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Emergence of MD type infectious hematopoietic necrosis virus in Washington State coastal steelhead trout

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ABSTRACT: Infectious hematopoietic necrosis virus (IHNV) occurs in North America as 3 major phylogenetic groups designated U, M, and L. In coastal Washington State, IHNV has historically consisted of U genogroup viruses found predominantly in sockeye salmon *Oncorhynchus nerka*. M genogroup IHNV, which has host-specific virulence for rainbow and steelhead trout *O. mykiss*, was detected only once in coastal Washington prior to 2007, in an epidemic among juvenile steelhead trout in 1997. Beginning in 2007 and continuing through 2011, there were 8 IHNV epidemics in juvenile steelhead trout, involving 7 different fish culture facilities in 4 separate watersheds. During the same time period, IHNV was also detected in asymptomatic adult steelhead trout from 6 coastal watersheds. Genetic typing of 283 recent virus isolates from coastal Washington revealed that the great majority were in the M genogroup of IHNV and that there were 2 distinct waves of viral emergence between the years 2007 and 2011. IHNV type mG110M was dominant in coastal steelhead trout during 2007 to 2009, and type mG139M was dominant between 2010 and 2011. Phylogenetic analysis of viral isolates indicated that all coastal M genogroup viruses detected in 1997 and 2007 to 2011 were part of the MD subgroup and that several novel genetic variants related to the dominant types arose in the coastal sites. Comparison of spatial and temporal incidence of coastal MD viruses with that of the rest of the Pacific Northwest indicated that the likely source of the emergent viruses was Columbia River Basin steelhead trout.

KEY WORDS: IHNV · Phylogenetic · Rhabdovirus · *Oncorhynchus mykiss* · *Oncorhynchus nerka* · Washington · North America

INTRODUCTION

Infectious hematopoietic necrosis virus (IHNV) is a species of single-stranded negative-sense RNA virus in the family *Rhabdoviridae* (Wolf 1988). It infects Pacific salmonids, primarily sockeye salmon *Oncorhynchus nerka*, Chinook salmon *O. tshawytscha*, and steelhead and rainbow trout (both *O. mykiss*). In these hosts, the virus typically causes acute disease in juvenile fish and asymptomatic infection in adults. Both juvenile and adult fish can transmit IHNV horizontally through water. Transmission from infected adults to progeny has also been observed via egg-associated virus (Amend 1975, Meyers 1998), but this is greatly reduced or eliminated in fish culture facilities by egg disinfection with iodophor. As observed

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in captive fish, acute IHN disease in juveniles is often associated with epidemic mortality, which can be up to 90% (Groberg 1983a,b, LaPatra et al. 1993a,b, Bootland & Leong 1999).

A viral genetic typing method has been established for molecular epidemiology and phylogenetic analyses of IHNV field isolates. It compares the consensus genetic sequence of a variable 303 nucleotide region in the middle of the viral glycoprotein gene (termed 'midG') (Emmenegger et al. 2000, Kurath et al. 2003). The midG sequencing method has been used previously to describe the genetic diversity of IHNV in Alaska (Emmenegger et al. 2000), British Columbia (Kurath et al. 2003), the Columbia River Basin (Garver et al. 2003) including the Idaho Hagerman Valley trout farming region (Troyer et al. 2000, Troyer & Kurath 2003), California (Bendorf et al. 2007, Kelley et al. 2007), and the coastal Washington and Puget Sound regions (Emmenegger & Kurath 2002). These studies revealed that IHNV in North America occurs as 3 phylogenetic genogroups designated U, M, and L (Kurath et al. 2003). Every unique midG sequence discovered by this method is now given a specific identifier referred to as a universal sequence designator (USD), in the format mG###, followed by U, M, or L to indicate the major genogroup to which the type belongs. IHNV field isolates from Alaska, Western Canada, Puget Sound, and coastal Washington prior to 2007 are in the U (upper region) genogroup and have relatively low genetic diversity. In contrast, isolates from Idaho trout farms in the Hagerman Valley are in the M (middle region) genogroup that has been reported to have more than 5 times the genetic diversity of the U genogroup, most likely related to the adaptation of the virus to a new host and a warmer environment (Kurath et al. 2003). Sequence types in the M genogroup have been reported as falling into several phylogentically defined subgroups designated MA to MF. In the Columbia River Basin of Idaho, Washington, and Oregon, the U and M genogroups co-occur. California isolates are all in the L (lower region) genogroup, which is also detected in the Southern Oregon coastal region.

In addition to geographic differences, there is some host specificity associated with the IHNV genogroups: U genogroup viruses cause high morbidity and mortality in sockeye salmon, but have little disease impact in rainbow and steelhead trout; M genogroup viruses cause high levels of mortality in rainbow and steelhead trout but not sockeye salmon (Garver et al. 2006, Peñaranda et al. 2009, Purcell et al. 2009); and L genogroup viruses cause mortality in Chinook salmon (Bendorf et al. 2007, Kelley et al. 2007). This host specificity is not absolute, and all 3 primary host species (as well as other more refractory species) can be infected by virus isolates from each genogroup (Garver et al. 2003, 2006, Kelley et al. 2007, Peñaranda et al. 2009, Purcell et al. 2009).

In Washington State, different genogroups of IHNV are considered endemic in different regions. The Columbia River Basin is a large complex watershed that extends throughout much of inland Washington, Oregon, and Idaho (Fig. 1B). Separate from the Columbia River Basin are the river systems of western Washington, which can be divided into 2 regions (Fig. 1A). The Salish Sea region is defined as any watershed draining to saltwater east of Cape Flattery and south

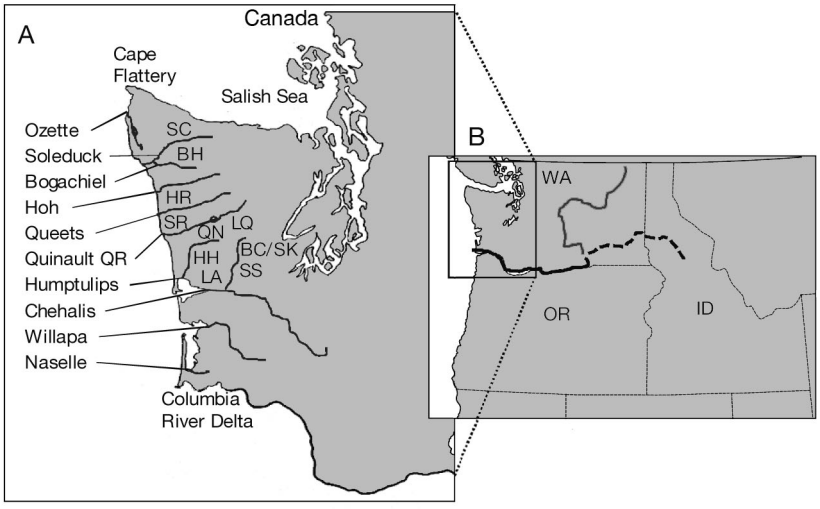

Fig. 1. Washington coastal rivers and relationship to the Columbia River Basin. (A) Coastal river names are shown at far left, and geographic features of western Washington are indicated as Canada (Canadian border), Salish Sea, Cape Flattery, and Columbia River Delta. The 11 fish culture facilities or fishery locations that were collection sites for coastal IHNV isolates are shown as: HH, Humptulips Hatchery; LA, Lake Aberdeen Hatchery; BC/SK, Bingham Creek Hatchery and associated Skookumchuck adult trap; SS, Satsop Springs Co-op; QR, Quinault River; QN, Quinault National Fish Hatchery; LQ, Lake Quinault Tribal Fish Hatchery; SR, Salmon River Tribal Fish Hatchery; HR, Hoh River; BH, Bogachiel Hatchery; and SC, Snider Creek Co-op. Both the Humptulips and Chehalis rivers drain into the large Grays Harbor estuary, which lies to the north of the Willapa Bay estuary. (B) The Columbia River Basin: the Lower Columbia River in solid black; the Upper Columbia in solid gray; and the Lower Snake River as a black dashed line

of the Canadian Desolation Sound (northern end of the Strait of Georgia). The coastal region is made up of rivers north of the Columbia River delta and south of Cape Flattery that drain directly into the Pacific Ocean (Fig. 1A). The western Washington rivers are shorter independent watersheds that are only linked via the ocean. All watersheds identified in this study have both hatchery and naturally produced salmonids, although to differing degrees. The Salish Sea and coastal regions have a long history of U genogroup IHNV detection (Emmenegger & Kurath 2002), while the Columbia River Basin is considered endemic for both U and M genogroup IHNV (Garver et al. 2003).

The rivers of coastal Washington have historically had a low but consistent prevalence of IHNV detection. Genetic typing of coastal IHNV isolates collected between 1976 and 1996 revealed that all were in the U genogroup (Table 1), primarily in sockeye salmon (6/12 isolates), but also in Chinook salmon (2/12 isolates), coho salmon *Oncorhynchus kisutch* (1/12 isolates) and steelhead or rainbow trout (2/12) (Emmenegger & Kurath 2002, R. Breyta & G. Kurath unpubl. data). These years of low detection levels of IHNV in coastal Washington were not due to low levels of IHNV surveillance. During these years, IHNV was a serious and growing problem for Columbia River Basin facilities operated by many of the same agencies operating in the coastal region, so screening was conducted at levels roughly equivalent across the entire state; incidence of IHNV in coastal salmonid species was lower than in the Columbia Basin for an unknown reason. As shown in Table 1, U genogroup IHNV has continued to occur in coastal watersheds with occasional detections through to 2011 (R. Breyta & G. Kurath unpubl. data).

Prior to 2007, the single known exception to the dominance of U genogroup IHNV in coastal Washington was one M genogroup isolate obtained in May 1997 from juvenile steelhead trout undergoing epidemic disease at the Salmon River hatchery in the Queets River watershed (Emmenegger & Kurath 2002). Genetic typing revealed that the epidemic was caused by an M group virus in the MD subgroup, specifically type mG111M (previously referred to as type 22). This incident was the first detection of an M genogroup IHNV outside of the Columbia River Basin. This epidemic of IHN in steelhead trout was highly unusual for a coastal watershed and all steelhead trout at the hatchery were euthanized to prevent the spread of the virus. For 10 yr following this eradication effort there was no subsequent detection of M genogroup IHNV in coastal watersheds, suggesting that the emergent mG111M virus did not spread or persist.

Table 1. General features of IHNV detections in salmonid species in the Washington coastal region from 1976 to 2011. Data shown is biological years (see 'Materials and methods'); only years for which IHNV isolates have been typed by midG sequencing are shown (missing years do not indicate a gap in IHNV surveillance). Data for 1976 to 1997 is from Emmenegger & Kurath (2002), and 2004 to 2011 data is from this study. Two archival isolates from 1989 (*) are also new in this article

Biological year	No. of detection events	No. of IHNV isolates	Host species[a]	Host life stage	Genogroup
1976	1	1	Unknown	Unknown	U
1977	1	1	Rainbow	Unknown	U
1988	1	1	Sockeye	Adult	U
1989	1	1	Coho	Adult	U
	1*	2*	Chinook	Adult	U
1990	1	1	Sockeye	Adult	U
1992	1	1	Sockeye	Adult	U
	1	1	Steelhead	Adult	U
1993	1	1	Sockeye	Juv	U
1994	1	1	Sockeye	Adult	U
1996	1	1	Sockeye	Adult	U
1997	1	1	Steelhead	Juv	M
2004	1	1	Steelhead	Adult	U
2007	5	9	Steelhead	Adult	M
	3	3	Steelhead	Juv	M
	1	1	Rainbow	Adult	M
	1	1	Rainbow	Juv	M
	1	1	Coho	Adult	M
	1	1	Chum	Adult	M
	1	1	Chinook	Adult	M
2008	3	13	Steelhead	Adult	M
	9	22	Steelhead	Juv	M
	1	1	Rainbow	Juv	M
	1	2	Coho	Adult	M
	1	1	Chinook	Adult	M
2009	6	28	Steelhead	Adult	M
	1[b]	2[b]	Steelhead	Juv	M
	1	1	Chinook	Adult	M
2010	9	72	Steelhead	Adult	M
	3	48	Steelhead	Juv	M
	1	1	Chinook	Adult	M
	3	4	Sockeye	Adult	U
	1	1	Sockeye	Juv	U
2011	2	4	Steelhead	Adult	U
	2	32	Steelhead	Adult	M
	3	31	Steelhead	Juv	M
	2	2	Sockeye	Adult	U
	2	4	Sockeye	Juv	U

[a]Host species: Rainbow, rainbow trout; Sockeye, sockeye salmon; Coho, coho salmon; Chinook, Chinook salmon; Steelhead, steelhead trout; Chum, chum salmon

[b]Events and isolates that are part of an event from a different biological year; see 'Materials and methods' subsection on 'Sampling and steelhead rearing schedule' for definitions

Starting in 2007 and persisting through 2011, IHNV has again emerged in steelhead trout populations of coastal Washington watersheds. In May 2007, an epidemic of IHN occurred in juvenile steelhead trout, again at the Salmon River hatchery. An isolate from this event was typed as an M genogroup virus in the MD subgroup (this article). Since then, M genogroup IHNV has been detected in coastal steelhead trout adults every year through to 2011, and confirmed epidemics or elevated mortality due to M genogroup IHNV has occurred in juvenile hatchery steelhead populations in 2008, 2010, and 2011. This article presents genetic typing data for 283 isolates collected from fish at 12 coastal sites in 7 different watersheds between 2007 and 2011. The majority of these virus isolates (260, 92%) came from steelhead or rainbow trout (*Oncorhynchus mykiss*) adult (59%) and juvenile (41%) fish (see Table 2). These isolates were nearly all in the M genogroup, indicating a major shift in the dominant genogroup of IHNV at coastal sites from U to M during these years (Table 1), along with the major increase of burden in steelhead trout hosts compared to sockeye salmon hosts. Genetic typing reported here revealed 2 waves of emergence between 2007 and 2011 that are genetically distinct from the first documented emergence of M genogroup IHNV (type mG111M) in coastal steelhead trout in 1997.

The emergence of M genogroup IHNV in coastal Washington suggests that the virus is expanding in geographic range and that other steelhead trout populations might be at risk for future emergence. At present, no M genogroup IHNV has ever been detected in the adjacent Salish Sea region (Fig. 1A). Since this region contains naïve wild and hatchery populations of steelhead trout, including some that are listed as threatened or endangered, a better understanding of the region-wide epidemiology of IHNV is needed. The purpose of this study is to document the emergence of M group IHNV in Washington coastal steelhead trout populations, and to use molecular epidemiology to gain insight into probable sources and transmission routes of the virus associated with this emergence.

MATERIALS AND METHODS

Virus isolates

The USGS Western Fisheries Research Center (WFRC) provides midG sequence analysis of IHNV 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/.

IHNV isolates and diagnostic records were obtained from fish health laboratory staff from the United States Fish and Wildlife Service, Washington Department of Fish and Wildlife, Northwest Indian Fisheries Commission, and from the archival collection at the WFRC. Samples were taken from dead, dying, or asymptomatic juvenile fish or from asymptomatic adult fish and processed for virus isolation in cell culture using standardized protocols (American Fisheries Society 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 283 IHNV isolates analyzed here were collected from fish at a total of 12 sites in 7 different coastal Washington watersheds between 2007 and 2011. The majority were obtained from steelhead or rainbow trout, but there were also 5 isolates from sockeye salmon, 3 from Chinook salmon, 4 from coho salmon, and 1 from chum salmon *Oncorhynchus keta*.

Sampling and steelhead rearing schedule

The majority of IHNV isolates in this report and in the WFRC database are from adult or juvenile hatchery fish (80 to 95%). Wild fish isolates are a minor fraction because they are less commonly sampled. The following rearing schedule refers to the focal host populations of this report, steelhead trout in coastal fish culture hatcheries. Winter-run steelhead trout adults return to their natal hatcheries or spawning grounds primarily between November and April (with small numbers of both early and late returnees). In this report, a fish is considered feral if it was released from a hatchery but then spawned naturally, outside a hatchery, or if it is progeny from such a naturally spawned hatchery-reared fish. At a hatchery, where steelhead trout are not allowed to be iteroparous (repeat spawners), the spawned adult population is collectively referred to as an adult Run Year, and the year designation refers to the calendar year in which the run ends. As part of ongoing active surveillance programs, a proportion of the spawned adults at all coastal hatcheries are screened for multiple pathogens at spawning, as articulated in the Salmonid Disease Control Policy of the Fisheries Co-Managers of Washington State (2006, access.nwifc.org). The proportion of adults screened for IHNV

infection varies according to regional resources and disease observation, among other factors.

Juvenile coastal steelhead trout begin hatching around February, and are reared at the hatchery for just over 1 yr before being released. Juvenile populations are referred to as a juvenile Brood Year, with the year designation referring to year that the eggs hatch. Thus, a given fish will be referred to by its Brood Year as a juvenile, and by its Run Year as an adult. The Brood or Run Year for a given population is referred to here as its biological year, and may not match the calendar year in which samples were taken.

Since the numbers of winter steelhead trout juveniles in a single Brood Year at a hatchery can range from several thousand to more than a million, nearly every hatchery divides the juveniles into multiple raceways, ponds, or lake net-pens for rearing. This subdivision strategy means that one Brood Year population consists of many sub-populations, each of which may have a unique pattern of disease incidence and prevalence. Juvenile sub-populations are monitored for IHNV in a screening or in a diagnostic manner. Brood Year populations are screened prior to transfer to other culture facilities to confirm that subclinical infection is not being disseminated with the juveniles. IHNV screening may also be performed prior to release in a site-specific manner at the discretion of a fish health specialist. Diagnostic testing occurs whenever one or more juvenile sub-populations suffers clinical disease. The Salmonid Disease Control Policy of the Fisheries Co-Managers of Washington State defines epidemic disease as mortality in a subpopulation of greater than 0.1% per day for 5 consecutive days. The frequency of diagnostic testing is not standardized, but it is performed by experienced fish health specialists according to site-specific criteria and may be performed on any number of sampled fish with or without disease signs, as well as on more than one sampling date. All screening and diagnostic testing is conducted by the fish health pathology laboratories of the agencies operating or serving the culture facility, and a subset of positive IHNV samples are submitted to WFRC for sequence analysis at the discretion of the fish health laboratory staff.

Bias and coding by detection events

The different testing strategies (routine adult screening and occasional juvenile screening and diagnostic testing) create 2 kinds of sampling bias. One, which is not strong in the present study, occurs when few samples are submitted for sequence analysis from specific geographic areas because the presence of IHNV there is not unusual. This pattern creates a surveillance-pressure bias: geographic areas with newly emergent IHNV are sampled and typed more heavily while fish in IHNV endemic areas are sampled and typed less intensely. The second form of bias, which is evident in the present study, is in the number of IHNV isolates submitted for typing. For example, 1 incident of juvenile epidemic IHN disease may be represented by only 1 or 2 isolates, while another may be represented by as many as 30. If detections were reported by the raw number of isolates, the sampling bias would mask basic patterns. To correct this bias, isolates have been coded into positive populations (by collection site, age, species, and seasonal timing) and each is assigned to one 'detection event'. If more than one genetic type (USD) was detected among multiple isolates from one sampling date and location, a separate event was assigned for each USD detected. In the current report, situations with more than one type are described as having a 'dominant variant' and 'non-dominant variant(s)'. Using this bias correction, Table 3 shows detection events, not numbers of isolates.

Viral RNA extraction and sequence analysis

Viral genomic RNA was extracted from 200 to 500 µl of virus culture supernatant with TriReagent (Sigma) according to the manufacturer's directions with tRNA (Promega) added to aid in RNA precipitation. Reverse transcription and amplification of a ~550 bp fragment containing the midG region was performed as previously described (Emmenegger et al. 2000) in one 50 µl reaction using avian myeloblastosis virus (AMV) reverse transcriptase, *Taq* polymerase, IHNV-specific primers and 30 cycles of amplification. The PCR product was purified away from amplification components using Strataprep PCR purification columns (Agilent Technologies), and 0.5 µl was used in each of two 10 µl Big Dye Terminator (Applied Biosystems) sequencing PCR reactions, using the same forward and reverse primers as above and 30 cycles as previously described (Emmenegger et al. 2003). All PCR reactions were performed in a PTC-100 thermocycler unit (Bio-Rad).

Phylogenetic analysis

Sequence data were assembled, edited, and trimmed to the established midG 303 nucleotide

fragment (Emmenegger et al. 2003, Garver et al. 2003, Kurath et al. 2003, Troyer & Kurath 2003) using Sequencher software v4.9. Consensus midG sequences for each virus isolate were aligned in ClustalX (Qt/QMake), and manually inspected and corrected for artefactual gap insertion. The term 'genotyping' is used to describe this process, and the term 'type' or 'genetic variant' is used to describe individual USD sequences (haplotypes). The sequence types are used as genetic tags for tracking transmission; the biological impact of each mutation separating the sequence types has not been determined. Newly generated sequences and previously known USD sequences representative of North American IHNV were used for phylogenetic analysis. Phylogenetic analysis was performed using coalescent Bayesian Markov chain Monte Carlo (MCMC) methods as implemented in the BEAST software package v1.6.1 (Drummond et al. 2002, Drummond & Rambaut 2007). Results were analyzed, annotated, and drawn using the complementary suite of programs, including Tracer v1.5 (beast.bio.ed.ac.uk/Tracer) and FigTree v1.3.1 (tree.bio.ed.ac.uk/software/figtree/). 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 (Biek et al. 2006, Drummond 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 3 separate MCMC analyses of 150 million generations that each achieved convergence and good mixing, and were subsequently combined, summarized, and analyzed.

RESULTS

Coastal IHNV detections and disease in 2007: emergence of type mG110M

In the late spring of 2007, Brood Year 2007 (BY07) juvenile winter steelhead trout at Salmon River hatchery (site SR in Fig. 1, Table 2) began suffering epidemic IHN disease. The parental Run Year 2007 (RY07) adults spawned earlier in the same year at this hatchery had been tested and there was no evidence of virus, but the hatchery water supply is not secure (it may contain anadromous fish). Genetic typing of an IHNV isolate from the epidemic among juvenile fish revealed type mG110M (Table 3). All the juvenile steelhead trout at this hatchery were euthanized to limit the spread of the virus to fish outside the hatchery.

The finding of an M genogroup IHNV prompted greater scrutiny of other IHNV isolates obtained in 2007 from coastal steelhead trout, especially since virus was found in more steelhead trout samples from more new sites in that year than in previous years (Table 1). In 2007, a total of 16 other IHNV isolates were collected from 4 other coastal facilities, all in the Humptulips or Chehalis River watersheds that both drain into the Grays Harbor estuary (sites BC/SK, SS, LA, and HH in Fig 1A and Table 2). Of these 16 isolates, 13 were from steelhead or rainbow trout and were isolated as follows. In March 2007, IHNV was detected for the first time in the Chehalis River Basin at the Skookumchuck Rearing Ponds in returning adult steelhead trout, whose fertilized eggs were transferred to the Bingham Creek hatchery for nursery incubation and rearing (BC/SK, Table 2). In July, juveniles at the Bingham Creek and Satsop Springs facilities developed IHN disease (Table 2); both facilities' water supplies may contain anadromous fish. In December, adults at Humptulips and Lake Aberdeen hatcheries were found to be infected with IHNV. Additionally, BY07 yearling juvenile steelhead trout at Humptulips suffered IHN disease in March and April 2008 (Tables 2 & 3). At the Humptulips hatchery, 1 sub-group of steelhead trout was euthanized, and 2 other sub-groups were reared and released.

A review of hatchery records indicated that during the summer of 2007, mortality was also elevated in sub-populations of juvenile steelhead and rainbow trout at Lake Aberdeen. The Lake Aberdeen hatchery has an unsecured water supply that may contain anadromous adult fish. Diagnostic testing of the steelhead trout juveniles revealed non-viral pathogens common to the hatchery but did not include virology, so presence of IHNV cannot be confirmed or ruled out. However, several months later, IHNV was found in juvenile rainbow trout at the facility, and this isolate was typed (Tables 2 & 3). The IHNV positive juvenile sub-groups at Lake Aberdeen were reared and released months later with the rest of their Brood Year populations. All the 2007 coastal isolates were typed as mG110M, and no U genogroup IHNV was found in 2007 in coastal watersheds (Table 1).

Table 2. Timeline of the occurrence of M genogroup IHNV in steelhead or rainbow trout at 11 Washington coastal sites from 2007 to 2011. The calendar year and month for virus isolation is listed on the left. Numbers in columns are number of IHNV isolates typed by midG sequencing. Juvenile columns are illustrated as follows: black borders indicate known epidemic/disease events; dashed borders indicate epidemic/disease events for which no virus isolates were available; and gray borders indicate isolates from juvenile survivors after mortality abated (post-epidemic populations). IHNV sequence types are not shown, see Table 3. Facility abbreviations: SR, Salmon River hatchery; BC, Bingham Creek hatchery; SK, Skookumchuck Spawning Site; SS, Satsop Springs Co-op; LA, Lake Aberdeen hatchery; HH, Humptulips hatchery; LQ, Lake Quinault hatchery; BH, Bogachiel hatchery; SC, Snider Creek Co-op; HR, Hoh River; QR, Quinault River; QN, Quinault National hatchery. Lifestage of the fish from which isolates were taken: adult (AD) or juvenile (JU)

Facility:	SR	BC/SK		SS		LA		HH		LQ		BH	SC	HR	QR	QN	
Lifestage:	JU	AD	JU	AD	JU	AD	JU	AD	JU	AD	JU	AD	AD	AD	AD	AD	JU
YEAR, month																	
2007																	
3		1															
5	1[a]																
7			2	1	1												
12						2	1	6									
2008																	
1								9									
2		1				3											
3									1								
4						2			1								
6										1[a]							
10							16										
11							1										
12						1	4										
2009																	
1											1						
2										3	1						
3		3				1				3							
12												18					
2010																	
1								10				5	3				
2													11				
3										10				3	1		
4	8[a]														1		
5	8[a]										2[a]						
7											24[a]						
8											6[a]						
11																16	
12																3	
2011																	
1										5					1	2	30[b]
2										15							
3										15							
4	1																
7	1[a]																

[a]Juvenile sub-group with epidemic IHN disease was euthanized
[b]Juvenile sub-group with elevated mortality was euthanized

Dominant type mG110M and non-dominant variants in 2008 to 2009

Three sites in the Grays Harbor area (Humptulips, Bingham Creek, and Lake Aberdeen hatcheries) also had IHNV detections in the next year, 2008 (Table 2). While the dominant type detected at all 3 sites was mG110M, increased genetic typing efforts at 2 of the sites revealed that non-dominant types were also present. In adults of RY08 at Humptulips, 15 out of 16 virus isolates were type mG110M, and 1 isolate was of a novel type, mG191M, that differed from mG110M at 1 nucleotide (Fig. 2A). The progeny BY08 eggs at Humptulips were disinfected according

Table 3. Genotype data showing emergence of 3 dominant types of MD genogroup IHNV and non-dominant variants. Numbers of detection events in trout of the coastal region, listed by universal sequence designator (USD) type. Across the top, the year reflects the biological year, not the calendar year of detection. Detection event abbreviations: AD, adult Run Year; D/EJ, diseased or epidemic juveniles; PE, post-epidemic juveniles. Facility abbreviations as in Table 2 with the addition of QR, Quinault River

Year:	1997	2007			2008			2009	2010			2011
Detection event:	D/EJ	AD	D/EJ	PE	AD	D/EJ	PE	AD	AD	D/EJ	PE	ADD/EJ
USD, Facility												
mG111M												
SR	1											
mG110M												
SR			1									
LA		1	1[a]	1	3	1[a]	2					
BC/SK		1	1									
SS		1										
HH			1[a]	2	2				2			
QR												1
LQ						1	2	1				
mG191M												
HH					1							
mG167M												
LA							1					
mG168M												
LA							2	1				
BC								1				
mG169M												
LA							1					
mG170M												
LA							1					
mG175M												
LA							1					
mG189M												
HH									1			
mG190M												
HH									1			
mG139M												
BH									1			
SC									1			
HR									1			
QR									1			
SR										1		1
LQ									1	2	2	1
QN										1		1
mG186M												
BH									1			
mG188M												
BH									1			
mG228M												
SR											1	

[a]Known or likely epidemic event with genetic type inferred from the virus isolated from the same population at a later time

to standard hatchery procedures and the juveniles were maintained on a secure water supply containing no anadromous fish; no IHN disease developed in these fish. At Lake Aberdeen hatchery, the 5 RY08 adult isolates tested were all type mG110M. Mortality was elevated in juvenile steelhead trout during the summer months and they were diagnosed with non-viral pathogens common to the hatchery. No virology was performed on juvenile samples until later in the year, when the mortality had subsided, and IHNV was detected between September and December of 2008 (Table 2). Due to the fact that these isolates came from juveniles after mortality abated, they are referred to as 'post-epidemic' isolates. The dominant type (13 of 23 isolates) in the Lake Aberdeen BY08 post-epidemic juveniles was type

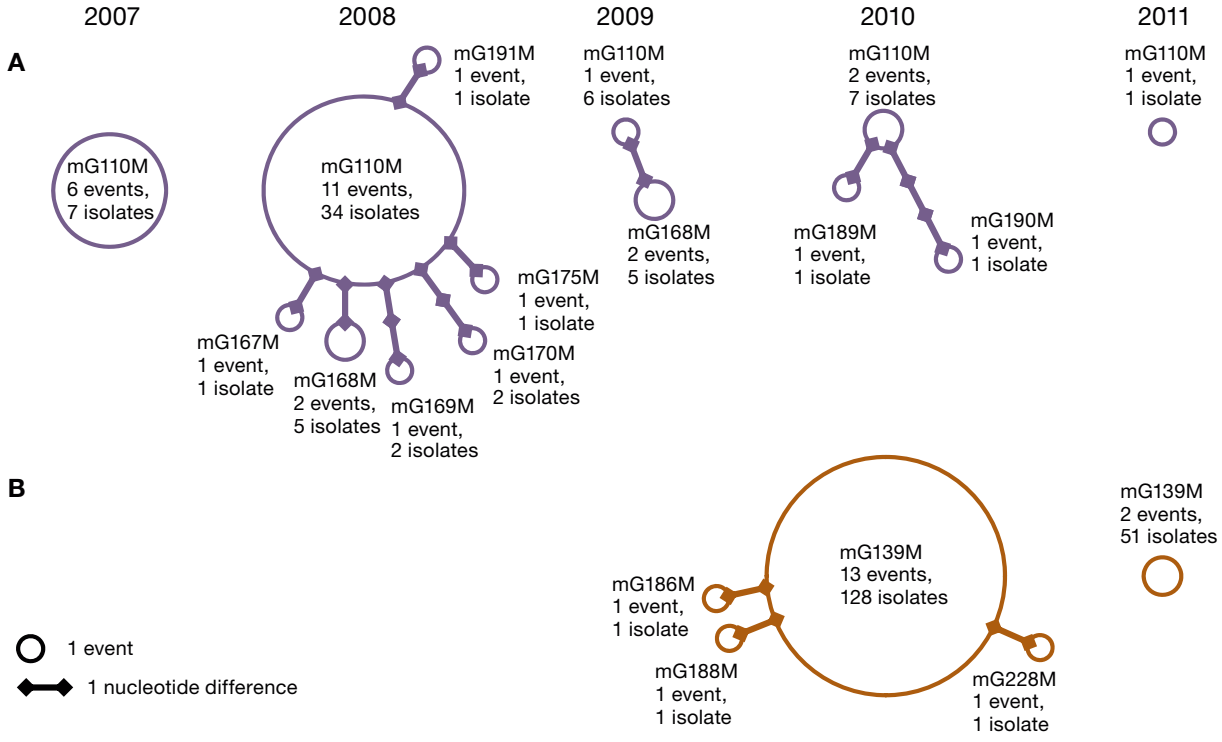

Fig. 2. Genetic relatedness and prevalence of IHNV types (A) mG110M and (B) mG139M and related non-dominant variants in coastal detection events, by biological year. Circles represent detection events, and are scaled in diameter to number of events, with number of isolates also indicated. Sequence differences in the midG region between types are indicated by connecting lines, scaled to indicate number of nucleotides that differ. The dominant variants mG110M and mG139M differ from each other by one nucleotide, but they are shown separately here due to strongly supported phylogenetic differentiation and epidemiological data indicating that they represent independent introductions into the coastal region (see 'Discussion')

mG110M and the remaining 10 isolates were of 5 other novel types: mG167M, mG168M, mG169M, mG170M, and mG175M (Table 3). These new types detected in 2008 differed from mG110M by 1 to 2 nucleotides (Fig. 2A). A single isolate collected from Bingham Creek 2008 adults in February was typed and found to be type mG110M (Table 2).

Also in 2008, IHNV was detected in net-pen-reared steelhead trout at Lake Quinault hatchery, which has an unsecured water supply. Although no virus was detected in the adult steelhead trout spawned earlier at this site in 2008, the progeny juvenile steelhead trout began to suffer epidemic disease in late spring and early summer (Table 2). There were 10 net-pens of BY08 juveniles being reared in the lake at the time IHN disease began and the virus was typed as mG110M. Three of the net-pen populations developed epidemic disease, and all fish in these 3 subpopulations were euthanized. The remaining subpopulations never developed epidemic disease, but several had low levels of IHNV detected later in the rearing period. This post-epidemic virus also typed as mG110M (Table 3). At this later point in rearing, adults for the next spawning year (RY09) were beginning to return and were present in the water supply of the hatchery, and they were found to have IHNV type mG110M as well. By the time juveniles were scheduled for release several months later, pre-release screening revealed virus in one sample of dead fish collected from 1 of 3 net-pens. However, no virus was found in healthy fish from any net-pen and there were no disease signs or elevated mortality, so the juveniles were released. The finding of type mG110M at Lake Quinault made the Quinault River the fourth positive coastal watershed and brought the number of coastal facilities with detections of MD types of IHNV to 7.

During 2009, one of the new non-dominant types detected in 2008 in post-epidemic juvenile steelhead at Lake Aberdeen, mG168M, was subsequently detected in 2 returning adult populations. The first was at Lake Aberdeen, in RY09 adults in the adult pond supplied by effluent from the post-epidemic juveniles where type mG168M was initially detected (Table 3). Shortly afterward, RY09 adults returning to Bingham Creek, in the same watershed, were also

found to be infected with mG168M (Table 3). In 2009, steelhead trout juveniles at Bingham Creek were being reared on a secured well-water supply. Despite the detections of IHNV in RY09 adults at Lake Quinault, Lake Aberdeen, and Bingham Creek, no juvenile epidemic events occurred in coastal facilities in 2009.

Emergence of type mG139M in 2010 to 2011

Between December 2009 and April 2010, IHNV was found in returning adult steelhead trout in 4 major coastal rivers north of Grays Harbor, including 2 new watersheds (the Quillayute and Hoh Rivers) farther north along the Washington coast (Table 2, Fig. 1A). Instead of the type mG110M that had been dominant for the preceding 3 yr, the majority of 2010 detections were a different type, designated mG139M. The new type mG139M differs from mG110M by 1 nucleotide in the midG region (see Fig. 2B and legend). The mG139M type was found in hatchery adults at Bogachiel (21/23 isolates) and Snider Creek Co-op (14/14 isolates) facilities, both of which are within the Quillayute River system. Type mG139M was also found in wild fish in the Hoh River (3/3 isolates), and wild fish in the Quinault River (2/2 isolates) (Table 3). These detections in wild adults were due to a concerted effort by fish health managers to increase the sampling effort on wild fish in the coastal region. In addition to the dominant type mG139M, 2 novel non-dominant variants were also detected at Bogachiel hatchery: types mG186M and mG188M. These variants each differed from mG139M by 1 nucleotide (Fig. 2B). No eggs were kept from the positive adults spawned at Bogachiel, and the Snider Creek Co-op adults were not spawned; no juvenile epidemic disease events were detected among progeny from virus-negative adults at Bogachiel hatchery, which has a secured water supply.

Also in 2010, Lake Quinault, which previously had type mG110M, was found to have fish infected with the new type mG139M. The dominant IHNV type in Lake Quinault RY10 steelhead trout adults was mG139M (10/10 isolates, one detection event, Table 3). Sockeye salmon adults in the lake at the same time were found to be infected with a U genogroup type IHNV (one isolate, Table 1). No eggs were reared from any positive steelhead trout adults at the Lake Quinault hatchery. In contrast, adults that returned to Humptulips in 2010 still had mG110M as the dominant type (7/9 isolates, Table 3) along with 2 novel non-dominant types, mG189M and mG190M. These variants differed from mG110M by 1 to 3 nucleotides (Fig. 2A).

Despite this widespread detection of IHNV in the adults at 6 coastal facilities, only 2 sites had IHN disease in juveniles in 2010. In both cases, disease developed in juvenile progeny from virus-negative adults that were reared in water that may have contained anadromous fish. Lake Quinault BY10 juvenile steelhead trout developed disease and were found to have type mG139M (Table 3). Juvenile steelhead trout at Salmon River hatchery also suffered epidemic disease in 2010 from type mG139M and all fish were euthanized (Table 3). At Lake Quinault juvenile sub-groups suffering epidemic IHN disease were euthanized, and no virus was detected several months later in pre-release screening of the rest of the Brood Year, so they were released.

Near the end of 2010, IHNV type mG139M was detected at a 5th new site, Quinault National hatchery. This site lies on a small tributary to the Quinualt River approximately 7 river miles below Lake Quinault, and its water supply is partially secured (anadromous fish in the water supply is greatly restricted but not completely blocked). Adult steelhead trout of RY11 returning to the this site tested positive for IHNV in November and December of 2010 (Table 2). This was the first time IHNV had ever been detected at this facility. Sequence analysis revealed that IHNV type mG139M was uniformly dominant (20/20 isolates, Table 3). Subsequent to these adult detections, one sub-population of yearling steelhead trout at Quinault National began suffering elevated mortality, and IHNV type mG139M was again uniformly dominant (29/29 isolates). The juveniles suffering disease due to IHNV type mG139M were not progeny of the positive RY11 adults, but rather yearlings from the previous RY10 adults which had no evidence of virus in screening tests. The mortality level in the yearling steelhead trout with IHNV type mG139M was well below the strict definition of epidemic disease, but it was significantly higher than that of the rest of the BY10 juveniles at the facility, so they were euthanized. No virus was detected in pre-release screening of the rest of the Brood Year, so the population was released several weeks later.

In 2011, IHNV type mG139M was dominant in the Quinault River system, but type mG110M was also evident. Adult steelhead trout positive for IHNV were detected at 3 locations in this watershed. At Lake Quinault hatchery, IHNV type mG139M was dominant (32/36 isolates), and the 4 remaining isolates were 1 of 2 different types of U genogroup IHNV (Table 1). At Quinault National, IHNV type

mG139M was dominant in RY11 adults (30/30 isolates). However, one adult sampled by the fishery (fish taken by fishermen in the lowest part of the Quinault River) was found to have IHNV type mG110M (Table 3).

While several non-dominant variants of mG139M were detected in 2010 (Fig. 2), the majority of them were found in adults. The only example of a variant within a juvenile population is the novel type mG228M detected in BY10 yearling steelhead trout at Salmon River, sampled in 2011 (Table 3). This variant differs from IHNV type mG139M in one nucleotide (Fig. 2B). Although these juveniles were sampled at the Salmon River hatchery, they originated from Quinault National. The juvenile fish were moved during the summer of 2010 to replace the fish that had been euthanized. The transfer occurred several months before any virus was detected at the National hatchery. These replacement fish were then reared without disease or elevated mortality for 7 mo at the Salmon River hatchery. Pre-release testing revealed IHNV in 1 of 2 pooled samples tested, and this isolate was type mG228M. These fish were released several weeks later.

The last coastal IHN epidemic at the time of this report was in BY11 juvenile steelhead trout at the Salmon River facility, in July 2011 (Table 2). As in the previous year, the virus typed as mG139M, and the fish were euthanized. This brought the number of documented IHN epidemics since 2007 to 8, with an additional epidemic event likely based on hatchery records. Ongoing surveillance detected no IHNV in coastal steelhead trout adult or juvenile populations during 2012.

Phylogenetic relatedness of IHNV MD types detected at coastal sites

All the M genogroup types isolated from fish in the coastal region were very similar in genetic sequence, differing from one another by ≤1% of the midG sequence. (Fig. 2). To infer whether these detections represented multiple introductions or the evolution of virus within fish populations at coastal facilities, we performed phylogenetic analysis including representative sequence types from the rest of the geographic range of IHNV (Fig. 3). All coastal M genogroup types were within the MD subgroup. Within the MD subgroup, 3 major polytomies with high posterior probability were evident (nodes X, Y, and Z, Fig. 3). Node X groups type mG111M and a non-dominant variant that was only detected in the Lower Columbia River Basin. Node Y contains type mG110M, a number of non-dominant variant types, and a subclade defined by node Z. Both IHNV types mG111M and mG110M were detected at the same coastal site, the Salmon River hatchery, but 10 yr apart, and they differ by 3 nucleotides in the midG region. The high posterior probability on node Y indicates that IHNV type mG110M is far more closely related to the non-dominant variants detected contemporaneously (Fig. 3, yellow stars) than with IHNV type mG111M. Similarly, node Z groups type mG139M and non-dominant variants detected contemporaneously (Fig. 3, red stars). The high posterior probability on node Z indicates that IHNV type mG139M and its related variants are phylogenetically distinct from IHNV type mG110M and its variants, even though IHNV types mG110M and mG139M differ by only one nucleotide in the midG region.

Non-dominant variants detected at coastal sites cluster with IHNV types mG110M and mG139M in a manner that matches with field epidemiology. None of the non-dominant variants described in this report have ever been detected outside the coastal region. The 5 non-dominant variants detected in 2008 (Fig. 2A) are all closely related to IHNV type mG110M (Fig. 3, yellow stars), which was the only dominant IHNV type at the time. In 2010, both IHNV types mG110M and mG139M were present, but in different coastal sites. The non-dominant variants mG189M and mG190M were detected at a site where IHNV type mG110M was detected in the majority of isolates; all fall into the polytomy with mG110M. Similarly, the non-dominant variants mG186M, mG188M, and mG228M that were detected at sites where IHNV type mG139M was dominant also fall within the polytomy with IHNV type mG139M (Fig. 3, red stars). The agreement of the timing and locations of field detections with the phylogenetic analysis indicates that there were 2 waves of virus emergence in coastal watersheds between 2007 and 2011 (IHNV types mG110M and mG139M), followed by the evolution of multiple new types in those watersheds.

DISCUSSION

The identification of MD genotypes of IHNV in fish of the coastal waters of Washington between 2007 and 2011 represents a dramatic range expansion (translocation emergence) of the trout-adapted M genogroup IHNV beyond its previous distribution

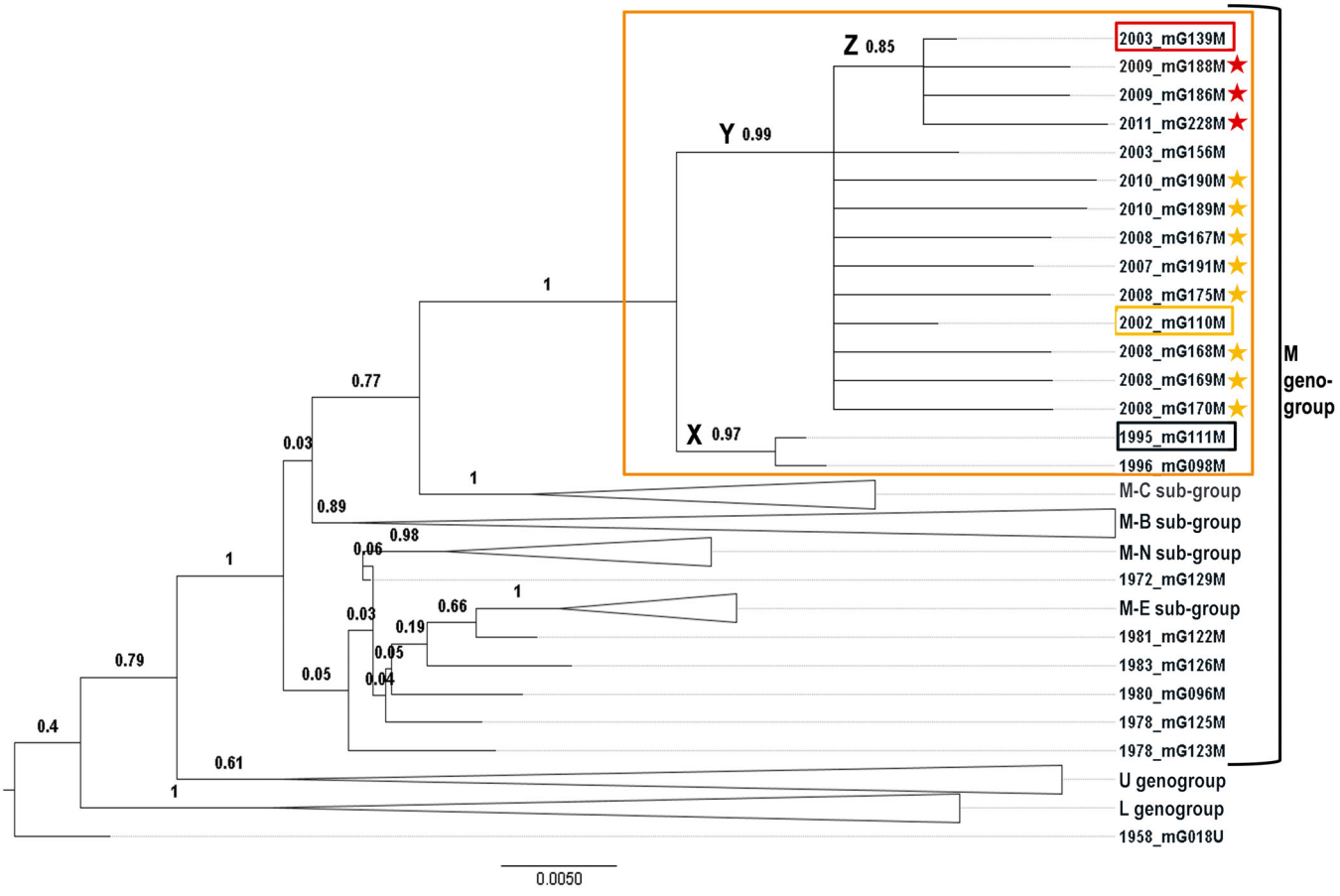

Fig. 3. Phylogenetic tree of IHNV midG types. Taxon names are aligned on the right, and consist of the earliest calendar year each type was detected in North America, followed by the universal sequence designator (USD). Genogroup and subgroup names are as previously published (Kurath et al. 2003). Branch lengths and scale bar are in substitutions per site. Posterior probabilities of each node are labeled on the branch leading to that node, in decimal format of 2 significant digits. Bayesian/coalescent methods calculate a rooted tree without the need for an outgroup. Major genogroups and sub-groups of IHNV other than the MD subgroup (orange box) are collapsed into labeled triangles. Within the MD subgroup, nodes with posterior probability with less than 0.85 were collapsed to polytomy and the remaining strongly supported nodes are designated X, Y, and Z. The dominant USDs in each of the 3 coastal incursions are boxed. Non-dominant genetic variants that were detected during the coastal events are indicated by a star with the same color as the dominant type

(Garver et al. 2003, Kurath et al. 2003). This expansion raises significant questions about the source(s) of the emergence, as well as concerns about the future risk of further expansion of MD group IHNV into as-yet-naïve regions. Genetic typing data presented here and published previously indicate that there have been 3 waves of IHNV emergence. The first was the previously reported emergence of type mG111M, which caused an epidemic in juvenile steelhead trout in 1997 (Emmenegger & Kurath 2002). This virus type did not persist in the coastal region, presumably due in part to the eradication effort of culling juveniles with epidemic disease, which were all the steelhead trout in the hatchery. The more substantial emergence of MD group IHNV in coastal steelhead trout since 2007 comprises the second and third waves of emergence: type mG110M and its associated variants in 2007 to 2009; and type mG139M and its associated variants from 2010 to 2011. The displacement of IHNV type mG110M by type mG139M was not absolute: during 2010 to 2011, IHNV type mG110M appeared to decrease in prevalence while type mG139M increased in prevalence (Fig. 2). This indicates that the 2 types coexisted in the same region, and possibly competed within specific populations. Thus 3 main types of MD subgroup IHNV have now emerged in Washington coastal steelhead trout: IHNV types mG111M in 1997, mG110M in 2007, and mG139M in 2010.

Source of emergent coastal MD virus types

One of the goals of this study was to determine the origin of viruses emerging in coastal steelhead trout. Comparisons of all the IHNV midG types detected in coastal steelhead with known midG types from throughout western North America (Troyer et al. 2000, Emmenegger & Kurath 2002, Garver et al. 2003, Kurath et al. 2003, Troyer & Kurath 2003, American Fisheries Society 2007, Bendorf et al. 2007, Kelley et al. 2007, R. Breyta & G. Kurath unpubl. data) revealed that the non-dominant variants reported here had never been observed before, but the 3 predominant IHNV types detected in Washington coastal waters (mG111M, mG110M, and mG139M) had all been previously detected in the Columbia River Basin. Within the Columbia River Basin, there is an extensive hatchery system for rearing of Pacific salmon and steelhead trout. A previously reported study of 120 IHNV isolates collected between 1973 and 2002 from throughout the Columbia River Basin provided evidence of 3 events in which M genogroup viruses emerged, spread, and persisted in the Lower Columbia River Basin, despite consistent presence of endemic U genogroup IHNV in the same region (Garver et al. 2003). The first emergence event pertinent to the coastal detections reported here involved virus type mG111M (previously referred to as Mcrb-2) that appeared in 1995 and spread and persisted in steelhead trout at multiple sites in the Lower Columbia River Basin until its last detection in 1999. The second emergence involved IHNV type mG110M (previously Mcrb-5), which was first detected in 2002 (Garver et al. 2003) and has since then become widespread and has remained dominant in Lower Columbia River Basin steelhead trout through to the time of this report (R. Breyta & G. Kurath unpubl. data).

Thus, IHNV types mG111M and mG110M that were associated with emergence of MD subgroup IHNV in coastal steelhead trout in 1997 and 2007 to 2011 were both dominant in the Lower Columbia prior to their emergence in coastal watersheds (Table 4), and they have not been detected elsewhere in western North America (Garver et al. 2003, R. Breyta & G. Kurath unpubl. data). Similarly, the most recent coastal emergence type, mG139M, was initially detected in the Lower Columbia River Basin between 2003 and 2005, and by 2008, it emerged and became the dominant M genogroup IHNV type in the Lower Snake River region, hundreds of miles upstream (R. Breyta & G. Kurath unpubl. data). Although it is theoretically possible that all 3 virus types reached coastal steelhead trout via an unknown external source that also transmitted these same types to fish in the Columbia River Basin, their prevalence and temporal occurrence in the 2 regions suggests that it is far more likely that each virus type was transmitted to coastal steelhead trout from Columbia River Basin steelhead trout (Table 4).

Since all 3 major types of coastal IHNV are within the MD subgroup, it is also theoretically possible that later types evolved from earlier types present in coastal fish following a single introduction event. In combination with the temporal and spatial patterns described above, phylogenetic analysis indicates that this is unlikely. The earliest coastal type, mG111M, differs from mG110M by 3 nucleotides. It is highly unlikely that the same exact 3 nucleotide mutations would occur independently in both the Lower Columbia River and coastal Washington regions. The single nucleotide difference between mG110M and mG139M is also highly unlikely to have occurred independently. This conclusion is supported by analysis of larger genome regions of numerous IHNV isolates from fish at coastal and Columbia River Basin

Table 4. Temporal occurrence of the 3 main IHNV types that have emerged in coastal steelhead, and their prior detection in Columbia River Basin salmonids. MidG universal sequence designators (USD) for virus types are on the left followed by columns indicating the calendar year of first detection, time span of detections, and relative dominance in the coastal region and the Columbia River Basin

USD	Columbia River Basin		Coastal region	
	First detection, sub-region	Time span, dominance	First detection, site	Time span, dominance
mG111M	1995[a], Lower Columbia	1995–1999[a], dominant	1997[c], Salmon River	1997[c], no recurrence
mG110M	2002[a], Lower Columbia	2002–2011[a,b], dominant	2007[d], Salmon River	2007–2009[d] dominant; then 2010–2011 waning
mG139M	2003[d], Lower Columbia, then 2008[d], Lower Snake	2003–2005[d], non-dominant 2008–2011[d] dominant	2010[d], Bogachiel H.	2010–2011[d], dominant

[a]Garver et al. (2003); [b]R. Breyta & G. Kurath unpubl. data; [c]Emmenegger & Kurath (2002); [d]this report

sites (R. Breyta & G. Kurath unpubl. data). The prior existence and phylogenetic distinctiveness of all 3 IHNV types in the Columbia River Basin suggests that the emergence of types mG111M, mG110M, and mG139M in coastal steelhead trout represent 3 independent incursions of MD genogroup viruses from the Columbia River Basin to coastal watersheds. The pattern of sequential emergence, dominance, and displacement of M genogroup IHNV types in the Columbia River Basin and coastal Washington is similar to patterns described in other RNA viruses such as dengue virus and rabies virus (Thu et al. 2004, Rico-Hesse 2007, Bull & Ebert 2008, Grinev et al. 2008, Rodríguez-Castillo et al. 2010).

IHNV transmission routes and mechanisms

While the genetic typing results indicate that the Columbia River Basin is the likely source of coastal translocation emergence events, probable transmission mechanisms between the 2 regions are more difficult to deduce due to the geographic distances involved. The 2 regions are separated by several hundreds of miles of river and ocean salmonid habitat, which contain both hatchery and wild fish of various species and ages. Therefore, we must consider potential transmission routes that are not strictly limited to cultured fish. Since the expansion of salmonid hatchery programs in the 20th century, IHNV has become a significant problem in cultured salmonid fish in the western United States. The virus has been found in numerous salmonid populations and epidemic disease has occurred in juveniles at various facilities nearly every year (Amend 1972, Amend & Wood 1972, Mulcahy et al. 1980, Garver et al. 2003, Kurath et al. 2003). Observation of increased pathogen incidence, prevalence, and severity in settings of animal rearing compared with wild or free-living animals is common in domestic animal systems and is largely attributed to greater animal densities and increased overlap between age or species classes (Daszak et al. 2000, Walker & Winton 2010, Kurath & Winton 2011). Since overall pathogen replication capacity is directly linked to the number of infected animals, greater incidence (especially of epidemic disease) within a farm or hatchery amplifies the infectious pressure on the surrounding populations of wild or free-swimming fish. For IHNV, hatchery amplification is of concern because there is no treatment for disease caused by the virus. Instead, IHN epidemic management strategies are limited to either allowing the mortality to run its course in order to preserve a surviving population for release, or destroying diseased and/or infected juveniles in the hopes of limiting subsequent transmission to other fish inside or outside the facility. Since hatchery effluent water is rarely treated to reduce pathogen load and many hatchery salmonids are released to the wild as juveniles, the impact of IHNV from hatchery systems on the health of other fish populations in the watershed is potentially important, but to date it is poorly understood.

Consideration of probable routes of virus transmission to Washington coastal steelhead trout populations should therefore include all free-ranging fish, even though wild and feral populations are difficult to sample at the same level as hatchery fish. Wild or feral spawning adults may pass their infection to progeny via egg-associated virus (pseudo-vertical transmission) or by shedding virus that infects sympatric juvenile fish by horizontal transmission. Both of these mechanisms can also introduce virus into a fish culture facility, despite the intervening infrastructure; both mechanisms may or may not occur at the same time. If errors are made in a hatchery during egg-disinfection, virus may pass from hatchery-spawned adults to juveniles (also pseudo-vertical transmission). Alternatively, horizontal transmission of virus into a hatchery can occur if adult fish pass above an unsecured hatchery water supply and shed virus. A hatchery water supply is considered 'unsecured' if free-ranging/anadromous fish are present in the water source and there is no disinfection of the intake water, such as ozone treatment. None of the coastal hatcheries described here have equipment for disinfection of intake water, and many use water supplies that are unsecured. Some sites have 'partially secured' water supplies, meaning they have some access to fish-free well or spring water for a portion of their juvenile rearing, or they have a barrier to decrease movement of adult fish upstream of the water intake.

Additional sources of horizontal transmission are unique to fish culture facilities and may include the importation of infected fish or contaminated equipment from another location, transfer of virus on the surface of personnel or hardware within a facility, and the re-use of effluent water from one fish population to supply other fish populations. Biosecurity measures designed to prevent virus transfer between Washington fish culture facilities are prescribed by the Salmonid Disease Control Policy of the Fisheries Co-Managers of Washington State, and each agency has biosecurity protocols to limit transmission within their own facilities. Due to the effectiveness of measures to limit anthropogenic transmission routes, the

least controlled sources of virus are the more natural horizontal transmission routes (Kurath & Winton 2011). We will refer to these routes relative to free-ranging fish populations as 'source' transmission to captive fish via unsecured water supplies and 'sink' transmission out of infected captive fish via effluent water. An anadromous hatchery salmonid will have 2 captive phases and 1 free-ranging phase during its lifetime, while wild fish would always qualify as free-ranging. At facilities with unsecured water supplies, the adult salmonids that are hatchery-spawned are often part of the same cohort of adults that bypass the hatchery, making the hatchery adults a proxy indicator of the type of IHNV that may be present in the 'source' fish.

'Source' transmissions into coastal fish culture facilities

The strongest example of 'source' transmission into a culture facility is the case of the Salmon River hatchery, which consistently suffered IHNV disease early in each of the 3 waves of coastal emergence. This hatchery had epidemic disease in juvenile steelhead trout in 1997 (mG111M), 2007 (mG110M), and 2010 (mG139M), each with the newly invading virus type. Each time, none of the sampled progenitor adults tested positive for IHNV, minimizing the probability of egg-associated transmission as the source. Therefore, these epidemics were likely due to horizontal transmission of virus in the hatchery water supply, presumably due to IHNV-infected free-ranging adult and/or juvenile fish above the hatchery water intake. The Salmon River facility uses river water as an 'unsecured' water supply and wild/feral steelhead trout adults were observed to be spawning upstream of the hatchery preceding each case of epidemic disease in the hatchery. Horizontal transmission via contaminated Salmon River water is also the best explanation for the case of virus type mG228M detected in yearling steelhead trout at the same tribal hatchery in 2011, since these fish were transferred from a facility with no known history of IHNV and there were no other steelhead trout at the receiving hatchery at that time. The detection of IHNV type mG228M occurred in April, when free-ranging adult steelhead trout could have been spawning upstream of the hatchery. Since it seems most probable that the route of transmission into the Salmon River hatchery was the unsecured water supply, the relative abundance of hatchery versus free-spawning fish could be an important risk factor for a hatchery. If a hatchery spawns a small fraction of the adults present in the river and the remainder of the run spawns freely upstream of the facility, the high number of free-spawning fish would be a significant 'source' exposure to the hatchery. This is the case for the Salmon River hatchery, where disease incidence in the hatchery was high, but it is also the case for the Lake Quinault hatchery, where the disease incidence was much lower. It is not clear why source wild/feral fish in the Salmon River would repeatedly provide enough virus to cause epidemic disease in the hatchery, when other coastal hatcheries with unsecured water supplies do not have this high incidence of disease.

Other sites involved in these MD expansion events that may have acquired virus in a similar manner share a combination of traits with the Salmon River site, namely that many have unsecured or partially secured water supplies and only fish that are collected for spawning are regularly tested for IHNV infection. This coexistence of hatchery and wild populations means that populations of free-ranging wild/feral anadromous fish present in the hatchery water supply are rarely if ever directly tested for infection, so this source of IHNV transmission remains largely inferred. The power of such inference is directly related to the size and composition of hatchery and natural fish populations because a hatchery may spawn a very small proportion of all fish of a given run/species in a river, or the hatchery fish may represent the majority of the river's population. For instance, the fraction of winter steelhead spawned at Salmon River is small compared to the free-ranging wild run, but the numbers of winter steelhead spawned at Lake Aberdeen hatchery constitute the majority of the population in that tributary. The inference about what virus type(s) might be present in the natural fish, and therefore what might be contaminating the hatchery water supply, is much stronger when the sampled fish are the majority of the population, as at Lake Aberdeen. Even in cases like Lake Aberdeen, detection of a uniform virus type makes it difficult to determine whether a juvenile epidemic was caused by horizontal transmission, pseudo-vertical transmission, and/or a breakdown in biosecurity.

Other coastal sites with unsecured or partially secured water supplies include the Humptulips, Lake Quinault, Lake Aberdeen, Bingham Creek, and Quinault National hatcheries. In these sites, at some point in the 5 yr of emergence events, IHNV was detected in hatchery-spawned adults before juvenile epidemic disease developed. Lake Aberdeen hatchery adults had type mG110M in 2008, and Lake

Quinualt had adults with type mG139M in 2010. Both sites were rearing juveniles on unsecured water supplies, so it is possible that wild/feral adult fish congregating upstream of the hatchery shed sufficient virus to contaminate the intake water. Even facilities with partially secured water supplies had the pattern of adult detections before development of juvenile epidemic disease: Bingham Creek hatchery adults had IHNV type mG110M in 2007, Humptulips Hatchery had adults with IHNV type mG110M in 2008, and Quinault National adults had IHNV type mG139M in 2010 (Table 2). In each case, the virus subsequently found in juveniles was the same genetic type found in the spawned adult fish. However, since no fish migrating or spawning naturally upstream of each hatchery were tested, this transmission route cannot be confirmed.

'Sink' transmissions from juvenile fish in coastal fish culture facilities

While 'source' transmission from free-ranging adult fish to captive juvenile fish seems to have occurred several times in the coastal region, 2 cases of 'sink' transmission from captive juveniles to adults were also evident in the coastal emergence events. Both cases demonstrate the greater infection pressure and transmission risk caused by hatchery amplification. First, the genetic type mG168M was detected in 2008 at Lake Aberdeen hatchery in adults after being captured to be held for maturation. Their migration into captivity and residence within the hatchery occurred in the effluent of the post-epidemic captive juveniles. Since type mG168M was detected as a minor variant within the post-epidemic juvenile population and it was not detected anywhere else previously, it likely arose within the juvenile population before being transmitted to adults. Second, the type mG168M was detected 5 mo later in 2009 adults returning to Bingham Creek hatchery, which lies further up the Chehalis watershed. The Bingham Creek adults would have passed through effluent from the Lake Aberdeen facility during their return migration. Therefore it is plausible that the Bingham Creek adults picked up type mG168M directly from the effluent of the fish at the Lake Aberdeen hatchery. This variant was the only emergent type unique to the coastal region to be detected beyond the site of its presumed emergence; no other variants that emerged in coastal steelhead trout between 2007 and 2011 were ever detected in a majority of isolates from a population. These transmission events observed during the emergence of IHNV in the Washington coast have highlighted the probable importance of both incoming and effluent hatchery water in IHNV transmission.

The emergence events reported here highlight the need for ongoing study of IHNV epidemiology. The M genogroup of IHNV has clearly expanded its geographic range, potentially threatening additional naïve steelhead trout populations. Regions like the Columbia River Basin with a high IHNV burden appear to be a repeating source of viral emergence into the previously naïve fish of the Washington coastal region. In the recent waves of IHNV emergence in coastal fish, culling of juveniles with epidemic disease at specific coastal sites has not been sufficient to protect those sites from recurrent IHN epidemics. Thus, containment of IHNV transmission may depend on larger scale control. The waning incidence of IHNV infection in fish of coastal sites in 2012 is similar to fluctuating levels of incidence at specific sites within endemic regions like the Columbia River Basin, so it is not clear what the future burden of IHNV in coastal steelhead trout might be; factors that drive waxing and waning levels of incidence are not well understood. Since these emergence and displacement events are ongoing in IHNV endemic regions, continued viral surveillance and molecular epidemiology based on genetic typing will serve to inform the development and refinement of control strategies aimed at effectively disrupting IHNV transmission.

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