1 Successful mitigation of viral disease based on a delayed exposure rearing strategy at a
2 large-scale steelhead trout conservation hatchery.

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Abstract

In 2009, the largest steelhead trout conservation hatchery in the state of Idaho, Dworshak National Fish Hatchery (NFH), lost over 50% of the juvenile steelhead trout (*Oncorhynchus mykiss*) population being reared for release. The causative agent of this high mortality was the viral pathogen infectious hematopoietic necrosis virus (IHNV). This was neither the first nor the worst epidemic of IHNV to occur at the hatchery, but it was the worst in over a decade. Genetic analysis of IHNV isolates taken from juveniles suffering epidemic IHN disease in 2009 revealed that the virus was of the M group of IHNV viruses, known to have high virulence for trout. The water supply for steelhead trout rearing at Dworshak NFH is untreated water taken directly from the Clearwater River. Further genetic analysis of IHNV isolates from adults spawned in 2009 indicated that adult steelhead trout in the river (in the hatchery water supply) were the most probable transmission source for the epidemic IHN disease in the juvenile fish. Previously, Dworshak NFH had been able to gain access to reservoir water from behind the Dworshak Dam for nursery egg incubation and the earliest stage of fry rearing, which nearly eliminated incidence of IHN disease in that stage of rearing. Additionally, the nearby Clearwater State Fish Hatchery (SFH), which operates entirely with reservoir water, has never had a case of IHN disease in juvenile steelhead trout. Therefore, staff at Dworshak NFH sought and obtained access to a limited supply of reservoir water for the first few months of outdoor rearing of juvenile steelhead trout, beginning in 2010. This strategy delayed the exposure of juvenile steelhead trout to river water for several months. The effects of this program change were: drastic reduction in IHN disease in juvenile steelhead trout; interruption in the transmission of highly virulent M group IHNV from adult steelhead trout; no interruption in the transmission of low virulent U group IHNV from adult Chinook salmon; and a shift of IHNV types in adult fish spawned at Dworshak NFH in subsequent years from M to U group viruses. While juvenile steelhead trout may still be infected via exposure to IHNV in river water, the disruption of virulent M group IHNV has been successful in dramatically reducing IHN disease in steelhead trout every year since 2010.

1. Introduction

Dworshak National Fish Hatchery is the largest steelhead trout conservation hatchery in Idaho. The US Army Corps of Engineers built it in 1969, before many important fish health pathogen control standards were developed. Juvenile steelhead trout at this facility suffered extremely high mortality in 2008 and 2009 when over 50% of the population was lost each year. Diagnostic tests revealed that the cause of the high mortality was infectious hematopoietic necrosis virus (IHNV), which had repeatedly caused disease outbreaks at the hatchery over the past 35 years, although not in every year. In response to these dramatic losses, hatchery staff designed a significant change to their rearing strategy for steelhead trout juveniles.

IHNV is a rhabdoviral pathogen that causes acute disease and mortality in salmonid fishes. It is endemic in western North America where it is a significant burden on hatchery-based salmon and trout conservation programs. IHN disease pathology is observed in the hematopoietic kidney and spleen tissues; disease occurs primarily in juvenile fish where it can cause mortality as high as 90%. Asymptomatic adult fish can have high viral loads at or near the time of spawning. Both adults and juveniles can transmit their infection (Mulcahy & Bauersfeld 1983; Mulcahy et al. 1983a; Mulcahy et al. 1983b; Traxler et al. 1993; Arkush et al. 2004). The primary route of IHNV transmission is horizontal, via waterborne virus, between fish of any age (Bootland & Leong 1999; Mulcahy et al. 1983b). Spawning adult fish may transmit IHNV to their offspring via egg-associated virus, but in hatcheries this route is usually blocked by egg disinfection, so hatchery fish generally start life free of IHNV infection (McDaniel, et. al., 1994).

Previous studies comparing genetic sequences of many isolates of IHNV from North America have revealed three distinct viral genetic groups (Kurath et al. 2003a). The M genogroup is found primarily in steelhead and rainbow trout (migratory and residential forms of *O. mykiss*, respectively) in Washington, Oregon, and Idaho, especially in the Columbia River Basin (Figure 1). The U genogroup of IHNV is found primarily in sockeye salmon (*O. nerka*) in Alaska, British Columbia, Washington, Oregon and Idaho, but also to a significant degree in Columbia River Basin Chinook salmon (*O. tshawytscha*) and steelhead trout. The L genogroup is found in Chinook salmon of

southern Oregon and northern California. Genetic typing of IHNV isolates based on sequence data from a variable 303 nucleotide segment of the virus glycoprotein gene, called the midG region, has been used for phylogeographic analyses. Each unique midG genetic sequence is called a ‘type’ and denoted by a universal sequence designator (USD) in the format mG###U/M/L, e.g. type mG001U. Many isolates may have the same type, and a single cohort of fish may have more than one type circulating among infected fish. When these midG types are combined with case-detection details like location, age, and species of the infected fish, IHNV transmission pathways can be inferred retrospectively (Breyta et al. 2013).

While egg disinfection protects hatchery juvenile fish from infection by their parents, horizontal transmission of IHNV is still a serious risk, as many hatcheries’ water supplies contain free-swimming and/or anadromous salmonids, which may be infected with IHNV. Resident, free-swimming fish like kokanee salmon or rainbow trout are less likely to become exposed to IHNV over their lifespan than anadromous fish, which may be exposed via numerous encounters with other salmonid populations during their lifespan. These unsecured water supplies have been described as likely sources of virus in juvenile IHN disease events (Wolf 1988); (Bootland et al. 1994; Bootland & Leong 1999; Anderson et al. 2000; Breyta et al. 2013)(Bendorf et al. 2007). Since the only truly ‘secure’ water supply is well or enclosed spring water, we define here alternative terms relative to the risk of IHNV in hatchery water supplies. The lowest IHNV-risk water supply is that with the lowest burden of IHNV infected fish, as defined by routine surveillance. A water supply with a low IHNV-risk includes surface water where migration of anadromous fish is prevented and resident fish are routinely tested and found negative for IHNV, as from behind a fish-impassable dam. Water supplies with a moderate IHNV-risk include those that lie upstream of fish barriers like weirs that can be breached occasionally by environmental conditions, or that use treatment systems that may fail, or those that alternate use of well and river water, for example. Alternating between water sources with different burdens of IHNV is usually the result of limited availability of the most secure water supply during summer and fall months. Unsecure water sources are those with the highest IHNV-risk such as surface water from lakes or rivers/creeks that can and frequently do contain migratory fish infected with IHNV.

Correlations between water supplies with a high virus burden and IHN disease emergence have been described in steelhead trout in coastal Washington in 1997 and 2007-2011 (Breyta et al. 2013). These emergence events were caused by a new incidence of trout-adapted M genogroup IHNV in a region where this virus had not been previously observed; genetic epidemiological analysis revealed that the most likely source of the M virus was populations of steelhead trout in the Columbia River Basin, including the Snake River watershed in northern Idaho (Breyta et al. 2013).

The Snake River is the largest tributary of the Columbia River Basin, and the Clearwater River watershed in north Idaho is the largest tributary of the Snake River (Figure 1). Ongoing IHNV surveillance (testing for virus presence and genetic typing of virus isolates) has detected fish infected with both U and M types in the Columbia River Basin as a whole, as well as within the Clearwater River (Table 1) (Garver et al. 2003; Kurath et al. 2003b). The Clearwater River is home to cultured and wild populations of steelhead trout and Chinook salmon, both susceptible to IHNV infection, as well as the more refractory coho salmon (*O. kisutch*). Of these species, steelhead trout are by far the most heavily burdened by IHN morbidity and mortality. There are a total of four federal, state, or tribal hatcheries on the Clearwater River system, rearing 1-3 populations of salmonids each (Figure 1, Table 2). The IHNV-risk of the water supply differs at each of the four hatcheries: the Nez Perce Tribal Fish Hatchery (TFH) uses a mix of river and well water year round, so its water supply is unsecure; Clearwater State Fish Hatchery (SFH) operates exclusively with water taken from the reservoir behind the Dworshak Dam, which is impassable to fish, so its water supply low IHNV-risk; Dworshak National Fish Hatchery (NFH) relied solely on unsecure river water when it was built in 1969 (more detail below); Kooskia NFH has a IHNV moderate IHNV-risk water supply, alternating between secure well water during March-October and unsecure creek water the rest of the year.

Within the Clearwater River watershed, Dworshak NFH has the largest summer steelhead trout program, and it is the largest in the state of Idaho. The primary water supply intake for Dworshak NFH is situated 1.3 river miles below the Dworshak Dam. The dam is impassible to fish, but spawning adult trout and salmon are routinely found in the river just below the dam, putting them in close proximity to the hatchery water intake.

From its first year of operation in 1969 until 1992, Dworshak NFH rearing relied on unsecure river water for all stages of juvenile fish husbandry. Since the first major IHNV epidemic at Dworshak NFH in 1982, juvenile steelhead trout there have suffered significant morbidity and mortality at every stage of rearing: in the nursery building where eggs are incubated and fry are reared until they are on feed, and in the outdoor ponds where juveniles are reared until release about one year after hatching. Between 1982-1989, 14 million fish died out of a total 21 million fish hatched, averaging 1,750,000 per year (Table 3). In 1992, the Army Corps of Engineers built a water line to supply low-risk reservoir water to the Dworshak NFH incubation and nursery building. From that time on, incidence of IHN disease in the nursery building was reduced to almost zero. The outdoor ponds still used the unsecure river water, however, and IHN disease was still a regular occurrence, causing mortality almost every year. In 2009, 6 of 10 rearing units of fish suffered epidemic IHNV, and mortality in these ponds reached 58%, which meant that the entire production year suffered 54.2% loss (Table 3). This level of mortality, which was the highest since the reservoir line to the nursery was installed, prompted Dworshak NFH staff to re-examine the established rearing practice for ways to improve steelhead trout juvenile survival. They implemented a complex re-design of water supply plumbing for outdoor rearing that delayed exposure of juvenile steelhead trout to unsecure river water. In addition, increased IHNV genetic typing surveillance was conducted, which revealed the epidemiological mechanism by which steelhead trout survival was improved.

While previous observations have suggested that hatchery water supply can be a significant source of waterborne virus into a hatchery, direct evidence of how the specific ecology can be manipulated is rare, though not unprecedented (Bendorf et al. 2007). In the study presented here, evidence for waterborne transmission of IHNV into juvenile steelhead trout through hatchery water supply is described, along with the effective alteration of this transmission route via a partial re-plumbing of the hatchery water supply. Only by including genetic surveillance of the virus in this strategy was it possible to conclusively demonstrate that the delayed exposure strategy was successful in protecting hatchery juvenile steelhead trout from adult-borne virus in the unsecure river water supply. The same genetic surveillance also indicated that while the delayed exposure

strategy significantly decreased IHN disease in juvenile steelhead trout, other strains of IHN present in other salmonid species in the river water were still able to infect hatchery juveniles.

2. Materials and Methods

2.1 Virus Isolates. The USGS Western Fisheries Research Center (WFRC) provides virus genetic typing by midG sequence analysis of IHN field isolates as a technical assistance service to fisheries managers throughout the Pacific Northwest. The data generated is maintained in a database at WFRC. This database has a freely accessible internet version at <http://gis.nacse.org/ihnv/>. For this study, IHN isolates and diagnostic records were obtained from fish health laboratory staff from the United States Fish and Wildlife Service, Idaho Department of Fish and Game, and from the archival IHN collection at the WFRC. Samples originated from dead, dying, or asymptomatic juvenile fish or from asymptomatic adult fish and were processed for virus isolation in cell culture using standardized protocols (Anonymous 2007). Virus culture supernatants of low passage number (≤ 3 passages for the majority of isolates) were sent to WFRC for analysis and archival storage at -80°C . The 609 IHN isolates analyzed in this study were collected from fish at a total of 9 sites in the Clearwater River Basin between 1984-2013; of those, 481 were isolated between 2008-2013. In addition to the 4 hatchery sites described in Figure 1 and Table 2, isolates also came from adult fish collected for spawning at 3 trap sites, and juveniles sampled at 2 acclimation sites. The majority of isolates were obtained from steelhead trout or Chinook salmon, but there were also 5 isolates from coho salmon, 5 from rainbow trout, and 3 from kokanee salmon.

2.2 Sampling and sample bias The majority of IHN isolates in this report are from adult or juvenile hatchery fish (97%). Wild fish isolates are a minor fraction because they are less commonly sampled. The majority of hatchery IHN surveillance comes from either screening or diagnostic testing strategies: routine adult screening, juvenile diagnostic testing, and a small amount of juvenile screening. These testing strategies create two kinds of sampling bias, both of which influence the data in this report. One occurs when few samples are submitted for sequence analysis from specific geographic areas because the presence of IHN there is not unusual. This pattern creates a surveillance-pressure bias: geographic areas with newly emergent IHN are sampled and

typed more heavily while fish in IHNV endemic areas are sampled and typed less intensely. The second form of bias is in the number of IHNV isolates submitted for typing. For example, one incident of juvenile epidemic IHN disease may be represented by only one or two isolates, while another may be represented by as many as thirty. If detections were analyzed by the raw number of isolates the two sampling biases would mask basic patterns. To correct both biases, isolates have been coded into positive fish cohorts (by year, collection site, age, species, and seasonal timing) and each is described as one ‘detection event’ (Breyta et al. 2013). If more than one genetic type (USD) was detected among multiple isolates from one cohort, a separate event was assigned for each USD detected. In the current report, situations with more than one type may be described as having a ‘dominant variant’ and ‘non-dominant variant(s)’. Using this bias correction, Table 4 shows detection events, not numbers of isolates.

2.3 Viral RNA extraction and sequence analysis Viral genomic RNA was extracted and analyzed according to established methods (Breyta et al. 2013). Briefly, viral RNA was phenol-chloroform extracted from 200-500µL of virus culture supernatant and a virus specific fragment amplified via RT-PCR. This specific DNA product was purified and used for sequencing.

2.4 Phylogenetic analysis Sequence data for positive and negative reads of each template were assembled, edited, and trimmed to the established midG 303nt fragment (Emmenegger et al. 2003; Garver et al. 2003; Troyer & Kurath 2003; Kurath et al. 2003a) using Sequencher software v4.9. Consensus midG sequences for each virus isolate were aligned in ClustalX (Qt/QMake), and manually inspected for artefactual gap insertion. The term ‘genotyping’ is used to describe this process, and the term ‘type’ is used to describe individual USD sequences (haplotypes). The term ‘genetic variant’ refers to a new type that is likely descended from a known, previously detected type; variants are often, but not always, initially detected in a minority of isolates within a positive population, with the parent type forming the majority. The sequence types are used as genetic tags for tracking transmission; any possible biological impacts of each mutation delineating the sequence types have not been determined. Newly generated sequences and previously known USD sequences representative of North American IHNV were used for phylogenetic analysis. Taxa with two USDs were used to indicate an isolate with

a single site of nucleotide sequence heterogeneity, indicating either a single fish with a dual infection or a pooled sample with two or more positive fish, each with different sequence types; these taxa were analyzed using the second USD sequence listed in the taxa name, the minority variant. The “HH” designation in a USD indicates an isolate with more than one nucleotide heterogeneity; this taxa was analyzed with the sites of heterogeneity treated as missing data.

Phylogenetic analysis was performed on midG sequence data either newly determined in this study or previously described (Kurath et al. 2003a; Breyta et al. 2013) and available at <http://gis.nacse.org/ihnv/>. Analysis was conducted using coalescent Bayesian MCMC methods as implemented in the BEAST software package v1.6.1 (Drummond et al. 2002; Drummond & Rambaut 2007). Results were analysed, annotated, and drawn using the complementary suite of programs, including Tracer v1.5 and FigTree v1.3.1 (Rambaut & Drummond 2003; Rambaut & Drummond 2007; Rambaut 2009). Dated taxa were used with a relaxed uncorrelated lognormal molecular clock prior, with a gamma-distributed rate prior extrapolated from the range of known error rates of RNA-dependent RNA polymerases (Drummond et al. 2006; Biek et al. 2006; Drummond & Suchard 2010). The relaxed clock was used for the midG analysis after it was confirmed that the strict clock could be rejected (95% confidence interval of the coefficient of variation and standard deviation of the relaxed clock did not span zero) and the relaxed clock yielded a positive Bayes Factor in comparison to the strict clock. The phylogenetic tree is derived from three separate MCMC analyses of 150 million generations that each achieved convergence and good mixing, and were subsequently combined, summarized, and analysed.

3. Results

3.1 High mortality in Dworshak NFH juvenile steelhead trout in 2009 associated with IHNV type mG139M. In 2009, Dworshak steelhead trout were reared according to the established schedule of hatching and early rearing within the nursery building, which was supplied by low risk Dworshak reservoir water. As rearing units (fish originating from a single spawn date) of juvenile steelhead trout reached target size between May and August, they were moved to outdoor ponds which were supplied with unsecure river water; one rearing unit is divided into several ponds. The numbers of fish per pond were set to allow the fish to stay in the same pond for the entire rearing period without reaching pond carrying capacity prior to release. This timing of exposure to unsecure river water will be referred to as ‘standard’ exposure. After an average of 32 days post-exposure to river water, ponds of steelhead trout began showing signs of IHN disease. Out of six ponds of fish in which virology tests confirmed the presence of IHNV (and likely cause of death), three had cumulative percent mortality (CPM) between 78-98% over the following months, and three were culled when CPM reached 48-79% (Figure 2A). Ponds where virus was either not detected (virology was performed and was negative) or not indicated (fish did not show symptoms and so no virology was performed) showed $\leq 20\%$ CPM over the same time period (Figure 2B). Among the 10 virus isolates analysed from this event, IHNV type mG139M was detected in 8 isolates while the remaining two isolates contained the new variant mG206M. Both IHNV types mG139M and mG206M are M group viruses in the well-supported MD subgroup (orange, Figure 3); mG139M has been described previously in steelhead trout mortality (Breyta et al. 2013).

3.2 Major virus type in Dworshak NFH adult steelhead trout in 2009 was mG139M. No IHN disease was observed in the nursery building in 2009, so egg-associated transmission due to a breach of biosecurity was not a likely cause of mortality in the juveniles that year. Thus the most likely source of transmission of mG139M into the ponds was via the hatchery intake of river water. Adult steelhead trout spawned at Dworshak NFH are a subset of the total population of steelhead trout adults in the river, making the virology and typing performed on the hatchery adults a proxy indicator of virus in adults contaminating the hatchery intake. From the 2009 hatchery spawned

steelhead trout adults, a total of 43 isolates were typed. Of these isolates, 38 (88%) were found to have mG139M, and 3 more isolates had types that were variants of mG139M, types mG229M and mG239M (Figure 3B). The remaining two isolates were U group IHNV type mG151U (star, Figure 3A).

3.3 Modified water supply and rearing practice to delay exposure to unsecure river water in Dworshak NFH steelhead trout juveniles in 2010. Since virus typing results from 2009 indicated that the unsecure water supply was the most likely source of IHNV in juvenile steelhead trout, major modifications were undertaken in 2010 to take advantage of available surplus Dworshak Dam reservoir water for the earliest weeks of outdoor rearing. To accomplish this, one of the four banks of ponds at Dworshak NFH was supplied with low IHNV-risk reservoir water only until September, when the reservoir water became unavailable and the ponds were transitioned back to unsecure river water. Fish lots coming out of the nursery, beginning in April/May, were initially placed in the low IHNV-risk water ponds, at higher than standard density. This meant that eventually the fish would become too crowded and require a second move, but kept the fish in the low risk water until that time. This modified timing of exposure to unsecure river water will be referred to as ‘delayed’ exposure.

In order to assess the effectiveness of this infrastructure change on the transmission of IHNV, juvenile fish were moved out of the nursery building using one of three ponding strategies: E) early hatching fish were moved first into low IHNV-risk reservoir water ponds to rear for ~2 months, and then were divided into ponds supplied by unsecure river water for the remainder of their rearing period; L) later hatching fish were moved first into the reservoir water ponds for a shorter time than group E, and then were slowly transitioned to unsecured river water; S) a small number of early hatching fish were moved directly into ponds of unsecured river water in June and July, to act as a control for ‘standard’ exposure to river water (Figure 4).

3.4 IHNV in standard exposed group S fish. For the control group S (Figure 5A, B) that was exposed to unsecure river water beginning in April/May, clinical signs of epidemic IHN disease were observed in 2 of 5 ponds, and mortality was observed at an average of 71 days post-exposure to river water. A total of 23 virus isolates from these fish were typed, and all were found to contain IHNV type mG139M. All fish in these two

ponds were culled before cumulative mortality passed 10% (Figure 5A, cross symbols). One of the remaining group S ponds was found to contain mG139M, but never developed IHN disease or elevated mortality (Figure 5A). The remaining two ponds of group S did not show signs of IHNV, and upon testing were found not to contain detectable virus (Figure 5B). Since the two ponds with disease were quickly culled, mortality levels cannot be compared to that of 2009 juveniles.

3.5 IHNV in delayed exposure groups E and L fish. Delayed exposure groups E and L were exposed to unsecure river water near the beginning of September. Surveillance for IHNV detected positive fish in 7 out of 23 of ponds tested (Figure 5C), at an average of 39 days after exposure to river water. Ten virus isolates from these ponds were all type mG174U, which is a U genogroup virus very different from the MD type mG139M (Figure 3A, C). Signs of clinical IHN disease were eventually observed, but mortality in delayed exposed ponds where mG174U virus was detected (Figure 5C) did not reach higher levels than randomly selected virus negative ponds (Figure 5D).

3.6 Virus types in adult steelhead trout and Chinook salmon in 2010. Virus isolated from spawned adult steelhead trout were typed to determine if they were the source of both M and U viruses detected in 2010 juvenile steelhead trout. Of 44 isolates typed, 10 were type mG139M, 33 were the variant type mG206M first detected in 2009 (Figure 3B), and the remaining isolate contained both types. Thus, all IHNV typed from adult steelhead trout in 2010 was of the MD subgroup, the subgroup that contains type mG139M. Since the 2010 juvenile fish in group S that were infected with M group IHNV were exposed to river water in June-July, and the juvenile fish in groups E and L that were infected with a U group IHNV were exposed in September-October, IHNV isolates from spring and fall Chinook salmon were also typed. These fish do not begin spawning in the Clearwater River until late summer/early fall, although they begin to arrive earlier. Of 47 isolates from Dworshak NFH Chinook salmon, 44 were found to have mG174U, and the remaining three isolates had unique mixed infections of mG174U and a variant of it: type mG174U/mG252U, type mG174U/ mG258U, and type mG174U/mG259U (Figure 3C).

3.7 Prevalence of U and M viruses in 2008 and 2009 adult fish at Dworshak NFH. To determine if U group IHNV, specifically type mG174U, had been present in similar

numbers in preceding years when mG174U was not detected in juvenile steelhead trout, additional isolates were typed. A total of 15 isolates were available for 2008 adult fish and 53 isolates were available for 2009 adult fish. Among isolates from 2008, no type mG174U was detected in either steelhead trout or Chinook salmon, but type mG139M was found in 11 steelhead trout isolates and 3 Chinook salmon isolates; the remaining isolate was type mG238M, a variant of type mG139M (Figure 3B). In isolates from 2009, there were also no detections of mG174U in Dworshak NFH adult steelhead trout or Chinook salmon, but type mG139M was found in 38 isolates of steelhead trout and 9 isolates of Chinook salmon (greater detail of 2009 adult steelhead trout isolates described above); remaining isolates were either type mG229M (2 isolates) or type mG239M (1 isolate), both types are variants of mG139M (Figure 3B). Also from 2009, U group IHNV was detected in one isolate of Chinook salmon and two isolates of steelhead trout adults, but these were type mG151U which is not related to type mG174U (star, Figure 3A).

3.8 Prevalence over time of U and M viruses in adult fish of the Clearwater River watershed. Since the adult fish spawned at Dworshak NFH represent only a portion of the adult fish in the wider Clearwater River watershed, typing was expanded to include a total of 413 isolates collected from adult fish returning to all four hatchery programs between 2008-2013. The number of isolates with type mG139M and its variants increased between 2008-2011, then fell sharply; type mG174U and its variants were not detectable in 2008, but built quickly to high levels between 2010-2012 (Figure 6A). There is a degree of host specificity evident, with the majority of type mG139M and its variants being found in steelhead trout (Figure 6B) and the majority of mG174U and its variants found in Chinook salmon (Figure 6C). The same pattern is evident in the number of detection events in each hatchery species population over the same time period (Table 4). The number of detections of M group IHNV (all were either type mG139M or its variants) in any species and any hatchery population fell dramatically in 2011, one year after the implementation of the delayed exposure rearing strategy at Dworshak NFH.

3.9 IHNV type mG174U detected in 2005 in the Clearwater River. Since type mG174U was detected in all years of the current study before becoming the dominant type, archival isolates were typed to determine where type mG174U may have originated.

IHNV type mG174U was first detected in late summer of 2005, in 2 isolates from adult Chinook salmon at Dworshak NFH and 1 isolate from adult Chinook salmon at Nez Perce TFH. During 2006-2007, it was detected in a total of 15 Chinook salmon isolates from these two sites as well as Kooskia NFH. This type has been detected elsewhere in the Snake River basin, both downstream and upstream of the confluence of the Clearwater River with the mainstem Snake River, since 2005. The type was not detected outside the Snake River watershed until 2013.

3.10 Incidence of IHNV remained detectable in adult fish but incidence of IHN disease fell in juvenile steelhead trout at Dworshak NFH in 2011-2013. Surveillance for IHNV in adult steelhead trout at Dworshak in 2011-2012 revealed a similar pattern as the wider Clearwater River. In 2011, 56 isolates from adult steelhead trout were positive with M (54 with type mG139M and 2 with variant type mG236M), and 6 isolates had a U (4 with type mG174U and 2 with variant type mG242U); in 2012 there were 3 isolates with M (all mG139M) and 56 with U (24 with mG174U, 30 with variant type mG242U, and one each with variant types mG266U and mG267U); and in 2013 only 5 isolates were positive, all with U type mG174U. Despite the significant numbers of virus-positive adult steelhead trout at Dworshak NFH, and presumably also in fish contaminating the hatchery water intake, incidence of IHN disease in juvenile steelhead trout was all but eliminated in 2011 and 2012 (Table 3). There was one case of IHN disease in several nursery tanks in 2011, but this was due to river water being mistakenly turned on for the tanks (two isolates from this event were both type mG139M); the fish were euthanized and no other cases of IHN were observed in the 2011 juveniles. In 2013, there was a low amount of mortality in one pond of juvenile steelhead trout, reared according to the delayed strategy described above. Five isolates from this group were typed, and 4 isolates were type mG174U and one was a mix of mG174U and new variant type mG301U (Figure 3C). During 2011-2013 when the delayed river water exposure was used for rearing steelhead trout, annual mortality was 2%, comprising a major improvement compared to years before implementation of the new rearing strategy (Table 3).

3.11 Prevalence of IHNV in free-swimming steelhead trout adults increases as maturity is reached. Naturally spawning fish reach the area of their spawning grounds weeks or months before they are sexually mature, and spend the intervening maturation

time selecting mates and spawning sites. In a hatchery, newly returning fish are usually attracted up a ladder and into a holding pond where they are held and monitored for maturation, at which time they are removed and spawned artificially by hatchery personnel. This holding period has been observed to amplify prevalence of IHNV in adult fish via horizontal transmission, possibly making hatchery adults a poor proxy of virus prevalence levels in free-swimming fish. Since free-swimming fish appear to be the source of transmission into Dworshak NFH, during 2011-2014 a subset of adult steelhead trout were sampled for IHNV immediately upon entry into the holding ponds in order to determine the prevalence of IHNV infection in free-swimming maturing fish. Fish were noted as immature or mature at the time of sampling. Before fish reached maturity, prevalence levels were below 15%, and after this time climbed steadily to 42-63% in 2011-2013, or to 18% in 2014 (Figure 7, A-D). Genetic typing was performed on a subset of positive isolates from 2011-2013, and all were either type mG139M or a variant type, or type mG174U or a variant type (Figure 7, A-C). Each year that free-swimming fish were sampled, the proportion of U versus M viruses did not change dramatically over the period of maturation, and each year there was a clear predominance of U or M viruses detected (Figure 7E). In 2011, the dominant IHNV type in free-swimming adult steelhead trout was the M group type mG139M and variants, but the U group type mG174U and variants dominated in 2012 and 2013 (Figure 7E), matching the observation of steelhead trout held and spawned traditionally (Figure 6B). Even without artificial hatchery holding, maturing fish clearly develop higher levels of virus prevalence as they approach sexual maturity, and during this time the ratio of U to M viruses in the fish did not change.

4. Discussion

Water quality is widely accepted as the single most important factor in maintaining good fish health. When considering horizontally transmitted infections like IHNV and conservation salmon/trout hatcheries, water quality equals water security. As defined here, unsecure water supplies contain susceptible migratory species infected with IHNV, which can expose all the juvenile fish in a hatchery and pose a significant health risk. The experiment in virus control undertaken in 2010 at Dworshak NFH to increase water security for early juvenile steelhead trout rearing has revealed several insights. Not only did the water supply changes at Dworshak NFH significantly reduce IHN disease in

juvenile steelhead trout, it also provided a rare insight into IHNV transmission mechanisms at work in an unsecure water supply. This partial switch to a water supply with a lower burden of IHNV was clearly successful, but the reservoir itself remains vulnerable to a change in IHNV burden as it contains susceptible species that could become infected if exposed to IHNV.

In the two years before the experiment was begun at Dworshak NFH, when juvenile fish were still reared with unsecure river water, adult steelhead trout were found infected primarily with IHNV type mG139M. Steelhead trout sampled anywhere in the Clearwater River those two years were also predominantly infected with type mG139M, indicating that the dominance of this M type was basin-wide, not just a Dworshak phenomenon. Also in 2008-2009, juvenile steelhead trout at Dworshak NFH suffered high mortality. Isolates from juvenile fish in both years had the same type mG139M as the adults. While it is not possible to rule out a breakdown of biosecurity (e.g. egg and equipment decontamination protocols) in causing the transmission of type mG139M into juvenile fish, it is unlikely due to the delay between the handling of infected adult fish (no later than April) and the onset of mortality in juvenile fish (no earlier than June). While there were small numbers of Chinook salmon adults positive with type mG139M in 2008-2009, the timing of these detections does not indicated these fish as likely transmission sources into the juvenile steelhead trout since onset of mortality occurred in June-July, before Chinook salmon adults are spawning in the Clearwater River. Therefore, the most probable transmission source for the juvenile epidemic IHN in 2008-2009 was free-swimming steelhead trout adults contaminating the hatchery intake.

In 2010, a major re-engineering of the hatchery water supply was implemented. This supplied one bank of outdoor rearing ponds with the more secure Dworshak Dam reservoir water, and allowed a delay in the timing of exposure of juvenile fish to unsecure river water. As in the previous two years, adult steelhead trout in 2010 were primarily infected with type mG139M. Unlike 2008-2009, however, high levels of mortality did not develop in juvenile steelhead trout in the delayed exposure groups. Two control ponds of fish (group S) developed IHN after being exposed to unsecure river water at the standard timing, in June/July; these juvenile fish had type mG139M and were culled immediately. This indicates that the risk of transmission of type mG139M via unsecure

river water was still present in 2010, and that the reason IHN disease was lower in 2010 compared to 2009 for the delayed exposure groups was due to the increased security of water during the summer of 2010. Among ponds exposed to unsecure river water at the delayed timing (groups E and L), IHNV was detected, but these fish were infected with a completely different virus type mG174U. Fish in these ponds developed a low-mortality presentation of IHN disease, which is consistent with U group IHNV in steelhead trout (LaPatra et al. 1993; Garver et al. 2006; Breyta et al. 2014). This type mG174U was not detected at all in adult steelhead trout in 2010, but it was detected in nearly all Chinook salmon adult isolates that year, both at Dworshak NFH and elsewhere in the Clearwater River. This suggests that the transmission route was still the unsecure river water, but that the source population had shifted to adult fish of the species spawning in the river later in the year. This also indicates that the larger/older juvenile fish are not entirely refractory to infection. So while the virulent trout-adapted IHNV type mG139M was effectively controlled in steelhead trout juveniles at Dworshak NFH, juvenile fish were still being infected with IHNV via unsecure river water. These results also indicate that in previous years, steelhead trout at Dworshak NFH were likely exposed to Chinook salmon virus types, but that those infections were masked by the virulent impact of trout-adapted viruses being transmitted earlier in the year by steelhead trout adults. Since juvenile Chinook salmon are also reared at Dworshak NFH unsecure river water poses a significant risk of transmission to two susceptible juvenile fish cohorts each year.

In 2011, the pattern was nearly the same as 2010 in that the majority of steelhead trout adults were found to contain type mG139M, but there were no cases of IHN disease in outdoor ponds of juvenile steelhead trout. There were also a significant number of type mG174U detections in Chinook salmon around the time that steelhead trout were being first exposed to unsecure river water, but no IHN disease was observed.

In 2012-2013, there was a shift apparent in the IHNV types present in returning adult steelhead trout. No longer predominantly the M group type mG139M, adult steelhead trout were instead primarily infected with mG174U. Adult Chinook salmon were also primarily infected with type mG174U during 2012-2013. Juvenile steelhead trout did not succumb to IHNV disease in 2012, of any type, but a small amount of mortality was observed in 2013; these juvenile fish were exposed at the delayed timing and were found

to have type mG174U. The U group IHNV type mG174U appears less capable of causing frequent or severe incidence of IHN disease in steelhead trout juvenile fish, as predicted from previous studies of U group IHNV in *O. mykiss* (LaPatra et al. 1993; Garver et al. 2006; Breyta et al. 2014).

The more recent preponderance of U group IHNV in the Clearwater River watershed, especially in Chinook salmon, may pose risk to future generations of that species. Dworshak NFH was the only hatchery in the watershed where steelhead trout juveniles suffered high mortality from the M group IHNV type mG139M because it was the only facility in the watershed that had both steelhead trout juveniles and an unsecure water supply for their rearing. Since type mG174U is transmitted by Chinook salmon adults and is now detected in the majority of isolates throughout the basin, the juvenile Chinook salmon programs with high or moderate low IHNV-risk water supplies at Nez Perce TFH and Kooskia NFH may face an increasing risk of infection, and potentially disease. In fact, Chinook salmon juveniles at Nez Perce TFH have had more incidence of IHN disease in the last 5 years than were observed in the preceding decade (personal communication, Marilyn Blair, USFWS). This increase of observed IHN disease in Chinook salmon juveniles is similar to observations in the wider Columbia River Basin, where IHN disease in Chinook salmon juveniles is becoming more noticeable (Breyta and Kurath, unpublished).

The incidence of IHN disease caused by M group IHNV in steelhead trout juveniles at Dworshak NFH has been dramatically reduced, but there is also a significant decline in detection of M group in steelhead trout adults. This might indicate that more has changed in the ecology of this virus than just transmission from adults into juveniles at Dworshak NFH. It may be that epidemic IHN disease in juvenile fish plays a critical role in a cycle of maintenance of M group IHNV: if infected juveniles shed virus into the river and increase the prevalence levels in returning adults, then disrupting the juvenile half of the cycle could effectively disrupt the adult half of the cycle as well. It may also be that prevalence of IHNV in returning adults is partly influenced by persistence of virus in survivors of epidemic IHN disease to the point that returning adults have the same type they were infected with as juveniles. Evidence for this is extremely limited, and whether or not salmonid fish can recover from IHNV infection but still contain viable virus that

may reactivate at a later time is therefore controversial, and is often referred to as a ‘life-long carrier state’. If this were the case, however, there should have been a detectable spike of type mG139M in survivors of the 2009 disease outbreaks returning to Dworshak NFH. Those fish would have returned in small numbers in 2012 and in greater numbers in 2013. Since type mG174U was dominant at Dworshak NFH in those years, a spike of type mG139M would have been noticeable among the large number of typed isolates from adult fish. The only detection of type mG139M in those years was in 4 of 62 isolates from adult steelhead trout in 2012 (6.5%), and there were none at all in 2013 (there were 17 isolates typed, making a lower limit of detection of 6%). While this suggests that persistently infected survivors of the 2009 epidemic did not comprise more than 6% of the returning population in 2013, there may have been some detected in 2012, which could also represent persistently infected survivors of the 2008 epidemic. Further analyses of those 4 isolates of type mG139M in 2012 may yield important clues about the role of persistence in IHNV transmission ecology.

Finally, it may be that the decline in M group IHNV detection in steelhead trout of the Clearwater River Basin since 2011 is linked to a similar pattern in the wider Columbia River Basin. Between 2011-2014, there have been few detections of any M group IHNV virus type in steelhead trout, of any age. In some segments of the Columbia River Basin, M group IHNV has not been detected at all during this time (Breyta and Kurath, unpublished). A period of waning of M group IHNV has been detected in the Columbia River Basin before, between 1999-2001, which was followed by an explosive emergence of M group IHNV viruses in 2002 at many locations (Garver et al. 2003)Breyta and Kurath, unpublished). Whatever mechanism drives these periodic cycles of emergence and waning in M group IHNV incidence in the entire Columbia River basin may also explain, at least in part, the observed pattern in the Clearwater River watershed. Thus, it is possible that the primary driver of this pattern is either unidentified, or that multiple mechanisms are at work.

This study has provided a nearly unparalleled insight into the mechanisms by which a virus control strategy was able to effect a major improvement in the health of hatchery steelhead trout juvenile fish. Without genetic typing surveillance, the results of the water security experiment at Dworshak NFH would have been difficult to interpret because

IHN disease was not eliminated by the water security program, it was altered. Genetic typing surveillance revealed that reduction in high mortality IHNV disease was due to the disruption of transmission of M group virus from adult steelhead trout and that lower mortality infection still occurred via infected Chinook salmon adults contaminating the water supply with U group virus. A previous study in California described a similar pattern: a large scale Chinook salmon hatchery suffered significant disease due to L genogroup IHNV, and mortality which was linked to a program of planting IHNV susceptible fish into the reservoir that served as the hatchery's water supply. When the planting program was discontinued, this resolved that hatchery's IHNV problems (Bendorf et al. 2007). Furthermore, this study at Dworshak NFH revealed a major shift in circulating IHNV viruses, from M group to U group viruses, within the Clearwater River watershed. Continuing virus surveillance will reveal how stable the new patterns described here will be in the future.

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Literature Cited

603 American Fisheries Society Suggested Procedures for the Detection and Identification of Certain
604 Finfish and Shellfish Pathogens. (2007) American Fisheries Society, Bethesda, Maryland

605 Anderson ED, Engelking HM, Emmenegger EJ, Kurath G (2000) Molecular Epidemiology
606 Reveals Emergence of a Virulent Infectious Hematopoietic Necrosis (IHN) Virus Strain in Wild
607 Salmon and Its Transmission to Hatchery Fish. *J Aquat Anim Health* 12:85-99

608 Arkush KD, Mendonca HL, McBride AM, Hedrick RP (2004) Susceptibility of captive adult
609 winter-run Chinook salmon *Oncorhynchus tshawytscha* to waterborne exposures with infectious
610 hematopoietic necrosis virus (IHNV). *Dis Aquat Organ* 59:211-216

611 Bendorf CM, Kelley GO, Yun SC, Kurath G, Andree KB, Hedrick RP (2007) Genetic diversity of
612 infectious hematopoietic necrosis virus from Feather River and Lake Oroville, California, and
613 virulence of selected isolates for Chinook salmon and rainbow trout. *J Aquat Anim Health*
614 19:254-269

615 Biek R, Drummond AJ, Poss M (2006) A virus reveals population structure and recent
616 demographic history of its carnivore host. *Science* 311:538-541

617 Bootland LM, Leong JC (1999) Infectious hematopoietic necrosis virus. In: Woo PTK, Bruno
618 DW (eds) *Fish diseases and disorders* CAB International, New York, pp 57-121

619 Bootland LM, Lorz HV, Drolet BS, Chen SJ, Fryer JL, Rohovec JS, Leong JC (1994) Current
620 status of studies on the life cycle of infectious hematopoietic necrosis virus (IHNV). In:
621 Anonymous International Symposium on Aquatic Animal Health University of California School
622 of Veterinary Medicine, Davis, CA, p W-4.1

623 Breyta R, Jones A, Kurath G (2014) Differential susceptibility in steelhead trout populations to an
624 emergent MD strain of infectious hematopoietic necrosis virus. *Dis Aquat Organ* 112:17-28,
625 DOI:10.3354/dao02781 [doi]

626 Breyta R, Jones A, Stewart B, Brunson R, Thomas J, Kerwin J, Bertolini J, Mumford S, Patterson
627 C, Kurath G (2013) Emergence of MD type infectious hematopoietic necrosis virus in
628 Washington State coastal steelhead trout. *Dis Aquat Org* 104:179 <last_page> 195,
629 DOI:10.3354/dao02596

630 Drummond AJ, Ho SY, Phillips MJ, Rambaut A (2006) Relaxed phylogenetics and dating with
631 confidence. *PLoS Biol* 4:e88

632 Drummond AJ, Nicholls G, Rodrigo A, Solomon W (2002) Estimating mutation parameters,
633 population history and genealogy simultaneously from temporally spaced sequence data. *Genetics*
634 161:1307-1320

635 Drummond A, Suchard M (2010) Bayesian random local clocks, or one rate to rule them all.
636 *BMC Biology* 8:114

637 Drummond A, Rambaut A (2007) BEAST: Bayesian evolutionary analysis by sampling trees.
638 BMC Evolutionary Biology 7:214

639 Emmenegger EJ, Troyer RM, Kurath G (2003) Characterization of the mutant spectra of a fish
640 RNA virus within individual hosts during natural infections. Virus Res 96:15-25

641 Garver KA, Troyer RM, Kurath G (2003) Two distinct phylogenetic clades of infectious
642 hematopoietic necrosis virus overlap within the Columbia River basin. Dis Aquat Organ 55:187-
643 203

644 Garver K, Batts W, Kurath G (2006) Virulence Comparisons of Infectious Hematopoietic
645 Necrosis Virus U and M Genogroups in Sockeye Salmon and Rainbow Trout. Journal of Aquatic
646 Animal Health 18:232-243

647 Kurath G, Garver KA, Troyer RM, Emmenegger EJ, Einer-Jensen K, Anderson ED (2003a)
648 Phylogeography of infectious haematopoietic necrosis virus in North America. J Gen Virol
649 84:803-814

650 Kurath G, Garver K, Troyer R (2003b) Infectious Hematopoietic Necrosis Virus Traffic in the
651 Columbia River Basin. Propagated Fish and Resource Management (American Fisheries Society
652 Symposium) 44:539-548

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

655 Mulcahy D, Bauersfeld K (1983) Effect of loading density of sockeye salmon, *Oncorhynchus*
656 *nerka* (Walbaum), eggs in incubation boxes on mortality caused by infectious haematopoietic
657 necrosis. J Fish Dis 6:189-193

658 Mulcahy D, Pascho R, Jenes CK (1983a) Mortality due to infectious hematopoietic necrosis of
659 sockeye salmon (*Oncorhynchus nerka*) fry in streamside egg incubation boxes.
660 Can J Fish Aquat Sci 40:1511-1516

661 Mulcahy D, Pascho RJ, Jenes CK (1983b) Detection of infectious hematopoietic necrosis virus in
662 river water and demonstration of waterborne transmission. J Fish Dis 6:321-330

663 Rambaut A (2009) <http://tree.bio.ed.ac.uk/software/figtree/>.

664 Rambaut A, Drummond AJ (2007) Tracer v1.4, Available from <http://beast.bio.ed.ac.uk/Tracer>.

665 Rambaut A, Drummond AJ (2003) Tracer [computer program].

666 Traxler GS, Roome JR, Kent ML (1993) Transmission of infectious hematopoietic necrosis virus
667 in seawater. Dis Aquat Org 16:111-114

668 Troyer RM, Kurath G (2003) Molecular epidemiology of infectious hematopoietic necrosis virus
669 reveals complex virus traffic and evolution within southern Idaho aquaculture. Dis Aquat Organ
670 55:175-185

671 Wolf K (1988) Infectious hematopoietic necrosis virus. In: Anonymous Fish viruses and fish viral
672 diseases Cornell University Press, Ithaca, NY, pp 83-114

673 McDaniel, et. al., 1994

674 http://www.adfg.alaska.gov/static/fishing/pdfs/hatcheries/sockeye_salmon_culture_manual.pdf

675