIC).

	Model 1		Model 2		Model 3		Model 4	
	Estimate	SE	Estimate	SE	Estimate	SE	Estimate	SE
β_0	-6.65192	0.73	-6.402	0.66	-6.89648	0.71	-6.6437	0.65
β_{dose}	1.66842	0.16	1.5996	0.16	1.64075	0.16	1.5747	0.15
α_4	0.67208	0.39	-	-	0.66162	0.39	-	-
α_3	-0.07554	0.39	-	-	-0.07434	0.39	-	-
α_2	-0.69391	0.40	-	-	-0.68281	0.39	-	-
γ_m	-0.33893	0.34	-0.3258	0.33	-	-	-	-
γ_u	-0.74167	0.34	-0.7127	0.34	-	-	-	-
AIC #	342.05		348.18		342.85		348.79	

Table 4.8. Parameter estimates, with standard errors (SE), for evaluation of viral loads using the full or reduced generalized linear models (GLMs). The complex GLM evaluated log viral load outcome against viral strain, exposure dose and host population (model 1). Reduced models omitted either host population (model 2), viral strain (model 3), or both (model 4). The complex GLM was favored, as indicated by the lowest observable Akaike's information criterion (AIC).

	Model 1		Model 2		Model 3		Model 4	
	Estimate	SE	Estimate	SE	Estimate	SE	Estimate	SE
β_0	5.56222	0.54	5.1885	0.54	5.4302	0.53	4.9953	0.53
β_{dose}	0.17506	0.11	0.1992	0.11	0.1692	0.11	0.1954	0.11
α_4	-0.60047	0.23	-	-	-0.6487	0.23	-	-
α_3	-0.47253	0.24	-	-	-0.4854	0.24	-	-
α_2	0.09666	0.26	-	-	0.0344	0.26	-	-
γ_m	-0.19495	0.20	-0.2507	0.20	-	-	-	-
γ_u	-0.4267	0.21	-0.4363	0.21	-	-	-	-
AIC #	459.94		466.55		460.28		466.93	

Table 4.9. The influence of IHN virus strain and exposure dose on infection status and log_{10} viral load was assessed using logistic regression and generalized linear models (GLMs), respectively. Each of the two models were refit three times, to allow comparisons between all pairs of levels. To account for multiple pairwise comparisons, the 0.05 significance level was Bonferroni-corrected to $\alpha = 0.0166$. Differences between all pair levels are reported as *P* values. Virus strain was not observed to influence the frequency of infection or viral load. In contrast, the logistic regression model indicated a statistically significant influence of exposure dose on the frequency of infection ($P < 0.001$).

	Infection status			Log_{10} viral load		
IHNV genogroup-subgroup (Experimental virus strain)	LII (FR0031)	UC (RB1)	MD (QTS07)	LII (FR0031)	UC (RB1)	MD (QTS07)
LII (FR0031)	—			—		
UC (RB1)	0.030	—		0.042	—	
MD (QTS07)	0.315	0.237	—	0.330	0.280	—
Exposure dose		0.001			0.115	

Table 4.10. The influence of experimental host population on infection status and log_{10} viral load outcome was assessed using logistic regression and generalized linear models (GLMs), respectively. Each of the two models were refit six times, to allow comparisons between all pairs of levels. To account for multiple pairwise comparisons, the 0.05 significance level was Bonferroni-corrected to $\alpha = 0.0083$. Differences between all pair levels are reported as *P* values. Experimental host population was observed to statistically influence frequency of infection ($P < 0.001$) and log_{10} viral load ($P < 0.005$), but only when contrasting upper CRB and lower CRB stream-type Chinook salmon. Asterisks denote significant differences.

	Infection status				Log_{10} viral load			
Chinook salmon population	Upper CRB Stream-type	Upper CRB Ocean-type	Lower CRB Stream-type	Lower CRB Ocean-type	Upper CRB Stream-type	Upper CRB Ocean-type	Lower CRB Stream-type	Lower CRB Ocean-type
Upper CRB Stream-type	—				—			
Upper CRB Ocean-type	0.056	—			0.576	—		
Lower CRB Stream-type	0.001*	0.119	—		0.005*	0.027[b]	—	
Lower CRB Ocean-type	0.085	0.847	0.08	—	0.008[a,b]	0.05[b]	0.707	—

[a]The *P* value shown as 0.008 was actually 0.00849, and thus not below the Bonferroni-corrected level of significance of 0.0083.
[b]As noted in the text, three *P* values were not below the Bonferroni-corrected level of significance of 0.0083, but were less than $P = 0.05$.

Table 4.11. Persistence of IHNV infection in juvenile Chinook salmon of the CRB at 30 days post exposure to L, UC and MD strains of IHNV at a single high dose of $2x10^5$ plaque forming units ml^{-1}.

	L (FR0031)			UC (RB1)			MD (QTS07)			
Host population (Genetic lineage)	Num. pos./tested[a]	Mean log viral load	SEM	Num. pos./tested[a]	Mean log viral load	SEM	Num. pos./tested[a]	Mean log viral load	SEM	Sum of fish pos.
Upper CRB Stream–type (Lineage 1)	4/8	6.67	0.56	4/8	5.77	0.031	2/8	5.34	0.23	10
Upper CRB Ocean–type (Lineage 2)	4/8	6.59	1.2	2/8	4.38	0.36	1/8	5.96	-	7
Lower CRB Stream–type (Lineage 3)	2/8	5.87	0.97	1/8	5.83	-	1/8	5.18	-	4
Lower CRB Ocean–type (Lineage 4)	5/8	5.22	0.51	3/8	4.99	0.16	1/8	5.87	-	9
Total of fish pos.	15			10			5			
Log total virus quantity	9.18			6.75			6.36			

[a]Infection prevalence is reported as the number of virus positive fish relative to the number of fish tested.

4.8 FIGURES

a)

b)

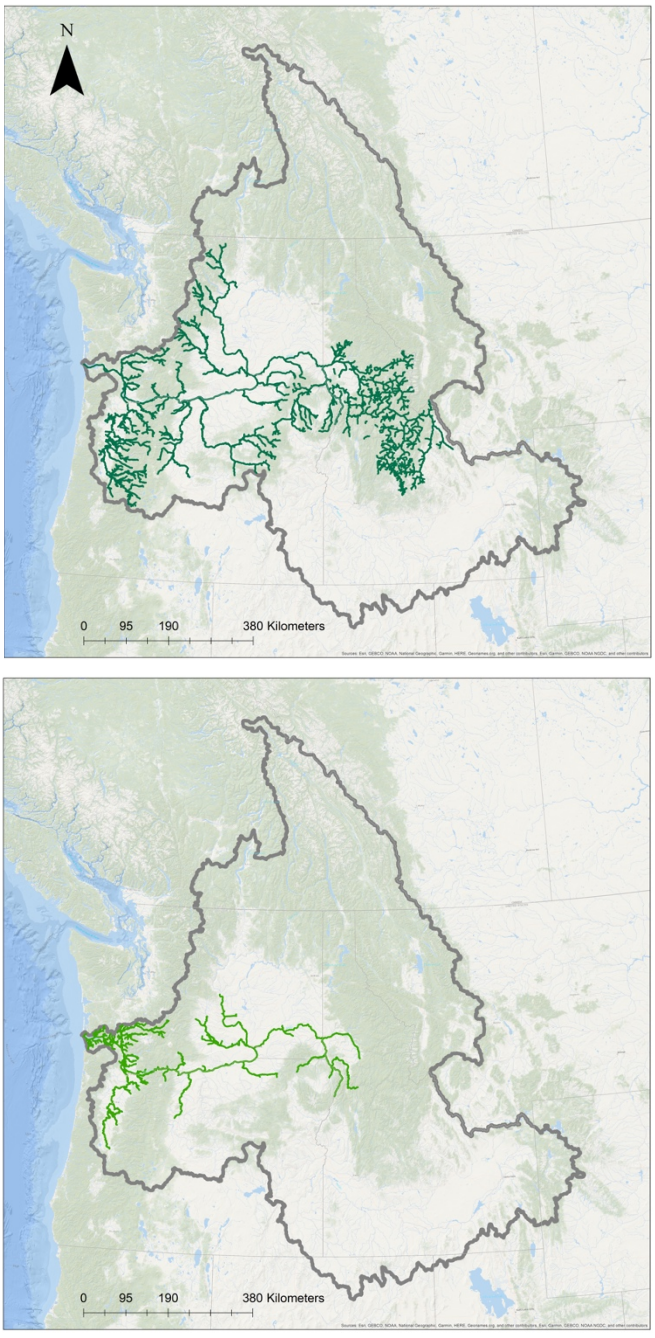


Figure 4.1. The Columbia River Basin (CRB) in the United States Pacific Northwest and the geographic distributions of spring-run (a) and fall-run (b) Chinook salmon populations. Spring-run (stream-type) Chinook salmon migrate to interior reaches of the CRB and spawn in smaller order, tributary streams, whereas fall-run (ocean-type) Chinook salmon spawn in larger order, mainstem rivers. Spatial data set for Pacific Northwest Chinook salmon fish distributions (StreamNet GIS Data, 2019).

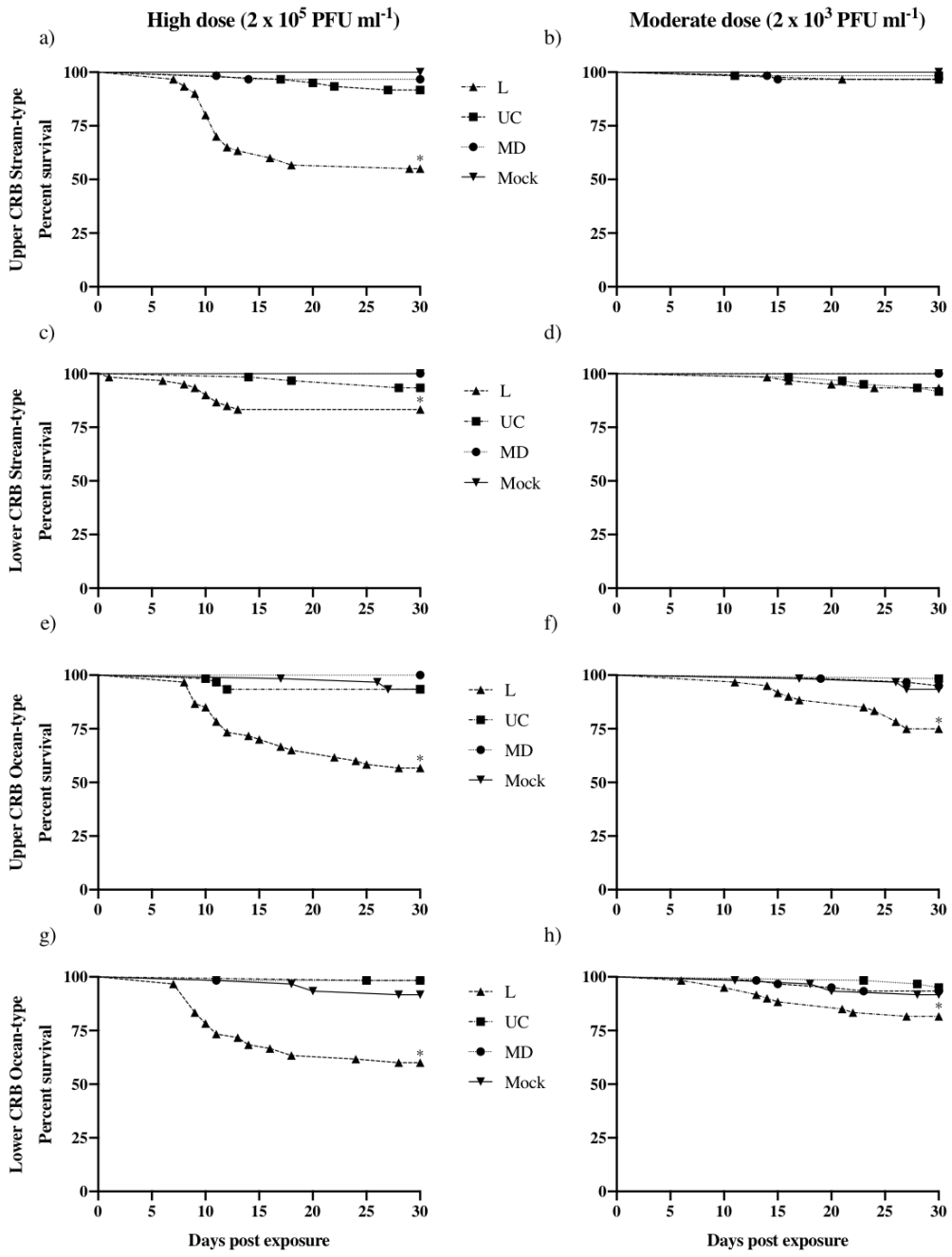


Figure 4.2. Daily cumulative percent survival of juvenile stream- (a-d) and ocean-type (e-h) Chinook salmon (*Oncorhynchus tshawytscha*) of the upper and lower Columbia River Basin (CRB) exposed to L, UC and MD strains of infectious hematopoietic necrosis virus (IHNV) or virus-free media (Mock). Experimental host populations were exposed to each virus strain at high (left) and moderate (right) doses by static immersion in 1 L. Survival curves were constructed by pooling data from triplicate groups of 20 fish per treatment. Asterisks indicate that fish in the positive control L genogroup (FR0031) IHNV treatment had significantly lower survival than fish in the other viral strain treatments ($P < 0.001$). No significant differences were observed between the MD and UC virus treatments within or across host populations.

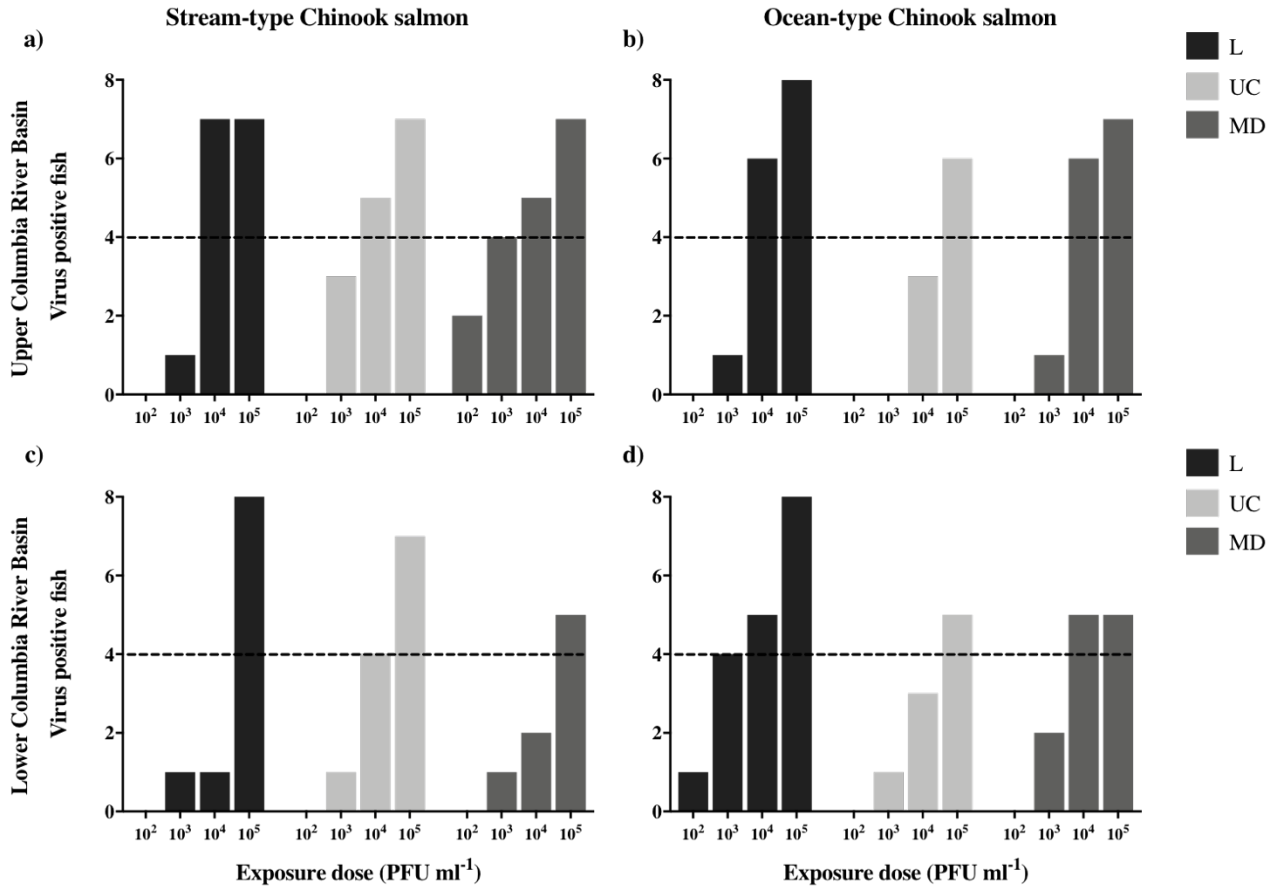


Figure 4.3. Virus positive stream- (a,c) and ocean-type (b,d) Chinook salmon of the upper (a, b) and lower (c, d) Columbia River Basin at 3 days post exposure to L, UC and MD strains of IHNV by static immersion. Exposure dosages of $2x10^2$ PFU ml^{-1} through $2x10^5$ PFU ml^{-1} are reported along *x*-axes as exponents of viral challenge concentrations. The *y*-axis indicates number of virus positive fish out of a total of 8 fish per treatment group, and dotted lines indicate 50% infection.

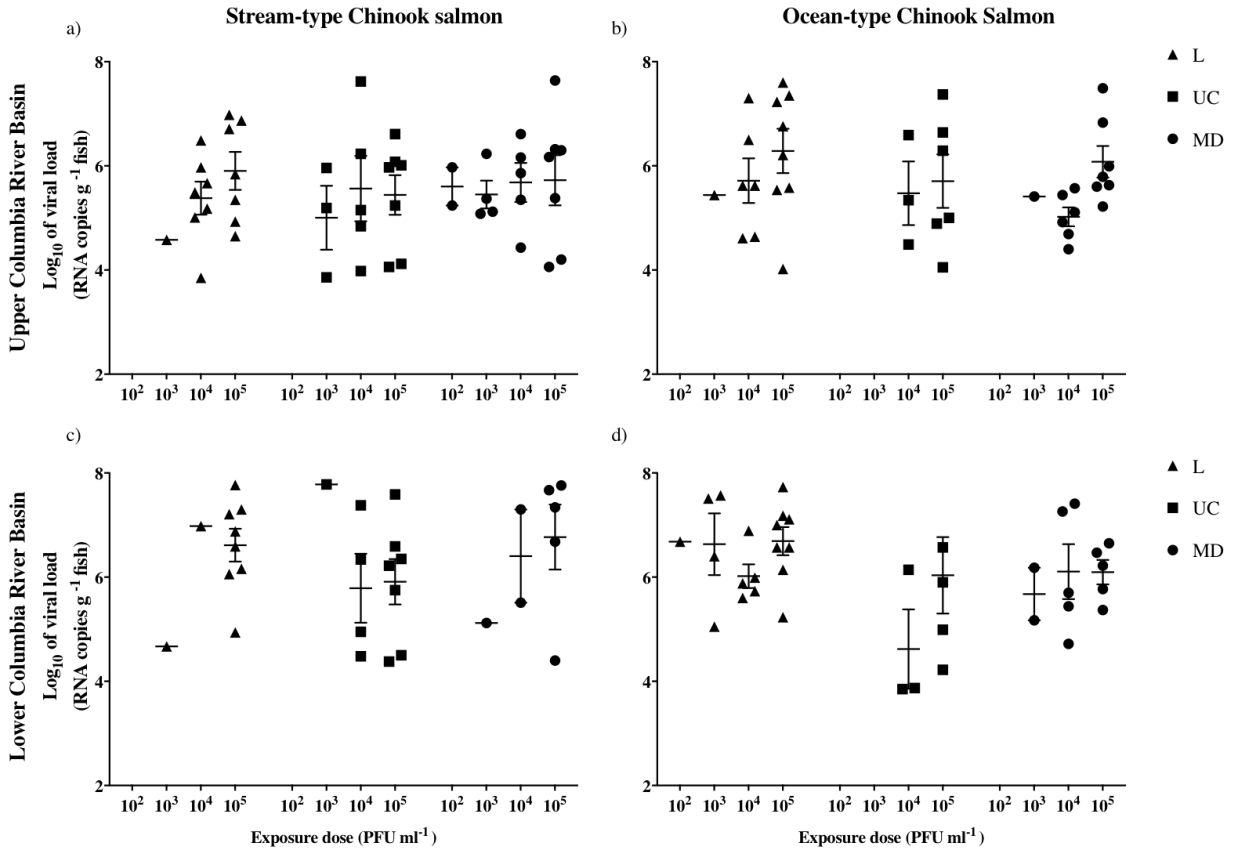


Figure 4.4. Viral load (log_{10} RNA copies g^{-1} of fish) of IHNV positive stream- (a,c) and ocean-type (b,d) Chinook salmon of the upper (a, b) and lower (c, d) Columbia River Basin at 3 days post exposure (dpe) to L, UC or MD strains of IHNV. Exposure dosages are reported along x-axes as exponents of viral challenge concentrations ($2x10^2$ - $2x10^5$ PFU ml^{-1}). Mean log_{10} viral loads (± SEM) are indicated for each group of virus positive fish. Quantities of IHN virus detected at 3 dpe are reported in Table 4.4.

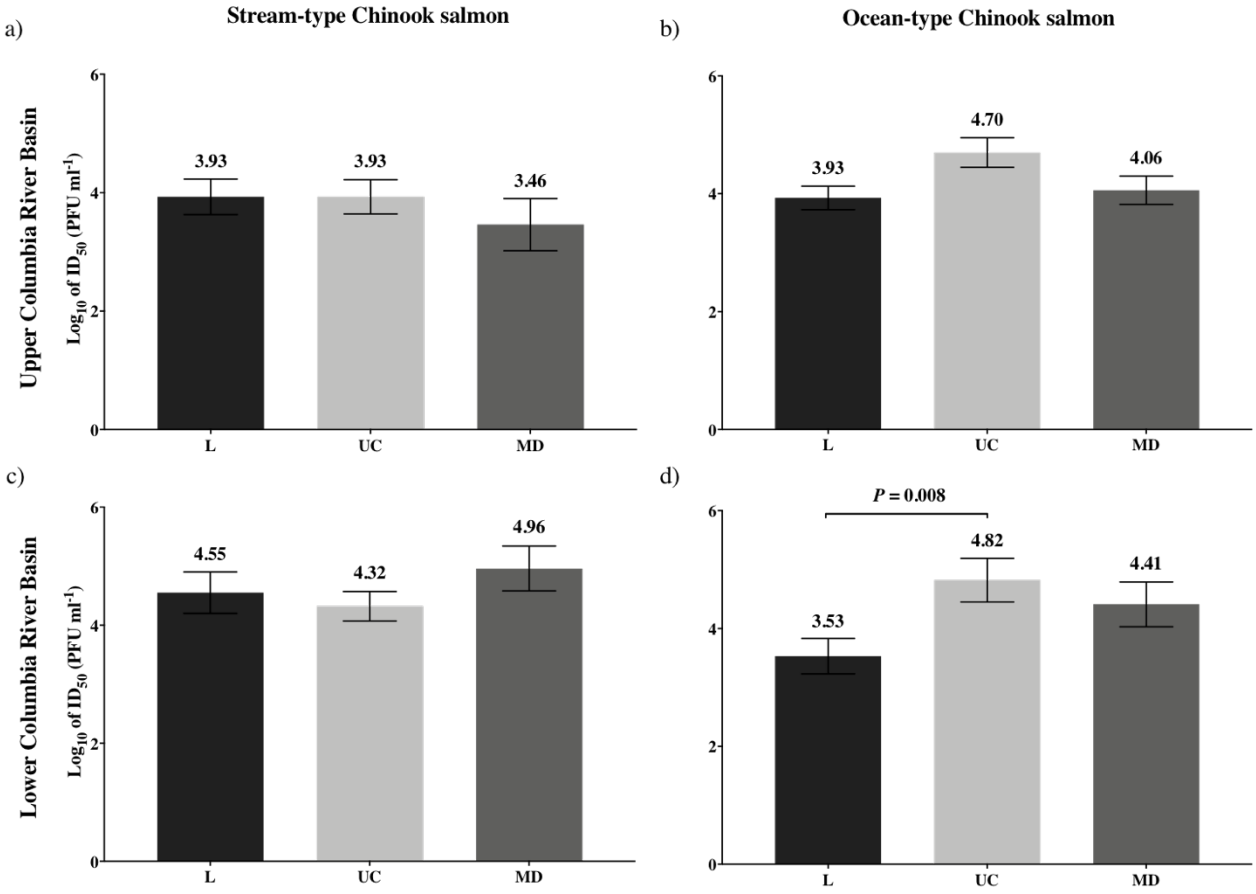


Figure 4.5. Estimates of viral dosage needed to infect 50 percent (ID_{50}) of juvenile Chinook salmon with L, UC, and MD strains of IHNV by batch immersion. ID_{50} estimates (log_{10} PFU ml^{-1}, ± SE) are reported for stream- (a,c) and ocean-type (b,d) Chinook salmon of the upper (a,b) and lower (c,d) Columbia River Basin at 3 days post exposure. Significant differences between the ID50 values generated are indicated as Welch-Satterthwaite 2-tailed t-test P values. To account for multiple pairwise comparisons, the 0.05 significance level was Bonferroni-corrected to $\alpha = 0.0166$.

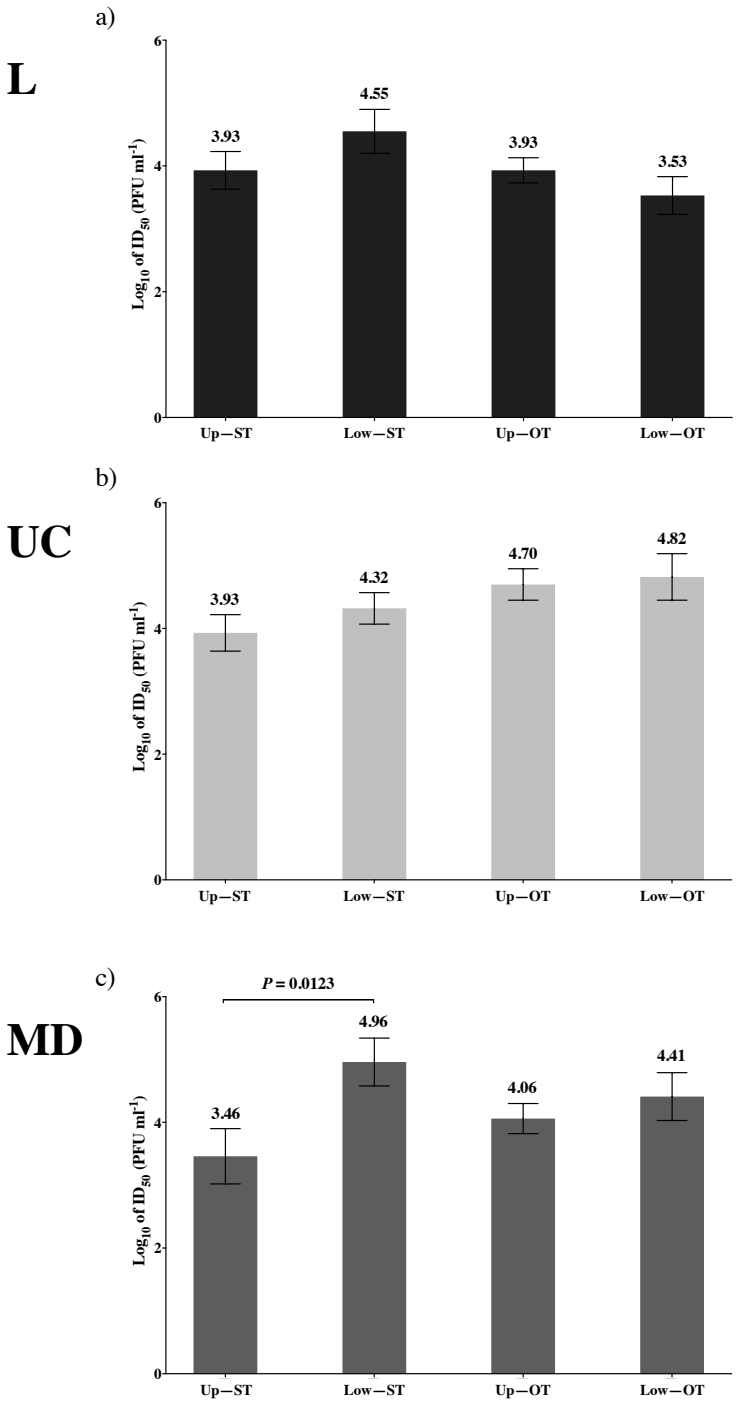


Figure 4.6. Comparison of ID_{50} values generated for the L (FR0031), UC (RB1) and MD (QTS07) strains of IHNV included in this investigation. ID_{50} values for the L- (a), UC- (b), and MD-IHNV (c) strains were assessed for differences in infectivity relative to experimental host population. Multiple pairwise comparisons of ID_{50} (log_{10} PFU ml^{-1}, ± SE) for stream-type (ST) and ocean-type (OT) *O. tshawytscha* of the upper (Up) and lower (Low) Columbia River were performed. Significant differences between ID_{50} values are reported as Welch-Satterthwaite 2-tailed t-test *P* values. To account for multiple pairwise comparisons, the 0.05 significance level was Bonferroni-corrected to $\alpha = 0.0083$.

4.9 LITERATURE CITED

ArcMap, E. S. R. I. Version 10.6.1. (2018). Redlands, California: ESRI.

Batts, W. N., & Winton, J. R. (1988). Enhancement of plaque assay detection of fish viruses by pretreatment of cells with polyethylene glycol. American Fisheries Society Fish Health Section Newsletter, 16(1), 4.

Bendorf, A. C. M. (2010). Phylogenetic analysis and virulence characteristics for Chinook salmon, rainbow trout, and steelhead of L genogroup infectious hematopoietic necrosis virus (IHNV) from the Feather River, California. University of California, Davis.

Black, A., Breyta, R., Bedford, T., & Kurath, G. (2016). Geography and host species shape the evolutionary dynamics of U genogroup infectious hematopoietic necrosis virus. Virus evolution, 2(2), vew034.

Bootland, L. M., & Leong, J. C. (2011). Infectious haematopoietic necrosis virus. Fish diseases and disorders, 3, 66-109.

Brannon, E. L., Powell, M. S., Quinn, T. P., & Talbot, A. (2004). Population structure of Columbia River Basin Chinook salmon and steelhead trout. Reviews in Fisheries Science, 12(2-3), 99-232.

Breyta, R., Jones, A., & Kurath, G. (2014). Differential susceptibility in steelhead trout populations to an emergent MD strain of infectious hematopoietic necrosis virus. Diseases of aquatic organisms, 112(1), 17-28.

Breyta, R., Samson, C., Blair, M., Black, A., & Kurath, G. (2016). Successful mitigation of viral disease based on a delayed exposure rearing strategy at a large-scale steelhead trout conservation hatchery. Aquaculture, 450, 213-224.

Breyta, R., Brito, I., Kurath, G., & LaDeau, S. (2017). Infectious hematopoietic necrosis virus virological and genetic surveillance 2000–2012. Ecology, 98(1), 283-283.

Columbia River DART Database (Data Access in Real Time), Columbia Basin Research. University of Washington, School of Aquatic & Fishery Sciences. Seattle (WA). Available at http://www.cbr.washington.edu/dart

Crawley, M. J. (2012). The R book. John Wiley & Sons.

Fleming, I. A., & Gross, M. R. (1994). Breeding competition in a Pacific salmon (coho: Oncorhynchus kisutch): measures of natural and sexual selection. Evolution, 48(3), 637-657.

Fulton, L. A. (1970). Spawning areas and abundance of steelhead trout and coho, sockeye and chum salmon in the Columbia River basin--past and present.

Garver, K. A., Troyer, R. M., & Kurath, G. (2003). Two distinct phylogenetic clades of infectious hematopoietic necrosis virus overlap within the Columbia River basin. Diseases of aquatic organisms, 55(3), 187-203.

Garver, K. A., Batts, W. N., & Kurath, G. (2006). Virulence comparisons of infectious hematopoietic necrosis virus U and M genogroups in sockeye salmon and rainbow trout. Journal of Aquatic Animal Health, 18(4), 232-243.

Healey, M. C. (1991). Life history of chinook salmon (*Oncorhynchus tshawytscha*). Pacific salmon life histories.

Hearsey, J. W., & Kinziger, A. P. (2015). Diversity in sympatric chinook salmon runs: timing, relative fat content and maturation. Environmental biology of fishes, 98(1), 413-423.

Hernandez, D. G., Purcell, M. K., Friedman, C. S., & Kurath, G. (2016). Susceptibility of ocean- and stream-type Chinook salmon to isolates of the L, U, and M genogroups of infectious hematopoietic necrosis virus (IHNV). Diseases of aquatic organisms, 121(1), 15-28.

Kurath, G., Garver, K. A., Troyer, R. M., Emmenegger, E. J., Einer-Jensen, K., & Anderson, E. D. (2003). Phylogeography of infectious haematopoietic necrosis virus in North America. Journal of General Virology, 84(4), 803-814.

Kurath, G. (2012). Fish novirhabdoviruses. In: Dietzgen RG, Kuzman IV, eds. Rhabdoviruses: Molecular taxonomy, Evolution, Genomics, Ecology, Host-Vector interactions, Cytopathology, and Control. Caister Academic Press; pp.89-117.

LaPatra, S. E. (1989). Strain differentiation and detection of infectious hematopoietic necrosis virus.

LaPatra, S. E., Fryer, J. L., & Rohovec, J. S. (1993). Virulence comparison of different electrophero-types of infectious hematopoietic necrosis virus. Diseases of Aquatic Organisms, 16(2), 115-120.

McKenney, D. G., Kurath, G., & Wargo, A. R. (2016). Characterization of infectious dose and lethal dose of two strains of infectious hematopoietic necrosis virus (IHNV). Virus research, 214, 80-89.

Myers, J. M. (1998). Status review of chinook salmon from Washington, Idaho, Oregon, and California.

Narum, S. R., Hess, J. E., & Matala, A. P. (2010). Examining genetic lineages of Chinook salmon in the Columbia River Basin. Transactions of the American Fisheries Society, 139(5), 1465-1477.

Penaranda, M. M. D., Purcell, M. K., & Kurath, G. (2009). Differential virulence mechanisms of infectious hematopoietic necrosis virus in rainbow trout (Oncorhynchus mykiss) include host entry and virus replication kinetics. Journal of General Virology, 90(9), 2172-2182.

Purcell, M. K., Thompson, R. L., Garver, K. A., Hawley, L. M., Batts, W. N., Sprague, L., ... & Winton, J. R. (2013). Universal reverse-transcriptase real-time PCR for infectious hematopoietic necrosis virus (IHNV). Diseases of Aquatic Organisms, 106(2), 103-115.

R Core Team (2013). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Available at http://www.R-project.org/.

StreamNet GIS Data (2017). Columbia River Watershed Boundary spatial data set. Portland (OR): StreamNet, March 2019. Available at https://www.streamnet.org/data/interactive-maps-and-gis-data/

StreamNet GIS Data (2019). Metadata for Pacific Northwest Chinook salmon fish distribution spatial data set. Portland (OR): StreamNet, March 2019. Available at https://www.streamnet.org/data/interactive-maps-and-gis-data/

Taylor, E. B. (1990). Phenotypic correlates of life-history variation in juvenile chinook salmon, *Oncorhynchus tshawytscha*. The Journal of Animal Ecology, 455-468.

Venables, W. N., & Ripley, B. D. (2013). Modern applied statistics with S-PLUS. Springer Science & Business Media.

Waples, R. S., Teel, D. J., Myers, J. M., & Marshall, A. R. (2004). Life-history divergence in Chinook salmon: historic contingency and parallel evolution. Evolution, 58(2), 386-403.

Waples, R. S., Pess, G. R., & Beechie, T. (2008). Evolutionary history of Pacific salmon in dynamic environments. Evolutionary Applications, 1(2), 189-206.

Wargo, A. R., Garver, K. A., & Kurath, G. (2010). Virulence correlates with fitness in vivo for two M group genotypes of Infectious hematopoietic necrosis virus (IHNV). Virology, 404(1), 51-58.

Wertheimer, A. C., & Winton, J. R. (1982). Differences in susceptibility among three stocks of chinook salmon, *Oncorhynchus tshawytscha*, to two isolates of infectious hematopoietic necrosis virus.

Winans, G. A. (1989). Genetic variability in Chinook salmon stocks from the Columbia River basin. North American Journal of Fisheries Management, 9(1), 47-52.

Workenhe, S. T., Rise, M. L., Kibenge, M. J., & Kibenge, F. S. (2010). The fight between the teleost fish immune response and aquatic viruses. Molecular immunology, 47(16), 2525-2536.

Wydoski, R. S., & Whitney, R. R. (2003). Inland fishes of Washington. American Fisheries Society.

World Organisation for Animal Health (2018). Aquatic Animal Health Code, Chapter 1.3, article 1.3.1, http://www.oie.int/index.php?id=171&L=0&htmfile=chapitre_diseases_listed.htm. World Organisation for Animal Health, Paris, France.

Chapter 5. SHEDDING KINETICS OF INFECTIOUS HEMATOPOIETIC NECROSIS VIRUS (IHNV) IN JUVENILE STREAM- AND OCEAN-TYPE CHINOOK SALMON OF THE COLUMBIA RIVER BASIN

5.1 ABSTRACT

This investigation sought to characterize the shedding of UC, MD and L genogroup IHN viruses in diverse populations of Columbia River Basin (CRB) Chinook salmon (*Oncorhynchus tshawytscha*). Juvenile ocean- and stream-type Chinook salmon were individually monitored for viral shedding following exposure to each strain of IHN virus. The kinetics of UC, MD and L virus shedding were determined for sub-groups of ten individual fish over the course of thirty days. Peaks in viral shedding and the overall quantities of virus shed were used to characterize differences in the shedding of UC, MD and L viruses between host populations. Survival curves were constructed for each Chinook salmon population and assessed to determine host mortality relative to the shedding kinetics of IHNV. Significant quantities of UC, MD and L IHN virus were shed by fish of each population. Viral shedding was observed to peak in all groups at 2-3 days following exposure to IHNV and declined to undetectable levels by 7 days. Viral treatment was observed to influence the total number of fish shedding and overall quantities of virus shed, however, host population also appeared to have an influence on the extent of virus shedding. Higher numbers of stream-type Chinook salmon shed UC, MD and L IHN virus than ocean-type fish. Most notably, six out of ten stream-type fish shed UC virus, relative to a single ocean-type fish. While the overall total quantities of MD and L IHN virus were comparable between the two host types, stream-type fish were observed to shed greater total quantities of UC virus. All viral

shedding occurred well before host mortality began. Together, results of this investigation showed juvenile Chinook salmon of the CRB can shed measurable quantities of UC and MD IHNV into the environment. A strong correlation between the magnitude and duration of IHNV shedding showed that with each consecutive day a juvenile fish sheds, the higher its overall transmission potential is. Results of this investigation showed that stream-type Chinook salmon of the CRB are more effective in shedding UC and MD IHN virus than ocean-type populations. To date, no studies have quantified the shedding of UC, MD and L IHNV in Chinook salmon. Intraspecific variation in the shedding of UC IHNV between Chinook salmon of differing life histories provide evidence for variation in the transmission potential of IHNV between stream-type versus ocean-type Chinook salmon of the CRB. Altogether, these data further our understanding of how IHNV may be transmitted and maintained in the complex Columbia River watershed.

5.2 INTRODUCTION

Infectious hematopoietic necrosis virus (IHNV) is an enveloped, single-stranded, negative-sense RNA virus that belongs to the taxonomic family *Rhabdoviridae* and is the type species of the genus *Novirhabdovirus* (species *Salmonid novirhabdovirus*). IHNV causes acute, systemic disease in Pacific salmon and trout populations (genus *Oncorhynchus*) and is the most economically significant viral pathogen affecting salmonid conservation hatchery programs and rainbow trout aquaculture (Bootland and Leong, 2011). Genetic sequencing of a variable region of the IHNV glycoprotein gene has defined three major genetic lineages or genogroups, designated U (upper), M (middle), and L (lower) for their relative geographic distributions across North America (Garver et al. 2003, Kurath et al. 2003). Field observations and controlled laboratory studies have shown distinct patterns of host specific virulence to occur with viruses belonging to each IHNV genogroup. U genogroup viruses, which comprise the largest geographic range of

IHNV, are highly virulent in juvenile sockeye salmon (*O. nerka*). M genogroup viruses, which have a smaller range and are detected predominately in the Columbia River Basin (CRB), are highly virulent in juvenile rainbow trout and anadromous steelhead (two life history forms of *O. mykiss*). Finally, L genogroup viruses, constituting the smallest and southernmost extent of IHNV, are highly virulent in juvenile Chinook salmon (*O. tshawytscha*). While U, M, and L IHN viruses appear to function largely as specialists, adapted to a single host species, a major exception is observed in the CRB.

The CRB, which spans portions of the US states of Washington, Oregon, Idaho, Montana, Wyoming, Utah, and Nevada, and the Canadian province of British Columbia, supports all three major IHNV host species (Breyta et al. 2017). Across the CRB, viruses from both the U and M genogroups are sympatric throughout much of the watershed (Garver et al. 2003; Breyta et al. 2016). The majority of M viruses detected in the CRB belong to the MD subgroup of the M genogroup and occur as specialists in steelhead and rainbow trout, with only a low frequency of detections in other host species (Breyta et al. 2016). In contrast, U viruses in the CRB are detected frequently in both Chinook salmon and steelhead trout, as well as in small numbers of sockeye salmon (Breyta et al. 2016). Outside of the CRB, U genogroup viruses occur as sockeye salmon specialists, rarely detected in sympatric populations of Chinook salmon or steelhead trout. The generalist host pattern associated with U genogroup viruses in the CRB was shown to comprise a novel U subgroup that has evolved in the Columbia River watershed and was designated UC (Black et al. 2016).

The evolution of viral pathogens is intrinsically owed to their short generation times, considerable replication numbers, and high mutation rates. However, as obligate intracellular parasites, viruses are maintained and evolve in accordance with their need to infect a new host or

vector. How an infected host controls or eliminates a viral infection heavily influences the effective rate with which a virus can infect another competent host (Purcell et al. 2009). As a result, for a viral pathogen to persist on a landscape scale, it must overcome formidable host-related constraints. While overcoming a host's antiviral immune response is a significant challenge faced by viruses (Oldstone 2006), the spatial and temporal distributions of new competent hosts impart a different selective pressure on virus evolution (Elena et al. 2009). It is unclear what selective pressures have led to the varying degrees of specialism and generalism that have evolved across the major genetic lineages of IHNV. However, the unique host association of UC viruses with Chinook salmon and steelhead trout populations raise questions about how the virus is maintained in the vast, multi-host CRB. Horizontal transmission of IHNV between host species is hypothesized as a driver of virus persistence in the CRB (Ferguson et al. 2018). While viral shedding is thought to dictate transmission of the virus, there are few published studies of IHNV viral shedding kinetics. Wargo et al. (2017) is the only controlled laboratory study that has assessed in vivo shedding kinetics of IHNV from individual fish, using M genogroup viruses in juvenile rainbow trout. Chinook salmon populations of the CRB are genetically diverse with expressed phenotypic differences in behavioral patterns, life histories, and geographic distributions (Waples et al. 2008). In Chinook salmon, two juvenile life history patterns have been defined (Gilbert 1913; Healey et al. 1991). Stream-type Chinook salmon reside in rivers for a full year prior to seaward migration, whereas ocean-type migrate to the ocean within their first year of life. These juvenile life history types are linked to a suite of environmental conditions affecting growth rate, and in many cases they co-vary with adult return migration timing and the spatial distribution of spawning (Brannon et al. 2004; Taylor 1990).

To date, no studies have quantified the shedding of IHNV in Chinook salmon, and no studies have compared shedding of UC, MD and L IHNV. This investigation aimed to characterize the shedding kinetics of IHNV in CRB Chinook salmon to evaluate the transmission potential of UC and MD virus by subclinically infected fish, in comparison with L virus as a positive control. Here, two populations of CRB Chinook salmon, representing distinct life history types, were exposed to high concentrations of UC, MD, and L IHNV by batch immersion. Following exposure to each IHNV strain, a subset of fish from each viral treatment group were isolated and individually followed for thirty days to quantify daily viral shedding.

5.3 METHODS

5.3.1 *Ethics statement*

All animal experiments were approved by the University of Washington Institutional Animal Care and Use Committee (UW IACUC 3042-12).

5.3.2 *Experimental host populations*

Chinook salmon of the Winthrop National Fish Hatchery (United States Fish and Wildlife Service) were selected as upper CRB stream-type fish (Table 5.1), whereas Chinook salmon of the Cowlitz Hatchery (Washington Department of Fish and Wildlife) were selected as lower CRB ocean-type population fish (Narum et al. 2010). A total of 2000 eyed eggs (developing embryo), sourced from a minimum of 12 parental spawning pairs, were obtained from each population. All Chinook salmon eggs were incubated and hatched at the USGS Western Fisheries Research Center (WFRC) laboratory in Seattle, WA where they were reared to approximately 1 g at a constant temperature of 10°C. Juvenile Chinook salmon were fed a semi-moist pellet diet (BioOregon) at a rate of 1.0 - 2.0% body weight per day. All fish rearing and experimental exposures were

conducted at the USGS WFRC using single-pass, flow-through, sand-filtered and UV-treated freshwater from Lake Washington.

5.3.3 *Experimental virus strains*

Three viral strains, representing the U, M and L genogroups of IHNV, were included in this investigation (Table 2). Strain RB1, representing the UC subgroup, is virulent in sockeye salmon (Garver et al. 2006), and has the most common UC sequence type detected in the CRB (type mG001U) (Garver et al. 2003; Breyta et al. 2016). Strain QTS07, representing the MD subgroup, has high virulence in steelhead trout (Breyta et al. 2014) and was selected because it has the most common MD IHNV sequence type (type mG110M) detected throughout the CRB (Breyta et al. 2016). Although L genogroup IHNV does not occur in the CRB, the California L genogroup strain FR0031 was included as a positive control known to have high virulence and infectivity in juvenile Chinook salmon (Bendorf 2010; Hernandez et al. 2016).

5.3.4 *Virus exposures*

All viral challenges were conducted at a constant water temperature of 10°C to mimic general conditions observed throughout the CRB (Brannon et al. 2004). Controlled laboratory challenges were performed on juvenile Chinook salmon at an average weight of 1 g. To assess the shedding kinetics of IHNV in stream- and ocean-type Chinook salmon, each host population was exposed to L, UC and MD genogroups strains of IHNV. Thirty fish were exposed to each viral strain or a virus-free media treatment (negative control) by static immersion for 1 hr in 1 L of water containing virus at dose 2×10^5 plaque forming units (PFU) ml^{-1}. Post immersion challenge, flow-through water was resumed and fish were rinsed in total volumes of 5 L for 1 hr. Following this rinse, 10 fish from each viral treatment (6 from the virus-free negative control) were placed into

individual 1.5 L tanks in a tower rack system (Aquatic Ecosystems) with water flowing to each tank at approximately 200 ml min^{-1}. A 1.4 ml sample of water was collected from each tank immediately after fish distribution. Water flow was then turned off for 23 hr to allow for accumulation of shed virus. A constant temperature of 10°C was maintained by circulating temperature-controlled water around the tanks. Following the 23 hr static hold, water was sampled from each tank after which flow was returned for 1 hr. This cycle of static periods was repeated daily and samples were collected 0, 1, 2, 3, 4, 5, 7, 8, 10, 14, 22 and 30 days post exposure (dpe). Individual water samples were stored in 2 ml graduated clear tubes (BioExpress) at -80°C for later RNA extraction and virus detection and quantification of viral RNA.

After the initial (time zero) sample was collected, the remaining 4 of the 10 total fish removed from the virus-free treatment group were euthanized using buffered Tricaine Methanesulfonate (Western Chemical, Inc.) at a concentration of 240 mg L^{-1}. The remaining 20 fish from each treatment group were held in batch and monitored daily over the course of the 30-day experiment for mortality. Kinetics of daily cumulative percent survival (CPS) were used to construct survival curves for each experimental host population following exposure to L, UC and MD strains of IHNV as described below. At the end of the 30-day observation period, all surviving fish were euthanized using buffered Tricaine Methanesulfonate, as described above.

5.3.5 *RNA extraction and cDNA synthesis*

Viral RNA was extracted from each individual water sample using the QIAamp® cador® Pathogen Mini Kit (QIAGEN®). Briefly, 200 µl of fluid sample were combined with 20 µl of proteinase K and 100 µl of Buffer VXL (QIAGEN®) and mixed by trituration. Following a 15 min incubation at 20-25 °C, 250 µl of Buffer ACB (QIAGEN®) was added to each sample and mixed thoroughly by pulse-vortexing. The lysate was then transferred to a QIAamp® Mini column

placed inside of a 2 ml collection tube and centrifuged at 6000 x g. The QIAamp® Mini column was treated and centrifuged sequentially with 600 µl of Buffer AW1 and AW2, before adding 100 µl of Buffer AVE to the center of the membrane and centrifuged at 20,000 x g (QIAGEN®). Viral RNA extracted from each water sample was assessed for quality and concentration by spectrophotometry before complementary DNA (cDNA) was synthesized using M-MLV reverse transcriptase with random hexamer primers (Wargo et al. 2010). A standard volume of 5 µl of RNA was used in each cDNA reaction and the final 20 µl of cDNA was diluted 1:2 by adding 20 µl of RNase-free water. Newly synthesized cDNA was stored at -80°C for polymerase chain reaction (PCR) analysis.

5.3.6 *Viral RNA quantification via reverse transcriptase real-time PCR*

Viral RNA was quantified using the universal IHNV N gene reverse transcriptase real-time PCR (RT-rPCR) assay as previously described (Purcell et al. 2013). Briefly, 5 µl of each diluted cDNA sample was combined with forward and reverse primers, TaqMan® FAM-labeled probe for the IHNV N gene, VIC® - labeled probe for the artificial positive control (APC) and amplified on an Applied Biosystems ViiA7™ real-time PCR machine. APC plasmid DNA was linearized and used to construct a standard curve (5×10^7 to 5 DNA copies) with which to quantify the absolute copy number of viral RNA (Purcell et al. 2013). Each sample was run in duplicate wells and interpreted as positive only when amplification was detected in both replicates within 40 cycles. Samples whose duplicate well results were not in consensus were considered suspect, requiring further analysis. For suspect samples, new cDNA was synthesized from RNA and assayed, once more, with the universal IHNV N gene RT-rPCR assay in 4 independent qPCR reactions, each in one well. Suspect samples whose secondary RT-rPCR assays resulted in 2 or more positive replicate wells, were confirmed positive and thereby included in data analysis as positive samples.

Suspect samples whose secondary RT-rPCR assays resulted in 1 or less positive replicate wells, were confirmed negative and not included in analyses of viral RNA quantities shed.

The analytical sensitivity of the IHNV RT-rPCR assay was determined based on the PCR efficiencies observed for the APC plasmid DNA standard curves for all assays included in this analysis (Purcell et al. 2013). The reaction efficiencies varied from −3.46 to −3.64 and the y-intercept values varied from 40.0 to 41.5, indicating similar limits of detection across the IHNV RT-rPCR assays. The detection limit of the IHNV N gene RT-rPCR assay was 566.9 viral RNA copies per ml of H_2O (2.75 $\log_{10}$ RNA copies ml^{-1}), referred to hereafter as viral load.

5.3.7 *Statistical analyses*

Viral shedding kinetics

Data generated from these controlled laboratory exposures were used to test hypotheses specific to IHNV shedding kinetics in CRB Chinook salmon. In this investigation, measures of mean viral shedding were compared between experimental groups under the null hypothesis of equality. One-way Analysis of Variance (ANOVA) was the analytical technique used to test for the equality of means, where rejection of the null hypothesis was based on the calculated F statistic being equal to or greater than the critical value (Zar 2010). Tukey's Honestly Significant Difference (HSD) a posteriori test for multiple comparisons was used to conclude between what means inequalities lie. All statistical analyses were conducted in GraphPad Prism®, version 8.1.0 (GraphPad Software, Inc.). From the host perspective, One-way ANOVA was robust in concluding whether host population had an effect on the shedding kinetics of IHNV. From the virus perspective, hypothesis testing was robust in determining whether virus strains had the same effect on viral shedding.

Survival of experimental host populations

To assess the influence of viral treatment on host survival, survival analysis was conducted using the Kaplan-Meyer method. Briefly, experimental host populations were monitored for mortality over the course of 30 days. Twenty fish from each treatment group were held together following immersion challenge. Survival curves were constructed for each group of twenty Chinook salmon held together and those fish individually monitored for viral shedding. Survival curves were compared between viral treatment groups, within and across host types, using a log-rank (Mantel-Cox) test (GraphPad Prism®, version 8.1.0).

5.4 RESULTS

5.4.1 *Viral shedding in stream- and ocean-type Chinook salmon*

Juvenile stream- and ocean-type Chinook salmon each shed detectible quantities of L, UC and MD IHN virus, with individual fish shedding kinetics illustrated in Fig. 5.1. Over the course of the 30-day observation period, no detectible virus was shed in mock-exposed fish (virus-free treatment) at any time point sampled. Across all 60 virus-exposed fish, 34 shed detectable virus over the 30-day course of infection. Among subgroups of ten stream-type fish a total of nine shed L virus, six shed UC virus, and seven shed MD virus at some point during the experiment. For ocean-type fish a total of seven, one and four fish shed L, UC and MD virus, respectively.

With regard to kinetics, no detectible virus was shed at time zero or 24 hr following exposure. Viral shedding was first detected 48 hr (2 days) following exposure, and was last detected 5 dpe. No viral shedding was detected in any treatment group at any time point sampled afterwards on days 7, 8, 10, 14, 22, and 30. The total number of days an individual fish shed detectable virus ranged between 1 and 5 days, with an overall mean of 2.24 days shedding.

The total numbers of fish shedding L, UC and MD virus were higher in stream-type Chinook salmon relative to ocean-type fish (Fig. 5.3a). Most notably, far more stream-type fish shed UC

virus relative to ocean-type fish. Over the 30-day course of infection, a total of one ocean-type fish shed UC virus, for only one day. Overall, the total quantities of L and MD virus shed by each treatment group of 10 virus-exposed fish were equivalent between stream- and ocean-type Chinook salmon (Fig. 5.3b). Consistent with the total numbers of fish shedding UC virus, stream-type Chinook salmon shed a greater total quantity of UC virus relative to ocean-type fish. Within stream-type Chinook salmon, fewer fish were detected shedding UC virus compared to the L virus. However, far more UC virus was shed than the L virus. Over the 30-day course of infection, stream-type Chinook salmon shed approximately 1 log more UC virus than the L virus (Fig. 5.3b).

5.4.2 *Viral shedding kinetics of individual fish*

For each of the 34 fish that shed detectible virus over the 30-day course of infection, the day and quantity of peak viral shedding was determined by the water sampling time point showing the highest viral load for each fish (Fig. 5.4). Across all virus-exposed fish, shedding increased rapidly, with the peak amount of shed virus occurring between days 2–3 in 88% of fish (44% day 2, 44% day 3). Viral shedding began to decrease from day 3 forward, such that by day 7, no fish shed detectible virus. There was no significant difference in the mean day of peak shedding between IHNV genogroup treatments within or across host populations (ANOVA, $P > 0.05$) (Fig. 5.4a). Assessment of the mean quantity of peak L, UC and MD virus shed (mean log virus RNA copies ml^{-1}) in all stream- and ocean-type Chinook salmon also showed no significant differences within or across host populations (ANOVA, $P > 0.05$) (Fig. 5.4b). The total quantities of L, UC and MD virus shed by individual juvenile stream- and ocean-type Chinook salmon over the 30-day course of infection are illustrated in Fig. 5.5. Assessment of the mean total quantities of each virus type shed by individual fish showed no significant differences within and across host

populations (ANOVA, $P > 0.05$) (Fig. 5.5). UC virus shed from ocean-type fish was excluded from this analysis because no mean could be calculated from one fish.

5.4.3 *Mean viral shedding kinetics*

Daily mean log transformed quantities of L, UC and MD IHN virus shed in stream- and ocean-type Chinook salmon of the CRB are illustrated in Fig. 5.6. Mean quantities of virus shed (log_{10} RNA copies ml^{-1} H_2O) were calculated from all fish that shed detectible virus daily within each treatment group over the 30-day course of infection. Across all viral treatments, shedding of L, UC and MD virus increased rapidly, reaching the highest observed quantities between days 2–3, and decreasing thereafter. Within and across host populations, the shedding kinetics of the L and MD virus were comparable over time. Within stream-type Chinook salmon, shedding of the UC virus was consistently 1 log higher over time than observed for the L and MD viruses (Fig. 5.6a). In contrast, within ocean-type fish the single fish that shed UC virus had a much lower peak magnitude than the L or MD shedding peaks. Between the stream- and ocean-type Chinook salmon, the UC virus was shed by greater numbers of stream-type fish over time, and in much higher mean quantities. Overall, stream-type Chinook salmon shedding UC virus represent the greatest amount of virus shed consistently over time.

The total numbers of fish shedding between days 2–5 in each viral treatment group (L, UC and MD IHNV) were analyzed using Fisher exact tests on a contingency table of the sum of the total numbers of fish shedding detectable virus on each day versus the total number of fish tested for the four days (n = 40 per each treatment). This test was performed separately for each host population between viral treatments, constituting 6 tests. This was also performed separately for each viral treatment between host populations, constituting 3 additional tests. To account for multiple pairwise comparisons, a Bonferroni correction of the 0.05 significance level was

performed by dividing it by the total number of comparisons (0.05/9) resulting in an $\alpha = 0.005$. Assessment of the total numbers of fish shedding L, UC and MD virus daily in stream-type Chinook salmon indicate no significant differences between viral treatments ($P > 0.005$). Within ocean-type Chinook salmon, the total numbers of fish shedding L and MD virus were not significantly different ($P > 0.005$). Similarly, total numbers of ocean-type fish shedding UC and MD virus were not significantly different ($P > 0.005$). The total number of ocean-type Chinook salmon shedding UC virus daily (1 out of 40) was significantly different than the total numbers of fish that shed L virus (14 out of 40) ($P = 0.0003$). Assessment of the total numbers of stream- and ocean-type Chinook salmon shedding L virus showed no significant difference ($P > 0.005$) between the populations. Similarly, the total numbers of stream- and ocean-type shedding MD virus were not significantly different ($P > 0.005$). Comparison of the total numbers of stream- and ocean-type fish shedding UC virus (17/40 and 1/40, respectively) were significantly different between the two populations ($P = 0.0001$).

To evaluate the transmission potential of IHNV in individual CRB Chinook salmon, the magnitude and duration of viral shedding was quantified for each fish in each host population. For each viral strain, the mean log total virus shed ($\log_{10}$ total viral RNA copies ml^{-1}) was calculated for all fish that shed detectable virus within each treatment group over the 30-day course of infection. One-way ANOVA analysis revealed significant differences between the means tested ($P < 0.0001$), while Tukey's (HSD) a posteriori test for multiple comparisons showed what population means differed (Fig. 5.7a). Within the ocean-type population, the mean total quantities of L, UC and MD IHN virus shed did not differ ($P > 0.05$). Within the stream-type population, the mean $\log_{10}$ total quantity of UC virus shed was significantly higher ($P = 0.00012$) than the mean $\log_{10}$ total quantity of L virus shed. Further, the mean $\log_{10}$ total quantity of UC virus shed in the

stream-type population was significantly higher than the mean $\log_{10}$ total quantities of L (P = 0.0014) and MD IHN virus (P = 0.0002) shed in ocean-type fish. Again, UC virus shed from ocean-type fish was excluded from this analysis, due to the inability to quantify variation in either measures from a single fish. Assessment of the mean number of days each virus strain was shed did not differ within or across host populations (ANOVA, $P > 0.05$) (Fig. 5.7b).

5.4.4 *Correlates of viral transmission*

Viral shedding has been described as a correlate of virus transmission, such that viruses which shed more infectious particles are predicted to have higher transmission rates (Wargo et al. 2017). In this investigation, two Chinook salmon populations were capable of shedding measurable quantities of L, UC and MD IHNV. Here, Pearson correlation analysis was used to characterize the relationship between the magnitude and duration of IHNV shedding in juvenile Chinook salmon. Sum total quantities of virus shed by individual fish ($\log_{10}$ virus RNA copies ml^{-1}) and the total number of days each fish shed virus were assessed for correlation, irrespective of virus strain and host population. The Pearson r coefficient reported (r = 0.741) indicated that a statistically significant correlation ($P < 0.0001$) exists between the total quantity of virus individual Chinook salmon shed and the total number of days a fish sheds (Fig. 5.8).

5.4.5 *Survival of experimental host populations*

Following exposure to L, UC and MD strains of IHNV by immersion challenge, juvenile stream- and ocean-type Chinook salmon were monitored for mortality over the course of 30 days. Twenty fish from each treatment group remained together post viral exposure to provide an indication of batch mortality, whereas ten of the initial thirty fish exposed (6 mock-exposed fish) were isolated to quantify the viral shedding of each individual as described above. Thus, survival

curves were constructed for each group of twenty fish per viral treatment and those fish individually monitored for viral shedding (Fig. 5.9). No mortality was observed in any mock-exposed fish (virus-free treatment). The positive control L IHNV treatment had significantly lower survival than batch held fish in the other viral treatments ($P < 0.001$), with mortality beginning on days 8-10 and occurring more rapidly in stream-type than ocean-type fish, reaching approximately 40% mortality in each host type by 30 dpe. No significant reductions in survival were observed in batch held fish exposed to the UC and MD virus treatments. Across fish held in isolation, mortality occurred as early as 12 dpe and as late as 30 dpe, ranging between 10% and 40% of fish in a given virus treatment group, comprised of a total of 10 fish per treatment. While ocean-type fish in isolation had more mortality events than stream-type fish in isolation, no statistically significant reductions in survival were observed within or across host populations held in isolation following exposure to L, UC and MD virus treatments. Together, all viral shedding occurred well before host mortality was observed in fish in isolation.

5.5 DISCUSSION

Viral pathogens can become highly specialized to infect a single host type in environments with homogenous host assemblages. However, multi-host landscapes provide an opportunity for viruses to evolve differentially across multiple hosts types (Elena et al. 2009). In the US Pacific Northwest, the aquatic viral pathogen IHNV is observed to infect multiple host species with differing levels of virulence. While host susceptibility to mortality with IHNV has been shown to differ relative to the innate immune response of an infected host (Purcell et al. 2009), an outstanding question is: Do hosts that successfully control or eliminate IHNV infections shed virus? This investigation sought to address this question with regard to the unique field occurrence patterns of IHNV in CRB Chinook salmon.

Across the CRB, IHNV is observed to infect multiple populations of Chinook salmon with high prevalence (Breyta et al. 2017). Consistent with field occurrence patterns, controlled laboratory studies examining the virulence and infectivity of UC and MD virus strains in four genetically distinct populations of CRB Chinook salmon showed high prevalence of IHNV infection with UC and MD virus strains and low levels of mortality (Chapter 4). While such observations suggest that Chinook salmon may be serving as a vector or reservoir of IHNV in the CRB, no study has tested this hypothesis directly. In this investigation, we assessed various aspects of IHNV shedding in juvenile Chinook salmon. The overarching goal of this investigation was to quantify the shedding of L, UC and MD IHN virus in CRB Chinook salmon and characterize the shedding kinetics of each virus type. Here, the two populations of CRB Chinook salmon tested were each capable of shedding detectible quantities of L, UC and MD IHN virus. Detectible virus shedding began 48 hr (2 days) after exposure. Viral shedding peaked 2–3 days after exposure, decreasing thereafter, and no longer detected by day 7. All viral shedding occurred well before host mortality began, with mortality ranging between 10% and 40% of fish in a given virus treatment group (n = 10). Together, these data showed that juvenile Chinook salmon of the CRB can shed detectible quantities of UC, MD and L IHN virus early in infection. The strong correlation between the magnitude and duration of IHNV shedding (r = 0.741) showed that with each consecutive day a juvenile fish sheds, the higher its overall transmission potential.

5.5.1 *Intraspecific variation in host shedding of UC and MD IHNV*

In this investigation, an L genogroup IHN virus strain was included as a positive control for its high virulence and infectivity in juvenile Chinook salmon (Bendorf 2010; Hernandez et al. 2016; Chapter 4). While L genogroup IHNV has never been detected in the CRB, previous controlled laboratory studies have shown the FR0031 L virus strain to infect and cause mortality

in juvenile stream- and ocean-type Chinook salmon populations of the CRB (Garver et al. 2005, IHNV DNA vaccine in Chinook salmon; Hernandez et al. 2016; Chapter 4). Here, the total quantities of L and MD IHN virus shed per population were equivalent between the stream- and ocean-type Chinook salmon tested. In contrast to the total virus shed by each population, statistical comparison of the mean total quantities for those fish that shed L and MD virus were significantly higher in stream-type Chinook salmon than in ocean-type fish. Further, in the stream-type population, the total quantity of UC virus shed was significantly higher than detected for the L genogroup IHN virus strain.

Overall, higher total numbers of stream-type Chinook salmon shed L, UC and MD IHN viruses relative to ocean-type fish. Most notable was the contrast between the number of stream- versus ocean-type Chinook salmon that shed detectable UC virus. A total of six stream-type Chinook salmon shed UC virus over the 30-day course of infection, whereas only one ocean-type fish was observed to shed the UC virus. Statistical comparison of the total number of stream-type Chinook salmon shedding UC virus was significantly higher than the total number of ocean-type fish shedding UC virus ($P = 0.0001$). This observation was consistent with the greater total quantity of UC virus shed in stream-type Chinook salmon, relative to the ocean-type population. Over the 30-day course of infection, stream-type Chinook salmon consistently shed approximately 1 log more UC virus than the L and MD virus. Although, no conclusion can be drawn by comparing only two host populations, our results suggest that stream-type fish may have greater shedding capability for UC virus than ocean-type fish. Further testing with additional populations would be needed to confirm whether this is consistently correlated with life history phenotype, or if it is due to some other attribute of the populations tested here (e.g. upper versus lower CRB).

5.6 TABLES

Table 5.21. Columbia River Basin (CRB) Chinook salmon (*Oncorhynchus tshawytscha*) populations obtained for experimental IHNV exposures.

Columbia River Basin Geographic region[a]	Juvenile life history (freshwater residence)	Adult migration timing	Chinook salmon Hatchery population[b]	Endangered Species Act Federal Listing Status
Upper	Stream–type (yearling)	Spring	Winthrop National Fish Hatchery	Endangered
Lower	Ocean–type (subyearling)	Fall	Cowlitz Hatchery	Threatened

[a]Populations sourced east of the Cascade Mountain Range represent upper CRB fish and those obtained west of the Cascade Range constitute lower CRB fish.
[b]Chinook salmon populations, from specific hatchery programs, were selected based on the analysis described in Narum et al. (2010).

Table 5.22. Infectious hematopoietic necrosis virus (IHNV) strains used in viral challenges.

Genogroup–subgroup	Virus strain	midG type[a]	Host	Isolation site	Year of Isolation
LII	FR0031	mG011L	Chinook salmon	Feather River Hatchery, California	2000
UC	RB1	mG001U	Steelhead trout	Round Butte Hatchery, Oregon	1975
MD	QTS07	mG110M	Steelhead trout	Salmon River Hatchery, Washington	2007

[a]Genotyping data based on 303 nt of the IHNV glycoprotein gene, referred to as the variable midG region (Kurath et al. 2003).

5.7 FIGURES

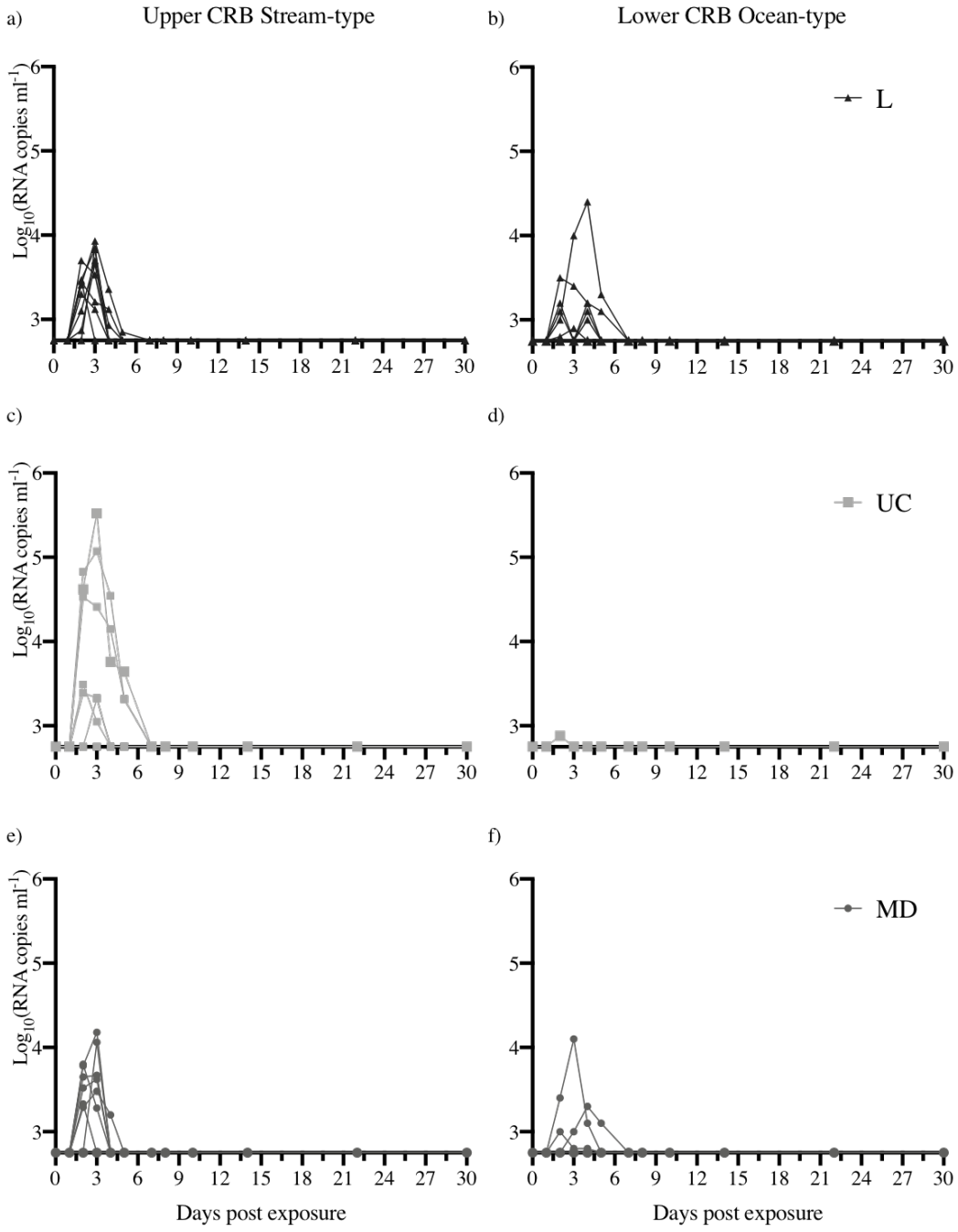


Figure 5.1. Daily quantity of virus shed by individual fish. Each panel shows the shedding kinetics of individual fish, per 23 hours, over the 30-day course of infection. Daily quantities of L (a, b), UC (c, d) and MD virus (e, f) shed from stream- (left) and ocean-type Chinook salmon (right) are reported as log10 virus RNA copies ml-1. The detection limit of the universal N gene IHNV reverse transcriptase real-time PCR (RT-rPCR) assay (2.75 $\log_{10}$ RNA copies ml^{-1}) is reflected on the y-axes. No fish in the virus-free treatment (negative control) shed detectible virus.

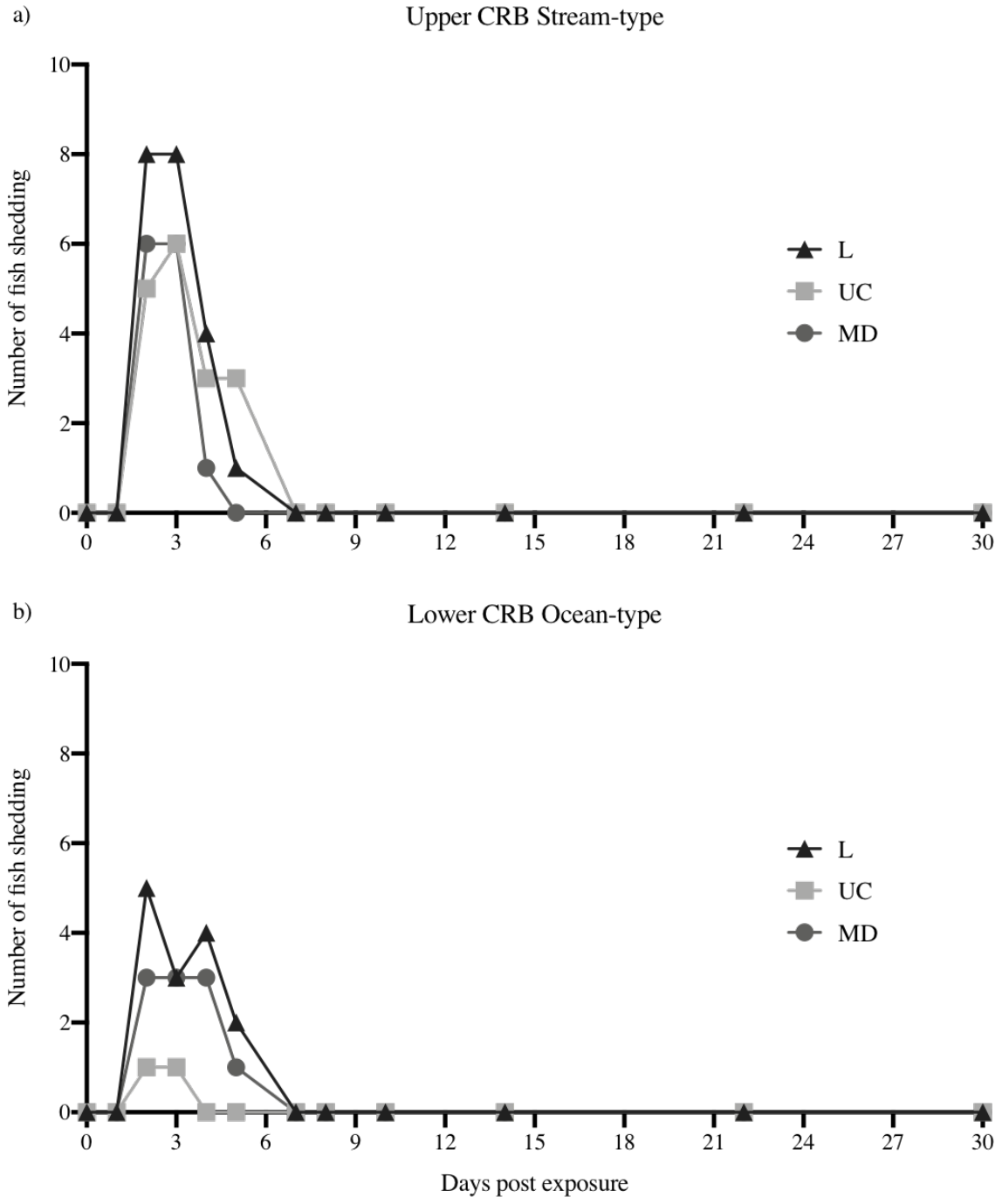


Figure 5.2. Daily number of fish shedding detectible virus over the 30-day course of infection. Stream- (a) and ocean-type (b) Chinook salmon shedding L, UC and MD IHN virus per day, out of 10 fish in each treatment group. No fish in the virus-free treatment (negative control) shed detectible virus over the course of the 30-day experiment.

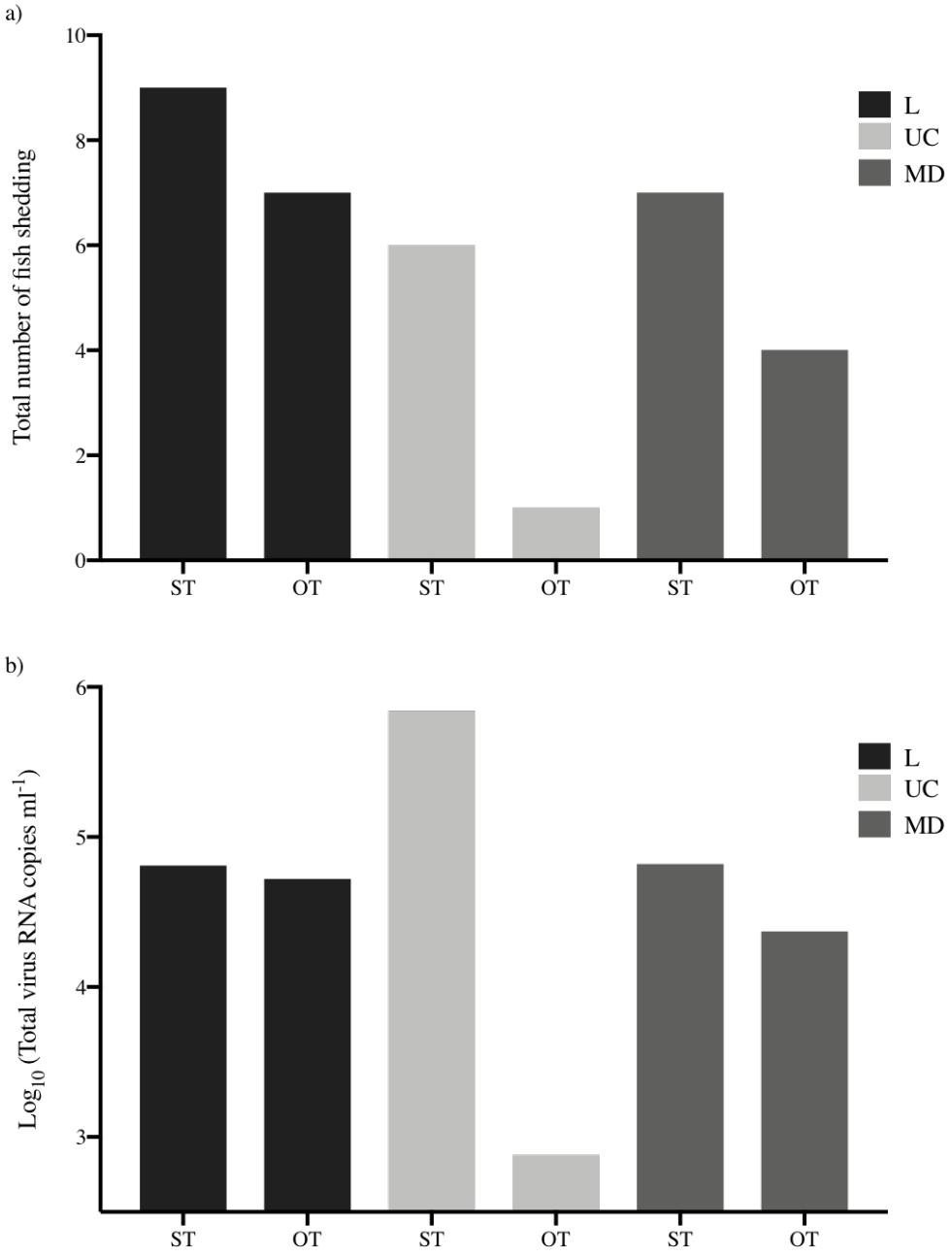


Figure 5.3.Total numbers of fish shedding detectible virus and the total quantities of virus shed in all fish per treatment group. (a) Total number of Stream-type (ST) and ocean-type (OT) Chinook salmon shedding L, UC and MD IHN virus over the 30-day course of infection. (b) Total log transformed virus quantities shed in all fish per treatment group.

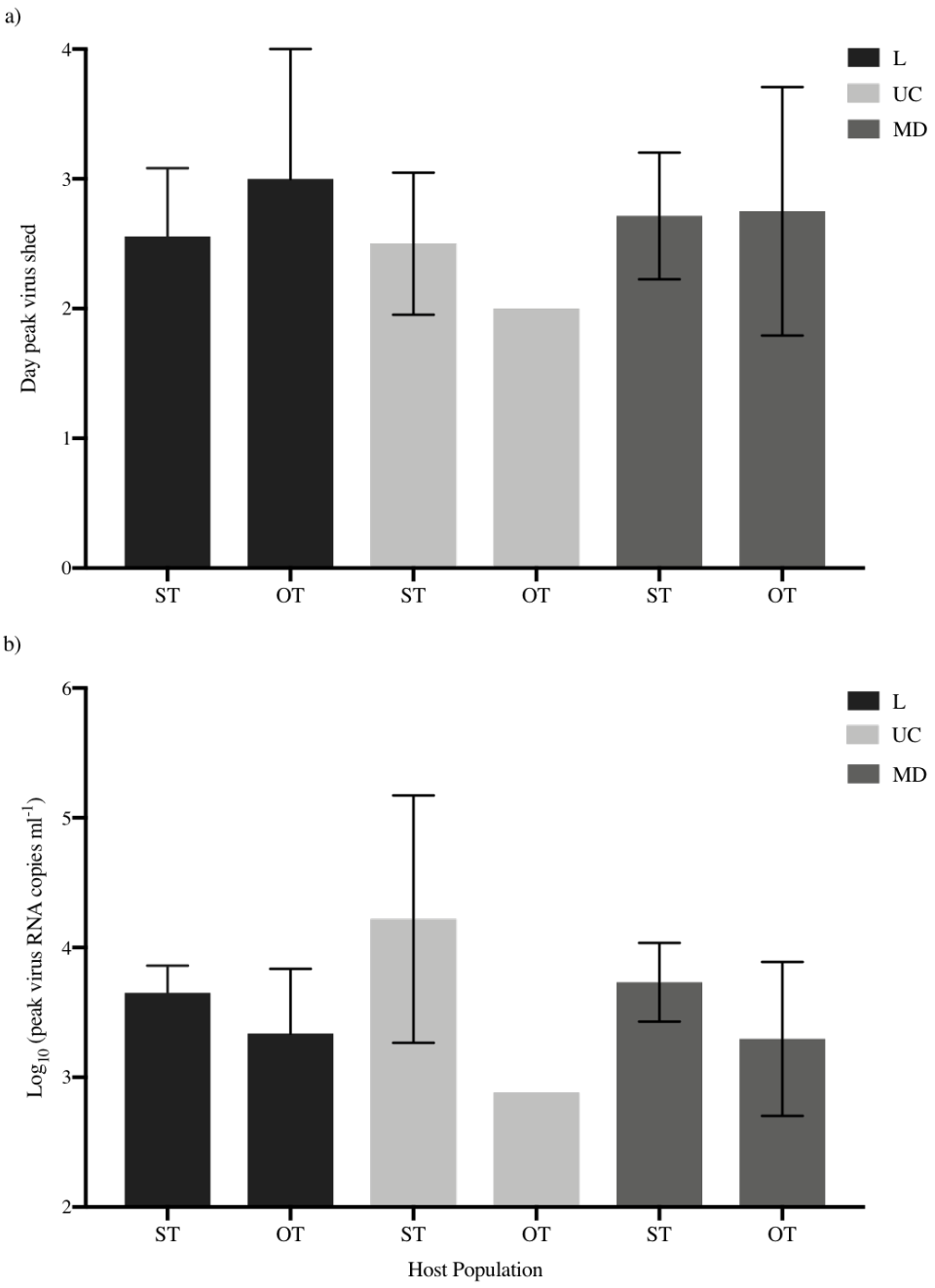


Figure 5.4. Shedding patterns of individual upper CRB stream-type (ST) and lower CRB ocean-type (OT) Chinook salmon. (a) Mean day (± 1 standard error) of peak shedding of L, UC and MD viruses in individual stream- and ocean-type fish. (b) Mean of log transformed (± 1 standard error) peak virus shed in each fish, irrespective of day.

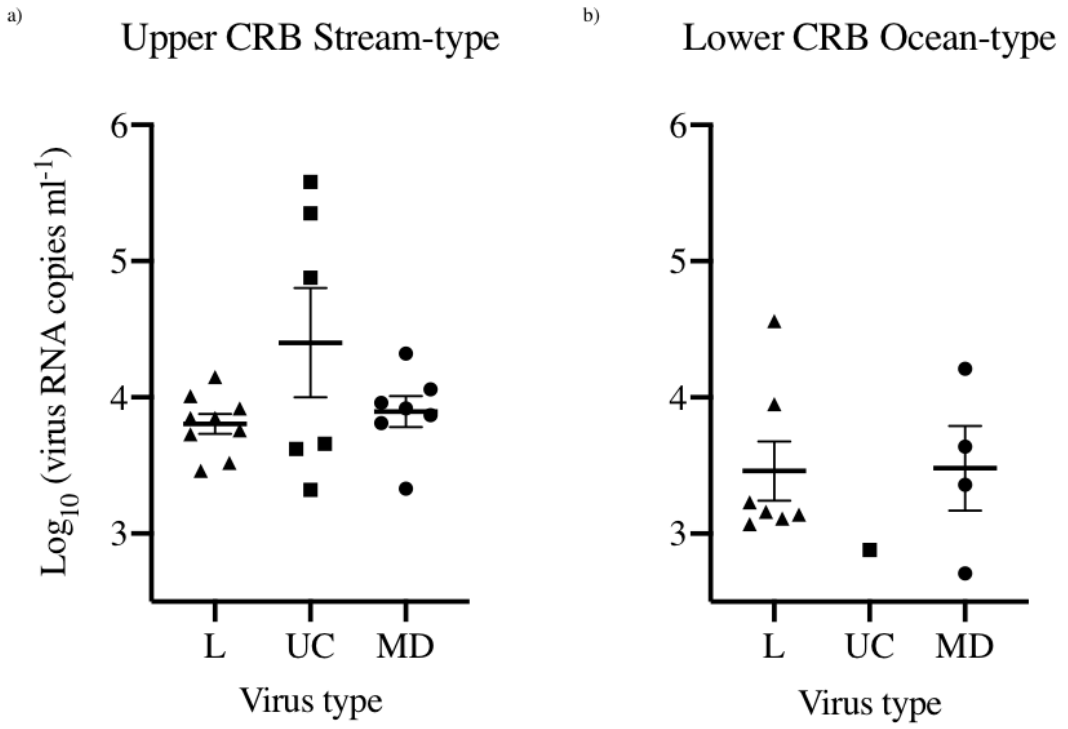


Figure 5.5. Individual quantities of L, UC and MD virus shed by juvenile stream- (a) and ocean-type (b) Chinook salmon. Mean (± 1 standard error) of the individual total quantities of log transformed virus shed by all fish in each treatment group over the 30-day course of infection.

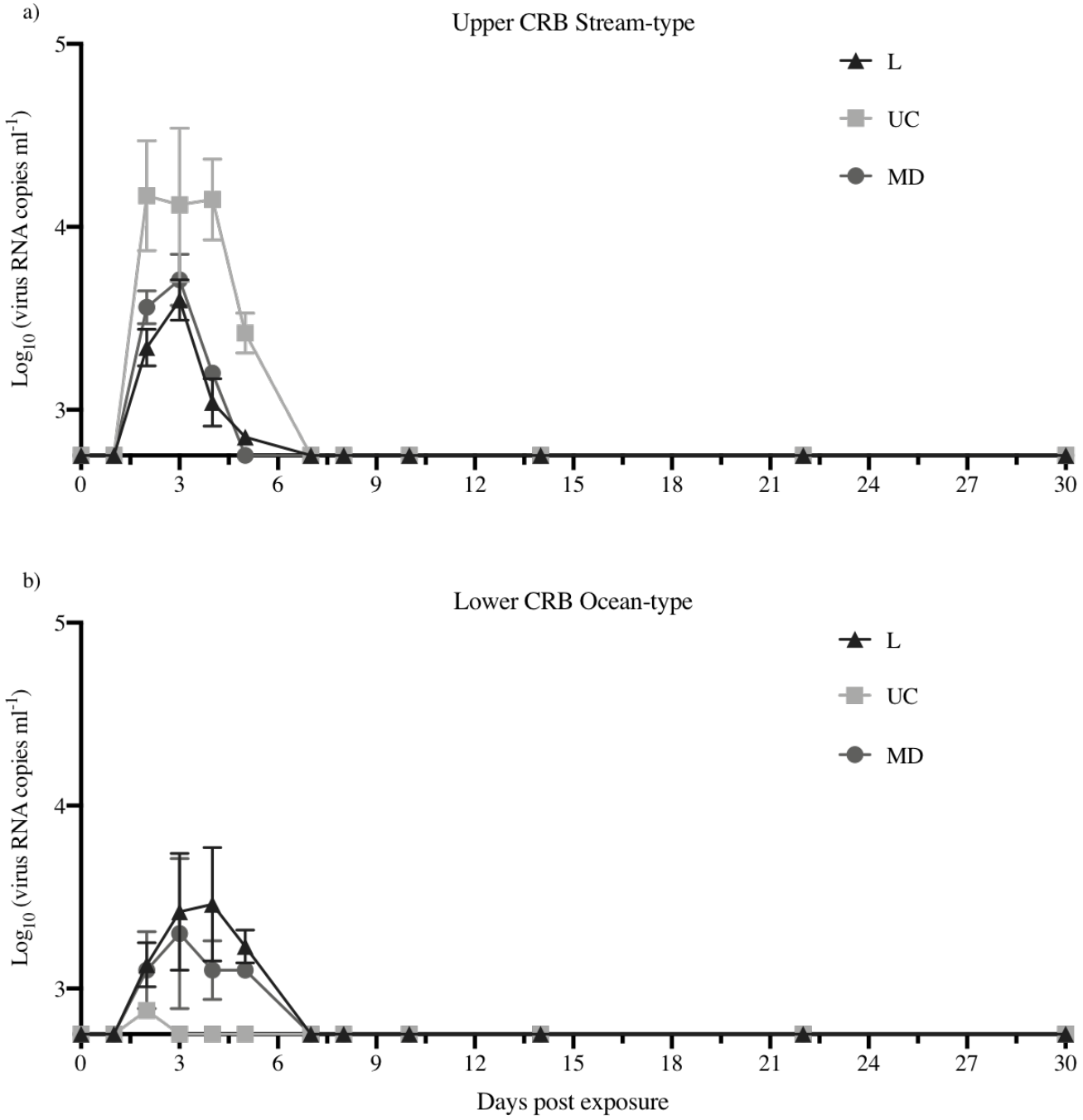


Figure 5.6. Shedding kinetics of L, UC and MD IHNV in (a) stream- and (b) ocean-type Chinook salmon of the CRB. Mean (± 1 standard error) log transformed virus quantities shed daily in all fish per treatment group. The detection limit of the universal N-gene IHNV rPCR assay (2.75 $\log_{10}$ RNA copies ml^{-1}) is reflected on the *y*-axes.

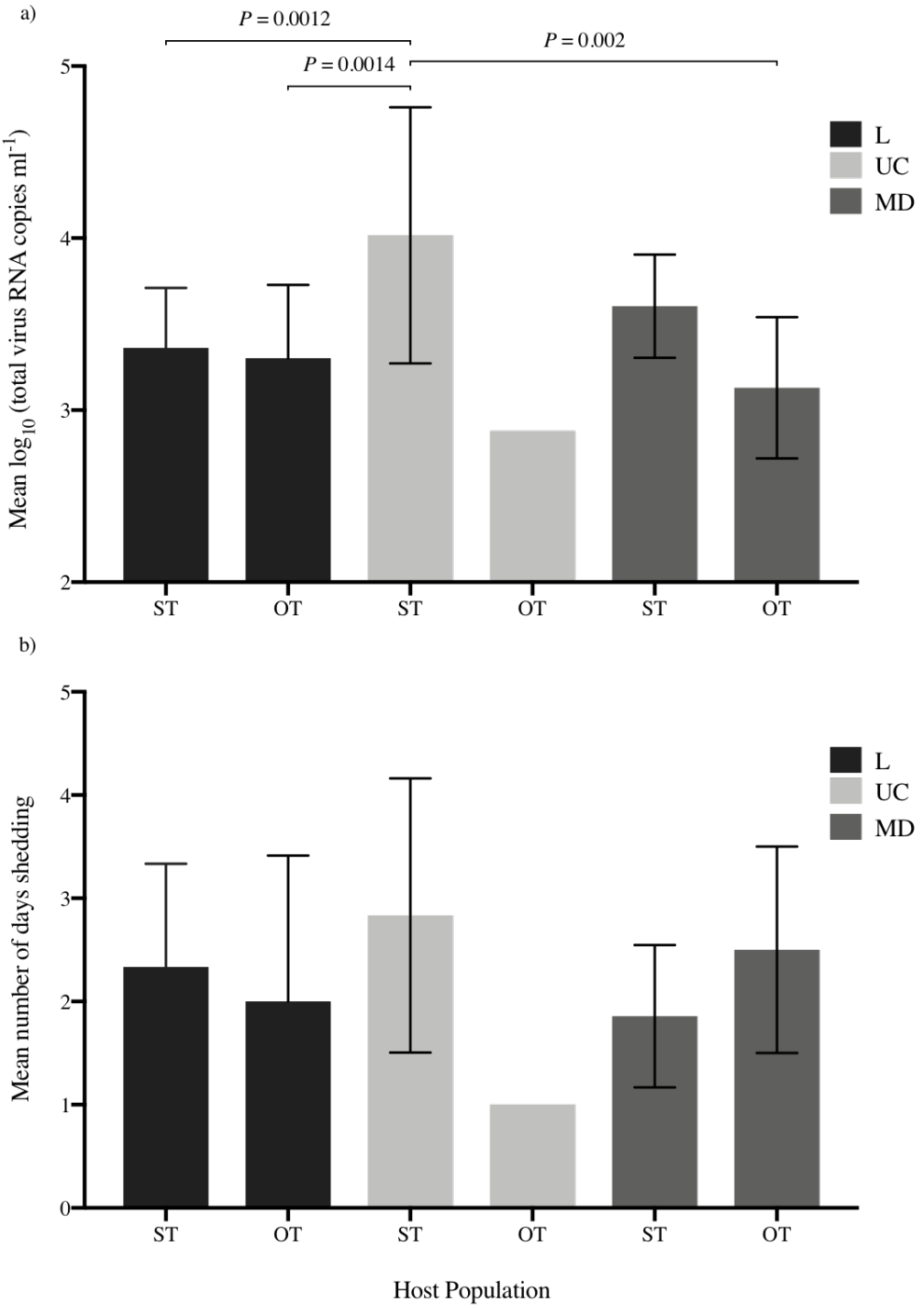


Figure 5.7. Mean total virus shed from all fish per treatment group over the 30-day course of infection and the mean number of days each virus strain was shed. (a) Mean (± 1 standard error) log transformed total virus quantities shed were compared within and across host populations. Brackets and reported *P* values indicate significant differences between two treatment groups. (b) The mean number of days (± 1 standard error) each virus strain was detectibly shed did not differ within or across host populations.

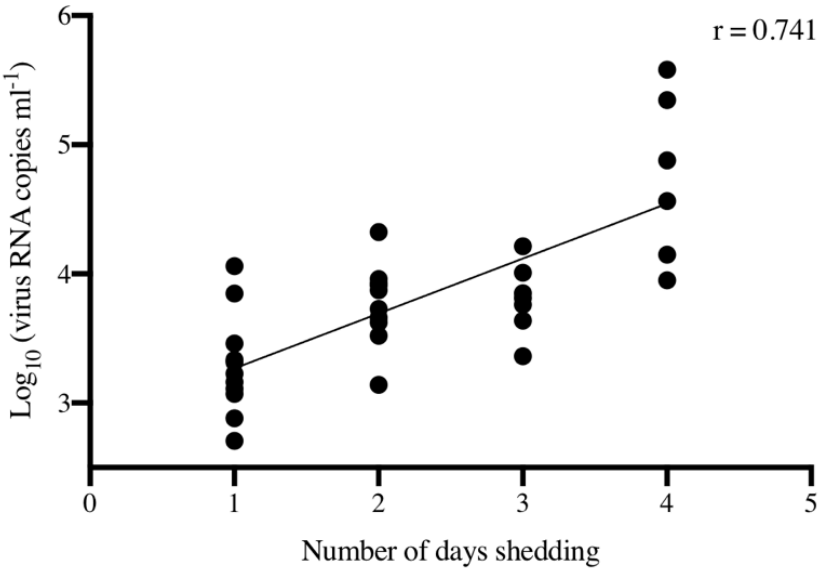


Figure 5.8. Correlation between the total quantities of log transformed virus shed by individual fish and the total number of days shedding detectible virus, irrespective of virus strain. Pearson r correlation coefficient is reported ($P < 0.0001$).

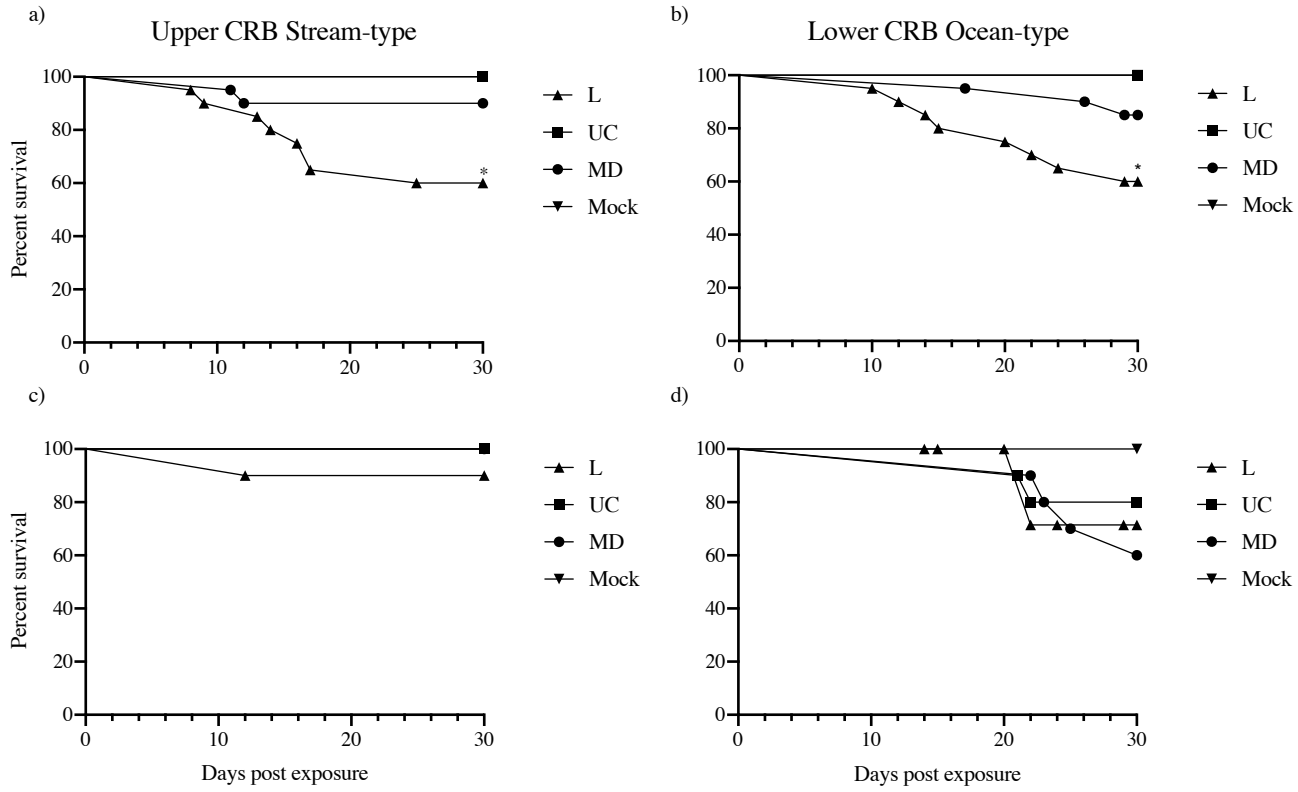


Figure 5.9. Daily cumulative percent survival of juvenile stream- (a,c) and ocean-type (b-d) Chinook salmon of the upper and lower CRB exposed to L, UC and MD strains of IHNV or virus-free media (Mock). Experimental host populations were monitored for mortality over the course of 30 days. Twenty fish from each treatment group remained together following immersion challenge (a, b), whereas ten of the initial thirty fish exposed were isolated to quantify the viral shedding of each individual (c, d). Survival curves were constructed for each group of twenty fish per treatment and those fish individually monitored for viral shedding. Asterisks indicate that fish in the positive control L genogroup IHNV treatment had significantly lower survival than batch held fish in the other viral strain treatments ($P < 0.001$). No significant reductions in survival were observed in batch held or isolated fish exposed to the UC and MD virus treatments.

5.8 LITERATURE CITED

Bendorf, A. C. M. (2010). Phylogenetic analysis and virulence characteristics for Chinook salmon, rainbow trout, and steelhead of L genogroup infectious hematopoietic necrosis virus (IHNV) from the Feather River, California. University of California, Davis.

Black, A., Breyta, R., Bedford, T., & Kurath, G. (2016). Geography and host species shape the evolutionary dynamics of U genogroup infectious hematopoietic necrosis virus. Virus evolution, 2(2), vew034.

Bootland, L. M., & Leong, J. C. (2011). Infectious haematopoietic necrosis virus. Fish diseases and disorders, 3, 66-109.

Brannon, E. L., Powell, M. S., Quinn, T. P., & Talbot, A. (2004). Population structure of Columbia River Basin Chinook salmon and steelhead trout. Reviews in Fisheries Science, 12(2-3), 99-232.

Breyta, R., Jones, A., & Kurath, G. (2014). Differential susceptibility in steelhead trout populations to an emergent MD strain of infectious hematopoietic necrosis virus. Diseases of aquatic organisms, 112(1), 17-28.

Breyta, R., Black, A., Kaufman, J., & Kurath, G. (2016). Spatial and temporal heterogeneity of infectious hematopoietic necrosis virus in Pacific Northwest salmonids. Infection, Genetics and Evolution, 45, 347-358.

Elena, S. F., Agudelo-Romero, P., & Lalić, J. (2009). The evolution of viruses in multi-host fitness landscapes. The open virology journal, 3, 1.

Ferguson, P. F., Breyta, R., Brito, I., Kurath, G., & LaDeau, S. L. (2018). An epidemiological model of virus transmission in salmonid fishes of the Columbia River Basin. Ecological modelling, 377, 1-15.

Garver, K. A., Troyer, R. M., & Kurath, G. (2003). Two distinct phylogenetic clades of infectious hematopoietic necrosis virus overlap within the Columbia River basin. Diseases of aquatic organisms, 55(3), 187-203.

Garver, K. A., Batts, W. N., & Kurath, G. (2006). Virulence comparisons of infectious hematopoietic necrosis virus U and M genogroups in sockeye salmon and rainbow trout. Journal of Aquatic Animal Health, 18(4), 232-243.

GraphPad Prism® version 8.1.0 (2019). GraphPad Software, Inc., La Jolla, CA, USA.

Gilbert, C. H. (1913). Age at maturity of the Pacific coast salmon of the genus Oncorhynchus (No. 767). Govt. Print. Off..

Healey, M. C. (1991). Life history of chinook salmon (*Oncorhynchus tshawytscha*). Pacific salmon life histories.

Hernandez, D. G., Purcell, M. K., Friedman, C. S., & Kurath, G. (2016). Susceptibility of ocean- and stream-type Chinook salmon to isolates of the L, U, and M genogroups of infectious hematopoietic necrosis virus (IHNV). Diseases of aquatic organisms, 121(1), 15-28.

Kurath, G., Garver, K. A., Troyer, R. M., Emmenegger, E. J., Einer-Jensen, K., & Anderson, E. D. (2003). Phylogeography of infectious haematopoietic necrosis virus in North America. Journal of General Virology, 84(4), 803-814.

Narum, S. R., Hess, J. E., & Matala, A. P. (2010). Examining genetic lineages of Chinook salmon in the Columbia River Basin. Transactions of the American Fisheries Society, 139(5), 1465-1477.

Oldstone, M. B. (2006). Viral persistence: parameters, mechanisms and future predictions. Virology, 344(1), 111-118.

Purcell, M. K., Garver, K. A., Conway, C., Elliott, D. G., & Kurath, G. (2009). Infectious haematopoietic necrosis virus genogroup-specific virulence mechanisms in sockeye salmon, Oncorhynchus nerka (Walbaum), from Redfish Lake, Idaho. Journal of fish diseases, 32(7), 619-631.

Purcell, M. K., Thompson, R. L., Garver, K. A., Hawley, L. M., Batts, W. N., Sprague, L., ... & Winton, J. R. (2013). Universal reverse-transcriptase real-time PCR for infectious hematopoietic necrosis virus (IHNV). Diseases of Aquatic Organisms, 106(2), 103-115.

Taylor, E. B. (1990). Phenotypic correlates of life-history variation in juvenile chinook salmon, *Oncorhynchus tshawytscha*. The Journal of Animal Ecology, 455-468.

Waples, R. S., Pess, G. R., & Beechie, T. (2008). Evolutionary history of Pacific salmon in dynamic environments. Evolutionary Applications, 1(2), 189-206.

Wargo, A. R., Garver, K. A., & Kurath, G. (2010). Virulence correlates with fitness in vivo for two M group genotypes of Infectious hematopoietic necrosis virus (IHNV). Virology, 404(1), 51-58.

Wargo, A. R., Scott, R. J., Kerr, B., & Kurath, G. (2017). Replication and shedding kinetics of infectious hematopoietic necrosis virus in juvenile rainbow trout. Virus research, 227, 200-211.

Zar, J. H. (2010). Biostatistical analysis fifth edition. Pearson Education Upper Saddie River, New Jersey, USA.

Chapter 6. CONCLUSION

Anadromy is a life history pattern characterized by emergence in freshwater, freshwater residence, a seaward migration, ocean residence, and a return to freshwater for reproduction (Quinn 2018). For Pacific salmon and trout (genus *Onchorhynchus*), what is known about the ocean phase of their life cycles is often inferred as opposed to directly measured, as is customary in the freshwater phases of their life cycles. Still, much is unknown about the freshwater ecologies that influence the survival of Pacific salmon and trout populations. One of those major unknowns continues to be the effect of infectious diseases on the survival of *Onchorhynchus* species to sexual maturity. Annually, conservation hatcheries across the US Pacific Northwest release millions of juvenile salmonid fishes. However, only a small proportion of those fish survive in the marine environment and return to freshwater to spawn. Once considered to have a minor role in the early mortality of juvenile salmonids, aquatic pathogens have been shown to regulate fish populations via host mortality (Sandell et al. 2015). Still, the impact of disease after release of juveniles from hatcheries is poorly understood. This investigation focused on the aquatic rhabdoviral pathogen infectious hematopoietic necrosis virus (IHNV) that causes acute disease in Pacific salmon and trout populations across western North America. While epizootic events occur primarily in juvenile fish in aquaculture facilities and freshwater conservation hatcheries, a comprehensive study of the spatial and temporal patterns of IHNV in watersheds of the US Pacific Northwest revealed a great majority of IHNV detections to be in adult fish at spawning. The high fidelity of distinct IHN virus genogroups to specific host species leaves many unanswered questions about how IHNV is maintained in a vast and multi-host watershed like the Columbia River Basin (CRB). This investigation aimed to build on previously published studies that characterized high prevalence of IHNV infection in adult Chinook salmon of the

CRB in the absence of disease in juvenile fish. Here, we evaluated field occurrence patterns of IHNV prevalence in Chinook salmon populations and conducted controlled virus exposures to characterize intraspecific variation in IHNV infection, disease and viral shedding in diverse Chinook salmon populations of the US Pacific Northwest. Our goal was to define the unique host-pathogen interactions of CRB Chinook salmon and U and M IHN viruses to better understand the role that Chinook salmon may have in the ecology and epidemiology of IHNV across the Columbia River watershed.

6.1 Differences in susceptibility to IHNV infection and mortality in juvenile stream- and ocean-type Chinook salmon

In our first study (Chapter 3, Hernandez et al. 2016), experimental exposures of two genetically and phenotypically distinct populations of Chinook salmon to U, M and L genogroup strains of IHNV showed observable differences in host susceptibility to infection and mortality at 1g. Although most differences were not statistically significant due to small samples sizes, infection prevalence was higher in the stream-type population with each of the U and M viruses when compared to the ocean-type population. Similarly, mortality due to L virus was also higher in the stream-type population when compared to the ocean-type population. While differences in host susceptibility may be due to differences in life history phenotype, these findings may instead have been linked to other differences in the origin of each host population. In this initial set of controlled virus exposures, one host population was sourced from outside of the CRB and the other was sourced from within the CRB. The ocean-type population was obtained from the Soos Creek Hatchery (WA State Department of Fish and Wildlife), located on the Big Soos Creek of Washington State, and represents a Puget Sound population of Chinook salmon. The stream-type population was obtained from the Little White Salmon National Fish Hatchery (U.S.

Fish and Wildlife Service), located on the Little White Salmon River of Washington State, and represents a CRB population of Chinook salmon. Assessment of the prevalence of IHNV infection in Chinook salmon populations from the CRB and coastal watersheds of Washington and Oregon (Chapter 2) showed a disproportionately low prevalence of IHNV infection in Chinook salmon populations outside of the CRB. This newly recognized pattern showed IHNV to be uniquely associated with Chinook salmon in host assemblages across the CRB. While differences in host susceptibility to IHNV infection and mortality between these stream- and ocean-type populations experimentally exposed may be linked to life history phenotype, additional experimentation with multiple Chinook salmon populations of the CRB (Chapter 4) showed juvenile susceptibility to IHNV infection and mortality may alternatively be linked to other host attributes.

6.2 Intraspecific variation in susceptibility to IHNV infection and disease in CRB Chinook salmon

To comprehensively characterize intraspecific variation in juvenile Chinook salmon susceptibility to IHNV infection and disease, four populations of CRB Chinook salmon were experimentally exposed to U, M and L genogroup strains of IHNV (Chapter 4). In an effort to maximize the genetic and phenotypic diversity between experimental host populations of Chinook salmon, and best represent the population structure of CRB Chinook salmon, each population was purposefully sourced from different specific hatcheries across the watershed. Host populations were selected based on genetic lineage, juvenile freshwater residence, adult migratory timing and geographic distribution at spawning. These controlled laboratory exposures showed little variation in susceptibility to IHNV infection between the four Chinook salmon populations. Each host population became infected with the U and M genogroup viruses, at

levels comparable to those observed with the positive control L genogroup virus. While infection prevalence and viral loads were comparably high for all 3 viral genotypes among the four host populations, mortality was observably low following exposure to the U and M genogroup viruses. Together, these experimental exposures empirically showed that juvenile Chinook salmon of the CRB can acquire U and M IHNV infections without the virulence observed with U virus in juvenile sockeye salmon or M virus in rainbow/steelhead trout. These findings suggest the relative absence of epizootic events in juvenile Chinook salmon of the CRB is not driven by the inability for U and M viruses to enter these host species, but rather the ability of juvenile Chinook salmon to effectively control viral infections. Further, this novel insight put into question the potential for Chinook salmon to transmit IHNV to susceptible, sympatric host species. Before this hypothesis was empirically tested, differences in the infectivity of U and M IHNV strains in CRB Chinook salmon were assessed quantitatively.

6.3 Infectivity of U and M viruses commonly detected across the CRB

The infectivity of U and M IHNV strains, commonly detected across the CRB, was characterized in each of the four CRB Chinook salmon populations described above. Virus strain infectivity was compared within a host population relative to a positive control L IHNV strain. The infectivity of each virus strain was also compared between host populations to characterize all possible differences owed to either the host or pathogen. The quantitatively rigorous metric used to compare virus strain infectivity was the dose needed to infect 50 percent of a host population (ID_{50}). Multiple comparisons of ID_{50} values showed little variation in the infectivity of the UC and MD IHNV strains within and across host populations. The U and M viruses were equivalently capable of infecting each Chinook salmon population, in the absence of disease in juvenile fish. U and M ID_{50} values were lowest in the upper CRB stream-type Chinook salmon

population, representing a higher overall infectivity in this host population. Further, the M IHN virus strain was significantly more infectious ($P = 0.0123$) in upper CRB stream-type Chinook salmon when compared to the lower CRB stream-type population.

6.4 The shedding kinetics of U and M viruses in two distinct CRB Chinook salmon populations

In an effort to empirically test the hypothesis that subclinically infected Chinook salmon serve as vectors of IHNV, controlled laboratory exposures were conducted to characterize the shedding kinetics of U, M and L genogroup strains of IHNV in two diverse Chinook salmon populations of the CRB. A proportion of fish from each host population shed detectable quantities of U, M and L IHNV, where virus shedding peaked between 2-3 days post exposure and was no longer detected after day 5. While each host population shed comparable quantities of M and L IHN virus, a notable difference in the number of fish shedding U virus was observed. A disproportionately low number of ocean-type Chinook salmon (one fish out of ten) shed detectible U virus relative to the stream-type population (six fish out of ten). Moreover, the stream-type population shed approximately 1 log more U virus consistently over the course of the infection, relative to the M and L IHN viruses. While our infectivity studies showed the U and M viruses to be equivalently infectious in four diverse populations of CRB Chinook salmon, results of our shedding studies report intraspecific variation in the shedding of U virus in CRB Chinook salmon. Of biological and epidemiological relevance, the same host population in which the M virus was most infectious was the population which was observed consistently shedding the high quantities of U virus. These findings may suggest that despite a lack of statistically significant differences, upper CRB stream-type Chinook salmon may be more closely linked to the ecology and epidemiology of IHNV in the CRB then ocean-type fish. Our

investigational approach made it possible to assess such findings from controlled laboratory exposures relative to field occurrence patterns of U and M IHNV infection in Chinook salmon of these life history phenotypes.

6.5 Prevalence of U and M IHNV infection in CRB spring-, summer-, fall-run Chinook salmon

The prevalence of IHNV infection in Chinook salmon populations of the CRB was assessed relative to adult life history phenotype using IHNV Virology, Genotyping and Surveillance database records available for CRB Chinook salmon between the years 2000-2012 (Breyta et al. 2017). Initial assessment of the infection prevalence in the two dominant life history phenotypes of CRB Chinook salmon appeared to be equivalent. Across spring-run Chinook salmon populations tested, infection prevalence was 27%, whereas for fall-run Chinook salmon infection prevalence was 25%. Infection prevalence was lower in summer-run Chinook salmon (13%). When assessed by both age class and life history phenotype, infection prevalence in spring- and fall-run Chinook salmon of the CRB appeared to be less comparable. Infection prevalence in adult spring-run fish was 43%, whereas in adult fall-run fish infection prevalence was 32%. While a difference of 11% may not appear significant or biologically relevant, one must consider the relative abundance of each host type across the CRB. Fall-run fish constitute 60% of adult Chinook salmon returning to the CRB, whereas spring-run fish comprise only 27% of the average annual returns of Chinook salmon. Alone, these data do not give a complete picture of the association of CRB Chinook salmon and IHNV, however, they do suggest that spring-run Chinook salmon may contribute disproportionately to the ecology of IHNV in CRB. While this notion is supported by the disproportionately high quantities of U virus shed by stream-type

(spring-run offspring) Chinook salmon, overall, host age appears to have a stronger influence on the prevalence of IHNV infection in CRB Chinook salmon.

6.6 The influence of age class on the prevalence of IHNV infection in CRB Chinook salmon

The prevalence of IHNV infection in Chinook salmon populations of the CRB was assessed relative to age class. Consistent with previously reported field occurrence patterns of IHNV infection in Pacific salmon and trout across the US Pacific Northwest (Breyta et al. 2017), infection prevalence was higher in Chinook salmon adults (36%) than in juvenile fish (9%) across the CRB. While disease associated with IHNV infection is observed principally in juvenile fish, prevalence of IHNV infection is higher in adult fish (Breyta et al. 2017). As anadromous salmonid fishes, anadromy confers a body size advantage onto anadromous fish, relative to non-anadromous salmonids, due to the increased prey availability in the marine environment. This body size advantage is most notable in Chinook salmon, which constitute the largest *Oncorhynchus* species at maturity (Quinn 2018). Increased body size at maturity is observed to correlate with increased reproductive fitness (Quinn 2018) allowing greater access to quality spawning grounds, larger egg sizes, and increased egg to fry survival. While anadromy is significantly advantageous for reproductive success, this life history strategy comes with physiological tradeoffs. Anadromous salmonid fishes are predominantly semelparous, spawning only once in their life time. Semelparous reproduction is owed to the physiological costs of changes in osmoregulatory function.

Anadromous salmonid fishes can inhabit the marine environment as a result of physiological changes in osmoregulation at the time of smolt transformation (Quinn 2018). Smolt transformation (smoltification) is driven by the endocrine system and consists of biochemical

and physiological changes in juvenile fish that increase salinity tolerance (Björnsson et al. 2011). At the time of smoltification and adult return to freshwater, the pituitary-interrenal axis becomes activated leading to hypertrophy of the interrenal tissue and increases in cortisol levels in the plasma (Hoar et al. 1997). During each of these periods in the salmonid life cycle, cortisol stress response is elevated increasing the overall susceptibility to infection (Hoar et al. 1997). The ability for adult fish returning to their natal spawning grounds to resist pathogens decreases as they near the time of spawning. At the time of spawning, Pacific salmon are reported to have hyperplasic interrenals, elevated corticosteroid concentrations, and degenerated spleen, thymus, kidney and thyroid tissues (Hoar et al. 1997). Further, adult Chinook salmon holding in freshwater for several months prior to spawning were observed to have elevated cortisol titers and few antibody-producing cells (Hoar et al. 1997). Together, the high prevalence of IHNV infection in adult Chinook salmon is consistent with the immunedeficiencies reported for Pacific salmon adults nearing spawn timing. When combined with previously published IHNV landscape epidemiological studies (Breyta et al. 2017; Ferguson et al. 2018), these findings suggest that adult fish greatly contribute to the maintenance and the potential transmission of IHNV across the Columbia River watershed.

Extended freshwater holding periods and farther upstream migrations, inherent to spring-run Chinook salmon of the upper CRB, increase the overall likelihood of these fish having elevated cortisol levels, potentially increasing the overall susceptibility of upper CRB spring-run Chinook salmon to IHNV infection. While few statistically significant differences where observed in the susceptibility of juvenile stream-type (spring-run offspring) versus ocean-type (fall-run offspring) Chinook salmon of the CRB to IHNV infection in controlled laboratory studies, assessment of the prevalence of IHNV infection in spring- versus fall-run Chinook salmon of the

CRB revealed a higher overall prevalence of IHNV infection in adult spring-run fish. Examination of the geospatial distributions of U and M IHNV positive cohorts of spring- and fall-run Chinook salmon across the CRB showed the geospatial distributions of IHNV positive cohorts of spring-run Chinook salmon to more closely coincide with the overall distribution patterns of U and M IHNV across the CRB (Chapter 2). Together, these observations suggest that spring-run Chinook salmon populations of the CRB may be disproportionately contributing to the successful maintenance of IHNV across the Columbia River watershed relative to fall-run Chinook salmon. This notion is further supported by differences in the prevalence of IHNV in Chinook salmon populations of the CRB versus coastal streams (Chapter 2) and differences in the overall abundance of spring- and fall-run Chinook salmon across the two geographic regions. Across coastal streams of Washington and Oregon, spring-run fish comprise a small proportion of the Chinook salmon that spawn in these watersheds (Healey 1991). Instead, coastal streams are more commonly inhabited by fall-run Chinook salmon (Healey 1991). If extended freshwater residence by adult Chinook salmon does contribute to increased susceptibility to IHNV infection, one would expect to see a higher prevalence of IHNV infection in spring- versus fall-run fish. Consistent with this hypothesis, a trend of higher infection prevalence is observed in spring- versus fall-run Chinook salmon across both geographic regions. While the disproportionately low prevalence of IHNV infection in Chinook salmon populations of the coastal watersheds relative to the CRB may be influenced by an overall lack of spring-run Chinook salmon across coastal streams, additional studies are needed to fully characterize the influence of host life history phenotype on Chinook salmon susceptibility to IHNV infection.

6.7 THE INFLUENCE OF LIFE HISTORY ON THE PREVALENCE OF IHNV INFECTION IN JUVENILE CHINOOK SALMON

It is necessary to clarify from the outset, that the prevalence of IHNV in adult Chinook salmon is disproportionately higher relative to juvenile fish in all life history phenotypes. Still, assessment of the influence of juvenile life history phenotype was worth examining, given observed differences in the prevalence of IHNV infection in juvenile Chinook salmon in our first study using controlled laboratory exposures. Assessment of the field prevalence of IHNV in spring-, summer-, and fall-run Chinook salmon relative to age class shows two notable differences in the prevalence of U and M viral infections in CRB Chinook salmon. While greater numbers of juvenile spring-run Chinook salmon cohorts were tested, IHNV prevalence was slightly higher in juvenile fall-run fish (Table 2.6). Infection prevalence was observed to be 8% in juvenile spring-run fish, whereas in juvenile fall-run fish infection prevalence was 12%. Most notable, was the lack of M IHNV detection in juvenile fall-run Chinook salmon, although this involved very low cohort numbers. All IHN virus detected in juvenile fall-run fish was U genogroup virus, whereas in juvenile spring-run fish both U and M viruses were detected. While caution must be taken when making inferences from such few records (Table 2.6), the following observations are worth noting. Whereas U genogroup viruses constitute 83% of IHNV detected in CRB Chinook salmon (Table 2.1), U viruses comprise 25% of IHNV detections in coastal spring-run Chinook salmon and 0% in fall-run populations (Table 2.3). While the absence of U virus detection in fall-run fish of the coastal watersheds is in stark contrast to the high prevalence of U virus in both fall- and spring-run Chinook salmon of the CRB, the converse is observed when examining juvenile fall-run Chinook salmon in the CRB. Numerous factors may be contributing to the observed differences in the prevalence of U and M IHNV infection across

Chinook salmon populations of these two geographic regions. While life history phenotype in adult Chinook salmon appears to be a possible contributing factor to the prevalence of IHNV in CRB Chinook salmon, there is less evidence to support such a clear linkage between a specific life history phenotype in juvenile Chinook salmon of the CRB. Additional exposure studies are needed to test whether there is an overall higher infectivity of U and M viruses in juvenile stream-type Chinook salmon. Further, additional studies are needed to confirm whether stream-type Chinook salmon do in fact shed U virus at a much higher magnitude than ocean-type Chinook salmon.

6.8 Novel insights gleaned about the host-pathogen interactions of CRB Chinook salmon and IHNV

Our investigational approach made it possible to empirically test hypotheses relating to IHNV infection, disease and virus shedding in CRB Chinook salmon populations. When combined with newly characterized field occurrence patterns of IHNV infection in naturally occurring Chinook salmon populations, the following novel insights were gleaned about CRB Chinook salmon and IHNV.

1. IHNV prevalence is disproportionately low across Chinook salmon populations of the coastal watersheds of Washington and Oregon relative to the CRB.

2. Similar to the Sacramento River watershed, the high prevalence of IHNV in Chinook salmon of the CRB appears to be associated with the diverse life history phenotypes of Chinook salmon across the Columbia River watershed and the multiple IHNV host species that can share or maintain the virus.

3. Virus exposure studies showed juvenile Chinook salmon of the CRB can become infected with UC and MD IHN virus, in the absence of disease.

4. Viral shedding studies showed juvenile Chinook salmon of the CRB can shed detectible quantities of UC and MD IHN virus for 2-4 days following exposure, in the absence of disease.

5. UC and MD viruses are equivalently high in infectivity in juvenile Chinook salmon populations of the CRB, representing diverse genetic backgrounds.

6. The relative lack of epizootic events in juvenile Chinook salmon of the CRB is not driven by the inability for U and M viruses to enter these fish, but rather the ability of juvenile Chinook salmon to effectively control viral infections of these genogroups.

7. The highest amount of virus shed per population was UC virus in upper CRB stream-type Chinook salmon, and the lowest was UC virus in lower CRB ocean-type fish.

8. UC and MD viruses appear to be more infectious in upper CRB stream-type Chinook salmon than in lower CRB stream-type fish and both upper and lower CRB ocean-type fish.

9. Field occurrence patterns of IHNV detections in Chinook salmon populations of the CRB showed an overall higher prevalence of IHNV infection in spring-run Chinook salmon relative to fall-run fish.

10. Despite a general lack of significant differences between spring- and fall-run Chinook salmon, several qualitative observations are consistent in suggesting that spring-run Chinook salmon adults and their corresponding juvenile stream-type off spring appear to be more closely linked to the ecology of IHNV in CRB Chinook salmon. Together, the longer freshwater residence times and spatial distributions of these populations increase their overall IHNV exposure and transmission potential.

6.9 LITERATURE CITED

Breyta, R., Brito, I., Kurath, G., & LaDeau, S. (2017). Infectious hematopoietic necrosis virus virological and genetic surveillance 2000–2012. Ecology, 98(1), 283-283.

Björnsson, B. T., Stefansson, S. O., & McCormick, S. D. (2011). Environmental endocrinology of salmon smoltification. *General and comparative endocrinology*, *170*(2), 290-298.

Ferguson, P. F., Breyta, R., Brito, I., Kurath, G., & LaDeau, S. L. (2018). An epidemiological model of virus transmission in salmonid fishes of the Columbia River Basin. *Ecological modelling*, *377*, 1-15.

Healey, M. C. (1991). Life history of chinook salmon (*Oncorhynchus tshawytscha*). Pacific salmon life histories.

Hernandez, D. G., Purcell, M. K., Friedman, C. S., & Kurath, G. (2016). Susceptibility of ocean- and stream-type Chinook salmon to isolates of the L, U, and M genogroups of infectious hematopoietic necrosis virus (IHNV). Diseases of aquatic organisms, 121(1), 15-28.

Hoar, W. S., Randall, D. J., Iwama, G., & Nakanishi, T. (1997). *The fish immune system: organism, pathogen, and environment* (Vol. 15). Academic Press.

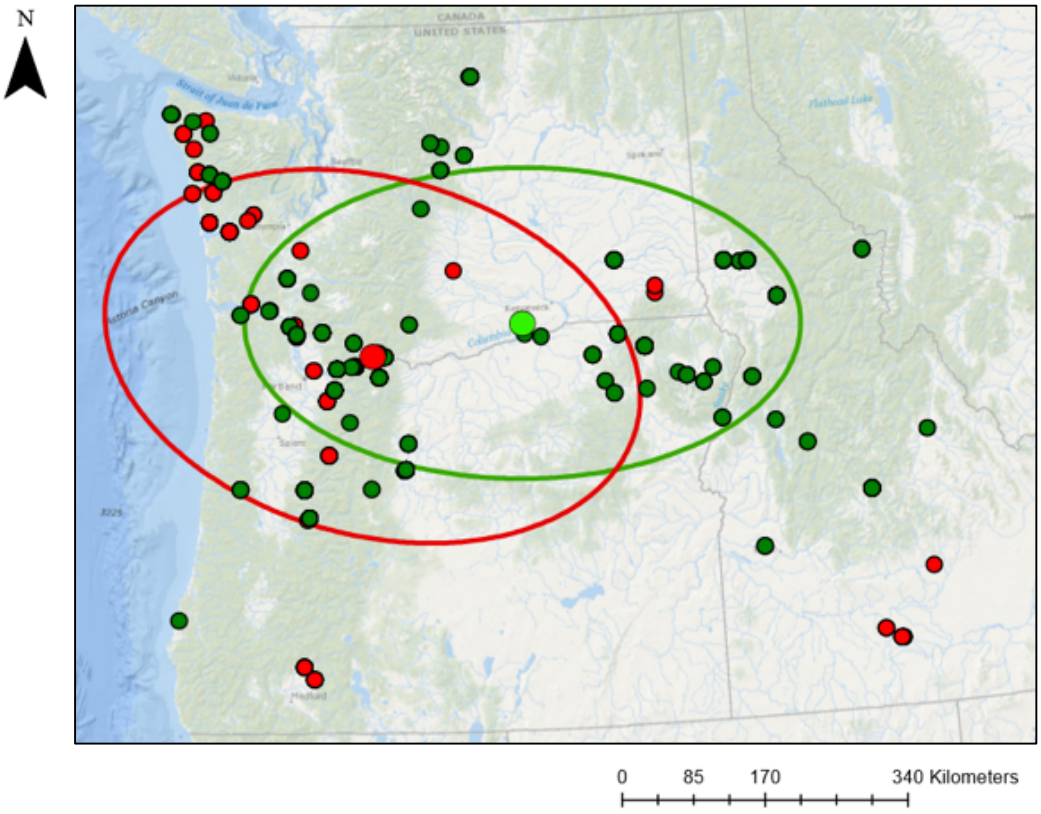
Quinn, T. P. (2018). *The behavior and ecology of Pacific salmon and trout*. University of Washington Press.

Sandell, T. A., Teel, D. J., Fisher, J., Beckman, B., & Jacobson, K. C. (2015). Infections by Renibacterium salmoninarum and Nanophyetus salmincola Chapin are associated with reduced growth of juvenile Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), in the Northeast Pacific Ocean. Journal of fish diseases, 38(4), 365-378.

6.10 APPENDICES A-F

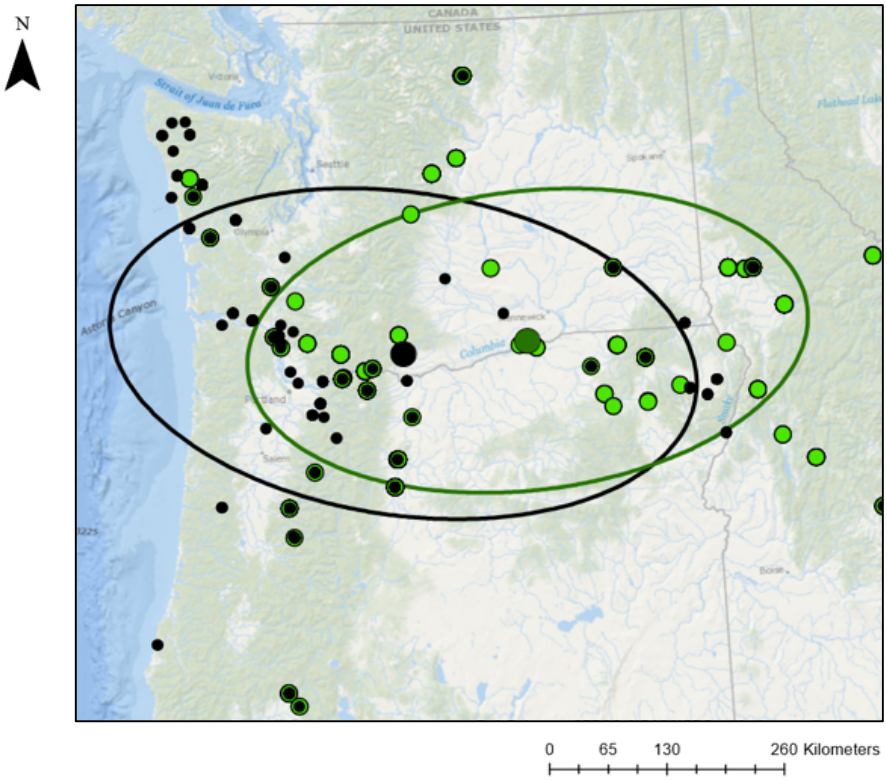
Prior to conducting the GIS analyses that comprise Chapter 2 of this thesis, a preliminary analysis was done based on all Chinook salmon records in the VGS database, including all coastal and CRB records together. These analyses are presented as appendices A-F. Subsequent to these findings, the CRB and coastal watershed subsets were analyzed separately, as presented in Chapter 2.

APPENDIX A



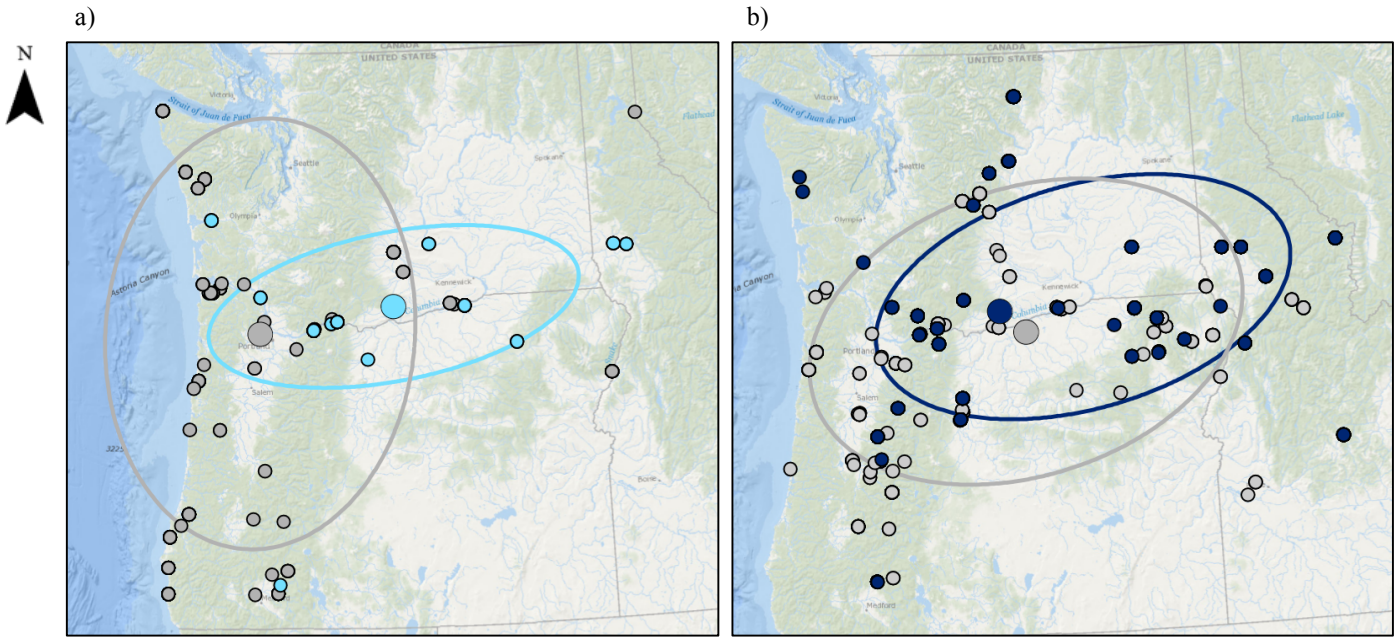
Appendix A. The geographic distribution of all M genogroup (red dots) and U genogroup (green dots) IHN virus positive cohorts of Pacific salmonids across the Columbia River Basin (CRB) and Coastal Watersheds of Washington and Oregon between the years 2000 - 2012. The Mean Center of M (large red dot) and U (large green dot) virus positive detections in the CRB-Coastal Watersheds and their Directional Distributions (Standard Deviational Ellipse) are reported. The mean center of M genogroup IHNV detections is observed to be westward toward the Pacific Ocean whereas for U genogroup IHNV detections the mean center is eastward toward the interior of the CRB. The directional distribution of M genogroup IHNV detections (red ellipse) is northwest to southeast in orientation whereas for U genogroup IHNV detections (green ellipse) the orientation is west to east within the CRB. Figure prepared using IHNV Virology, Genotyping and Surveillance (VGS) database records (Breyta et al. 2017). Analysis did not include M genogroup IHNV detections in rainbow trout aquaculture facilities in the Hagerman Valley, Idaho.

APPENDIX B



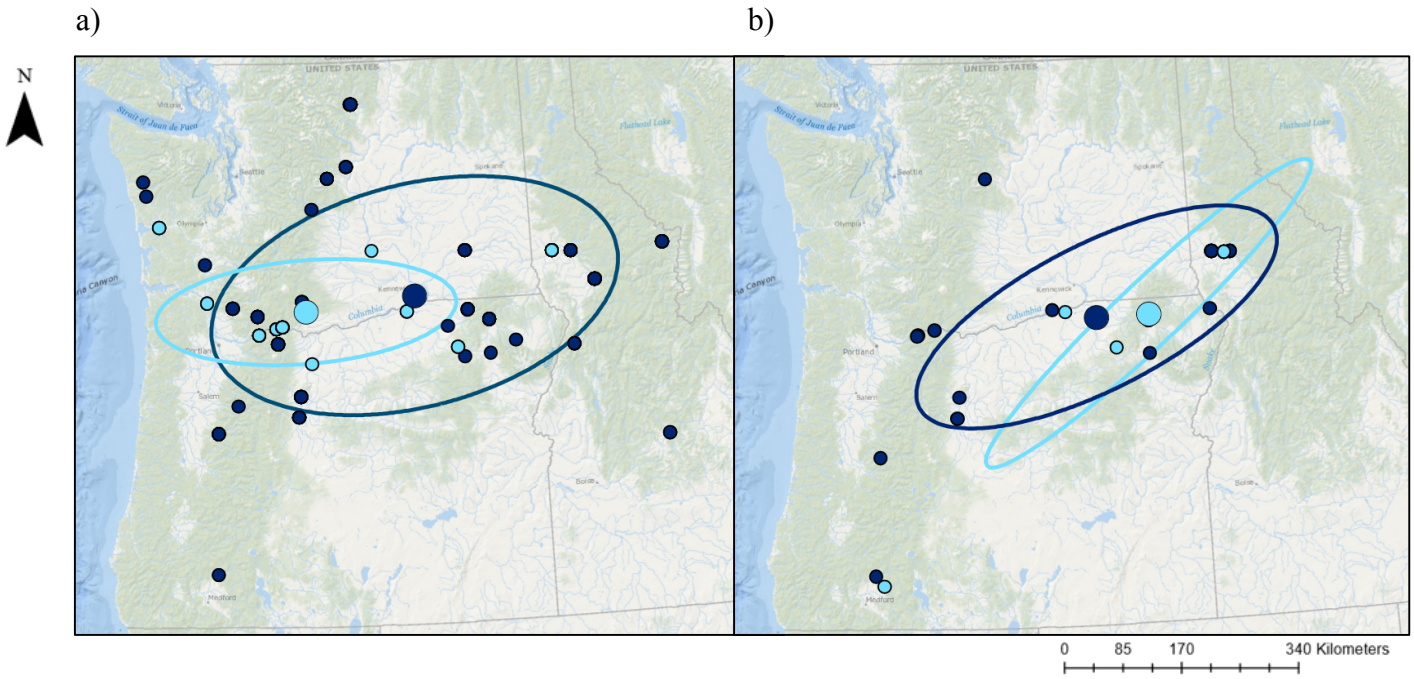
Appendix B. IHNV positive steelhead trout (black dots) and Chinook salmon (green dots) cohorts across the Columbia River Basin (CRB) and Coastal Watersheds of Washington and Oregon between the years 2000 - 2012. The mean center of IHNV positive cohorts of steelhead trout (large black dot) is observed to be westward toward the Pacific Ocean whereas for Chinook salmon (large green dot) it is eastward toward the interior of the CRB. The directional distributions of IHNV positive cohorts of each species is depicted by the black (steelhead trout) and green (Chinook salmon) ellipses. The mean center and directional distribution of IHNV positive steelhead trout cohorts closely corresponds with that of M genogroup IHNV events (Appendix A). Similarly, the mean center and directional distribution of IHNV positive Chinook salmon cohorts closely corresponds with that of U genogroup IHNV events.

APPENDIX C



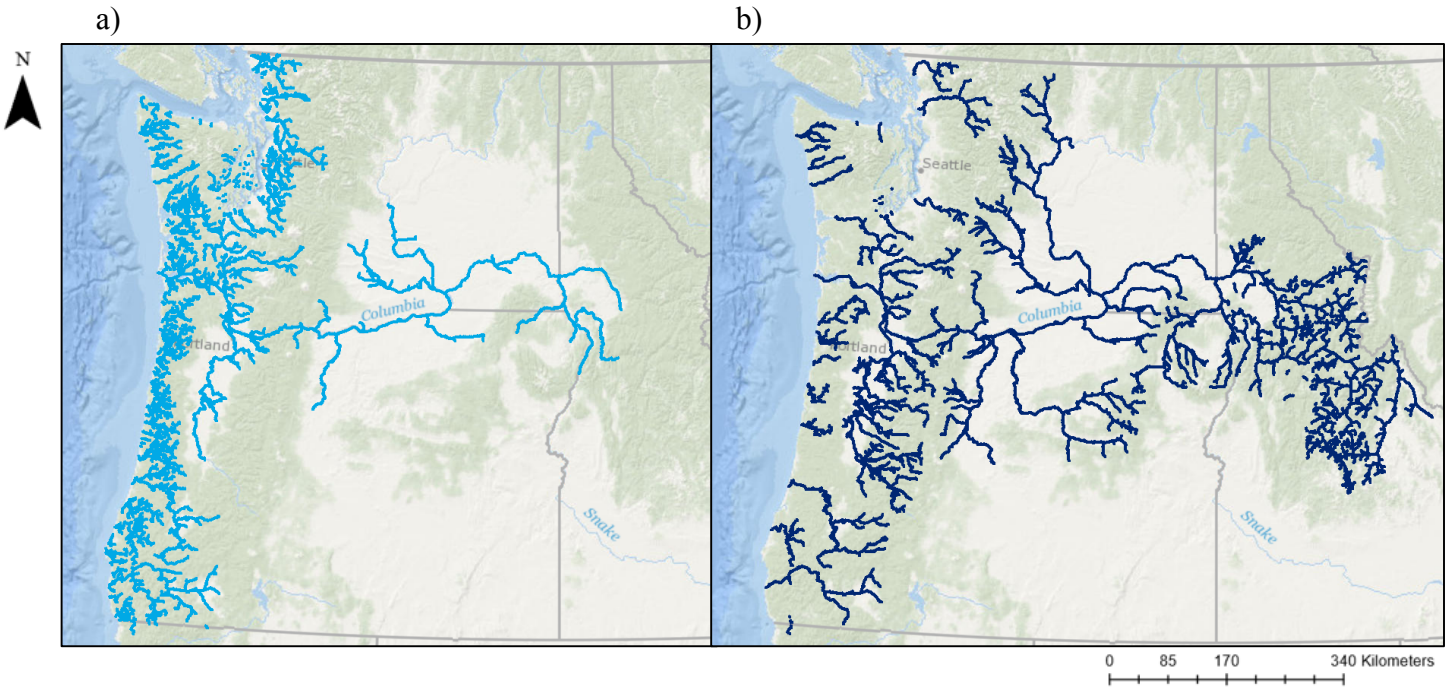
Appendix C. The mean centers of IHNV negative (large grey dots) and positive (large blue dots) fall- (a) and spring-run (b) Chinook salmon cohorts across the Columbia River Basin (CRB) and Coastal Watersheds of Washington and Oregon between the years 2000 - 2012. The calculated centers of concentration for IHNV positive fall- (large light blue dot) and spring-run (large dark blue dot) Chinook salmon cohorts coincide in the middle Columbia River main stem. For both IHNV positive fall- and spring-run Chinook salmon cohorts, the orientation of their standard deviational ellipses are equivalent, but their sizes differ. The standard deviational ellipse of IHNV positive spring-run fish is longer (*x*-axis) and wider (*y*-axis) than for fall-run fish, representing a broader distribution within the CRB. The standard deviational ellipse of IHNV positive fall-run Chinook salmon is shorter (*x*-axis), more narrow (*y*-axis) and encapsulates the lower main stems of the Columbia and Snake Rivers. For IHNV positive spring-run Chinook salmon, the standard deviational ellipse is observed eastward encapsulating a greater and more interior expanse of the CRB. IHNV negative fall- and spring-run Chinook salmon cohorts were also included in this analysis (grey dots). The mean center of IHNV negative Fall-run fish (a) is observed west of the Cascade Range whereas for IHNV negative spring-run fish (b) it is east of the mountain range. The standard deviational ellipse of IHNV negative fall run Chinook salmon cohorts is similar in size to that of IHNV negative fall-run Chinook salmon cohorts, but its orientation is North to South whereas for IHNV negative spring run Chinook salmon cohorts it is Northeast to Southwest.

APPENDIX D



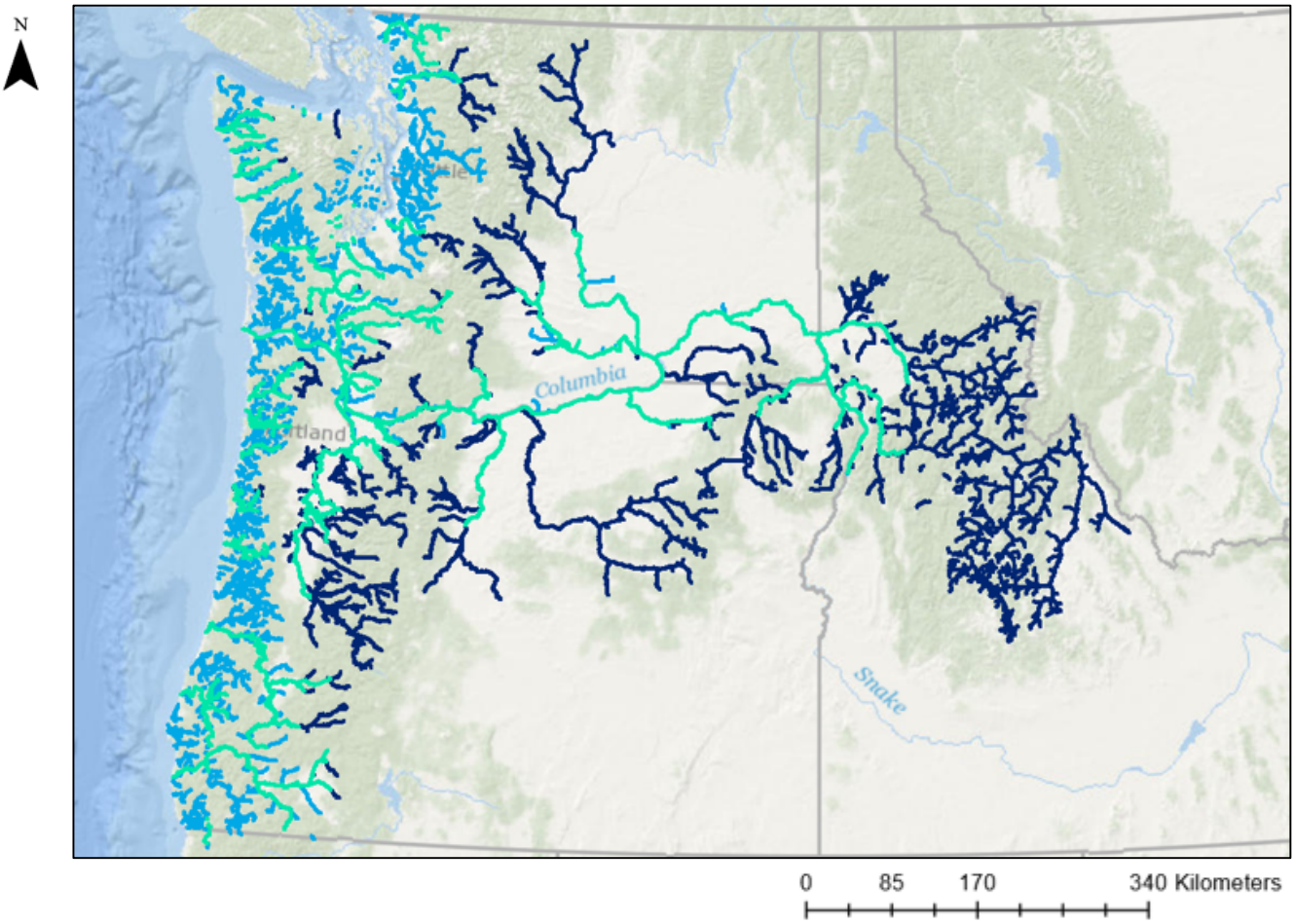
Appendix D. Mean centers and standard deviational ellipses for IHNV positive cohorts of adult (a) and juvenile (b) fall-run (light blue) and spring-run (dark blue) Chinook salmon across the Columbia River Basin (CRB) and Coastal Watersheds of Washington and Oregon between the years 2000 - 2012. Following patterns previously described for IHNV positive cohorts of fall- and Spring-run Chinook salmon (Appendix C), the standard deviational ellipse of IHNV positive adult spring-run Chinook salmon is longer (*x*-axis) and wider (*y*-axis) than for fall-run fish, representing a broader distribution within the CRB. For IHNV positive adult spring-run fish, the mean center is observed eastward within the CRB and their standard deviational ellipse encapsulates a greater and more interior expanse of the CRB. The standard deviational ellipse of IHNV positive adult fall-run Chinook salmon is shorter (*x*-axis), more narrow (*y*-axis) and encapsulates the lower main stems of the Columbia and Snake Rivers. Further, the mean center and standard deviational ellipse of IHNV positive cohorts of adult fall-run Chinook salmon are westward toward the Pacific Ocean. Conversely, for IHNV positive cohorts of juvenile fall-run Chinook salmon, the mean center and standard deviational ellipse is observed eastward toward the interior of the CRB with a Northeastern to Southwestern orientation. Similarly, for IHNV positive cohorts of juvenile spring-run Chinook salmon, the mean center and standard deviational ellipse are also observed east toward the interior of the CRB but with a broader distribution than for IHNV positive cohorts of fall-run juvenile fish. Paradoxically, the mean center of IHNV positive juvenile spring-run Chinook salmon is observed westward more than calculated for IHNV positive fall-run juvenile fish.

APPENDIX E



Appendix E. The distributions of fall- (a) and spring-run (b) Chinook salmon across the Columbia River Basin (CRB) and coastal watersheds of Washington and Oregon. The distribution of fall-run Chinook salmon (light blue) is more closely associated with coastal watersheds and Columbia River tributary streams west of Cascade Range. Fall-run Chinook salmon are also distributed east of the Cascade Range in the main stems of the lower and middle Columbia River, the Yakima River, the Snake River and its major tributaries. Spring- run Chinook salmon (dark blue) are observed to have more interior distributions within the CRB where they are more closely associated with the upper reaches of the Columbia River, Yakima River, Snake River and their smaller order tributary streams. Figures produced using StreamNet (2017) fish distribution data for the Pacific Northwest.

APPENDIX F



Appendix F. The combined distributions of fall- (light blue) and spring-run (dark blue) Chinook salmon across the Columbia River Basin (CRB) and coastal watersheds of Washington and Oregon. While fall-run Chinook salmon are observed to have more coastal distributions (west of the Cascade Range) and spring-run Chinook salmon are observed more eastward toward the interior CRB, the spatial distributions of fall and spring-run Chinook salmon coincide (teal) in some coastal streams and portions of the CRB. Spatial overlap is observed primarily in the main stem of the Lower Columbia River, Snake River, Yakima River and Willamette River of the CRB. Figure produced using StreamNet (2017) fish distribution data for the Pacific Northwest.