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A Systematic Surveillance Program for Infectious Salmon Anemia Virus Supports its Absence in the Pacific Northwest of the United States

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Abstract

In response to reported findings of infectious salmon anemia virus (ISAV) in British Columbia (BC), Canada in 2011, U.S. national, state, and tribal fisheries managers and fish health specialists developed and implemented a collaborative ISAV surveillance plan for the Pacific Northwest region of the United States. Accordingly, over a 3-1/2 year period, 4,962 salmonids were sampled and successfully tested by real-time reverse-transcription PCR. The sample set included multiple tissues from free-ranging Pacific salmonids from coastal regions of Alaska and Washington and farmed Atlantic salmon (*Salmo salar* L.) from Washington, all representing fish exposed to marine environments. The survey design targeted physiologically compromised or moribund animals more vulnerable to infection as well as species considered susceptible to ISAV. Samples were handled with a documented chain of custody and testing protocols, and criteria for interpretation of test results were defined in advance. All 4,962 completed tests were negative for ISAV RNA. Results of this surveillance effort provide sound evidence to support the absence of ISAV in represented populations of free-ranging and marine farmed salmonids on the northwest coast of the United States.

Keywords: Infectious salmon anemia (ISA), Pacific salmon, Atlantic salmon, surveillance

Introduction

Infectious salmon anemia (ISA) is a World Organization of Animal Health (OIE)-listed disease of serious international concern. The etiologic agent, a virus in the family *Orthomyxoviridae*, is known for disease outbreaks causing high economic losses in Atlantic salmon (*Salmo salar* L.) farming regions of Norway, eastern Canada, eastern United States, Faroe Islands, the United Kingdom, and Chile (OIE, 2016). Though principally a pathogen of marine farmed Atlantic salmon, the virus is periodically detected in freshwaters, free-ranging populations, and in alternate species such as brown trout (*Salmo trutta* L.) (Plarre, Devold, Snow & Nylund 2005) and experimentally infected rainbow trout (*Oncorhynchus mykiss* Walbaum) and Atlantic herring (*Clupea harengus* L.) (Nylund & Jakobsen 1995; Nylund, Devold, Mullins & Plarre 2002; Biacchesi, Le Berre, Le Guillou, benmansour, Bremont, Quillet & Boudinot 2007). In the eastern United States (Maine), ISA outbreaks affecting Atlantic salmon farms between 2001 and 2006 were eliminated through a state/federal program harmonized with parallel efforts in New

Brunswick, Canada. Virus elimination efforts were based on early detection, early removal of affected cages, movement controls, and functional separation between farming neighborhoods and year-classes (Ellis, Gustafson, Giray, Robinson, Marenghi & Merrill 2006). The last occurrence of a pathogenic strain of ISAV in Maine was documented in 2006, though a non-pathogenic (HPR0) strain is occasionally detected on routine surveillance (http://www.oie.int/wahis_2/public/wahid.php/Diseaseinformation/statusdetail; https://www.aphis.usda.gov/aphis/ourfocus/animalhealth/animal-disease-information/aquaculture/ct_general+_information). HPR0, whose nomenclature denotes a full-length sequence in a highly polymorphic region (HPR) of segment 6 of the ISAV genome, is considered a plausible progenitor to outbreak (HPR-deleted) strains of ISAV (Cunningham, Gregory, Black, Simpson & Raynard 2002). HPR0's transient and non-clinical occurrence, coupled with its predilection for gills (a surface tissue) and its non-cultivability, makes its detection less reliable than that of HPR-deleted strains (Christiansen, Østergaard, Snow, Dale & Falk 2011). However, to date, no ISAV (of any form) has ever been detected in the United States outside of the affected region in Maine.

In October 2011, researchers from Simon Fraser University in British Columbia (BC), Canada, announced in a press release the detection of ISAV genetic sequences in samples from two of 48 free-ranging juvenile sockeye salmon (*Onchorhynchus nerka* Walbaum) collected from marine waters near the central coast of BC (SFU 2011). Testing was conducted in a research laboratory at the University of Prince Edward Island, Canada, using a real-time reverse-transcription polymerase chain reaction (real-time RT-PCR) assay. In 2016, additional ISAV sequence detections in salmon from fish markets and wild populations in British Columbia were reported by the same laboratory (Kibenge, Iwamoto, Wang, Morton, Routledge & Kibenge 2016). These reports have not been confirmed by the Canadian Food Inspection Agency, Canada's Veterinary Competent Authority (http://www.inspection.gc.ca/animals/aquatic-animals/diseases/reportable/2017/eng/1339174937153/1339175227861) but the resulting concerns led to additional investigation and surveillance in both Canada and the United States.

In response to the 2011 report, the U.S. Aquatic Animal Health Task Force collaborated with regional and local fishery management entities to develop and carry out enhanced surveillance to investigate the potential presence of ISAV in salmon and trout in northwestern U.S. waters. The Task Force is led by the U.S. Department of Agriculture's Animal and Plant Health Inspection Service-Veterinary Services (USDA APHIS VS) and includes the Department of Commerce's National Marine Fisheries Service (DOC, NOAA, NMFS) and the Department of the Interior's U.S. Fish and Wildlife Service (DOI, USFWS). Regional and local fishery management entities included Alaska Department of Fish and Game (ADFG), Washington Department of Fish and Wildlife (WDFW), Northwest Indian Fisheries Commission (NWIFC), and U.S. Geological Survey (USGS) Western Fisheries Research Center. A paper describing the collaborative effort and preliminary results following the first year of sampling and testing was published in 2014 (Amos, Gustafson, Warg, Whaley, Purcell, Rolland, Winton, Meyers, Stewart, Kerwin, Blair, Bader & Evered 2014). We present here the final report and conclusions for the full multi-year multi-agency surveillance effort.

Materials and Methods

Sampling strategy

The U.S. Pacific Northwest sampling strategy was designed to provide a high probability (≥ 0.95) of detecting ISAV present at a threshold prevalence of 1% in any one of the regional populations sampled over the study period (OIE Aquatic Animal Health Code Chapter 1.4 recommends 2% or lower), for any one of the most susceptible species populations (Atlantic salmon and steelhead trout) sampled over the study period. Sample size estimates presumed mixed populations, 90% test sensitivity (Abayneh, Toft, Mikalsen, Brun & Sandberg 2010), and confirmatory protocols used as needed to approach perfect specificity. However, test accuracy estimates, benchmarked for virulent strains of ISAV, are not available for HPR0. Consequently, surveillance results can only infer apparent, rather than true, prevalence for HPR0 or similar variants. Our approach to HPR0 evaluation is described further in Data Analysis.

The sampling design ensured broad species, temporal and geographic coverage of anadromous Pacific salmon and trout indigenous to the U.S. Pacific Northwest, as well as marine-farmed Atlantic salmon. Sampling was distributed over three and one half years, multiple seasons, six free-ranging Pacific salmon or trout species, and all active marine net-pen Atlantic salmon farms. Pacific salmonid species included coho (*O. kisutch* Walbaum), Chinook (*O. tshawytscha* Walbaum), chum (*O. keta* Walbaum), pink (*O. gorbusha* Walbaum) and sockeye salmon, and steelhead trout (anadromous form of resident rainbow trout, *O. mykiss*). Sampling was further structured to preferentially select fish with greatest expected susceptibility to ISAV. This objective was achieved by (1) conducting quarterly sampling at all active marine net-pen Atlantic salmon farms in the region, prioritizing collection of moribund fish (or fresh mortalities) more likely to succumb (or have succumbed) to pathogens if present, and by (2) also ensuring strong representation of steelhead trout, a species whose freshwater resident form (rainbow trout) has demonstrated infection susceptibility to select strains of ISAV, though less so than Atlantic salmon (Nylund, Kvenseth, Krossøy & Hodneland 2003; Kibenge, Kibenge, Groman & McGeachy 2006; Biacchesi, Berre, Guillou, Benmansour, Bremont, Quillet, & Boudinot 2007). Furthermore, steelhead trout and Pacific salmon were sampled during spawning, a physiologically taxing period for these fish, with potentially higher virus loads or increased susceptibility to infection.

Testing Laboratories

Prior to sampling, four laboratories were designated to test tissues for the presence of ISAV RNA. Samples from Alaska were submitted for testing to the ADFG Anchorage Fish Pathology Laboratory or to the USDA APHIS National Veterinary Services Laboratories (NVSL), samples collected by officials at the WDFW and NWIFC were submitted to the Washington Animal Disease Diagnostic Laboratory (WADDL) at the Washington State University College of Veterinary Medicine, and samples collected by officials of the USFWS were submitted to the USFWS Idaho Fish Health Center. NVSL created control materials and working in cooperation with the testing laboratories developed a set of standardized operating protocols, all of which were distributed to the testing laboratories prior to testing. These protocols describe the procedures for tissue collection and processing, RNA extraction, and real-time RT-PCR, and included the criteria for test acceptance, data analysis, and interpretation. The utilization of the

standardized operating protocols by each laboratory was evaluated through comparison of limit of detection data on virus stocks across testing laboratories (data not published), completion of a proficiency examination administered by the NVSL, and inclusion of positive amplification controls on all test runs.

Sample Collection and Processing

Samples collected within Washington State consisted of heart, gill, and kidney tissues from individual Pacific salmonids, and heart and kidney tissues from individual marine farmed Atlantic salmon. In Alaska, gill and kidney were sampled from individual Pacific salmonids. Within both states, tissue samples from each individual fish were pooled and preserved in RNAlater® in a 1:10 tissue to preservative ratio. Samples not processed within 48 hours were held at 4-8°C overnight and then transferred to temperatures at or below -20°C for storage until processed. Officials from the USFWS, WDFW and NWIFC collected duplicate samples from fish; officials in Alaska collected one sample of each tissue type from each fish. Once testing was completed, the duplicates, or remaining original samples in the case of those from Alaska, were provided to the USGS laboratory at the Western Fisheries Research Center for additional testing for RNA viruses (Purcell, Powers, Evered, Kerwin, Meyers, Stewart & Winton, 2017).

Following collection, samples were submitted to one of the designated testing laboratories as described above. Tissues were homogenized by micro-bead beating (Hick, Tweedie & Whittington, 2010; Hope, Casey, Groocock, Getchell, Bowser & Casey et al, 2010). Briefly, equal-size pieces of each tissue were removed from the RNAlater® and rinsed in phosphate buffered saline (PBS) or cell culture medium (100 ± 10 mg of combined tissues). Tissues were added to a tube containing zirconia/silica or chrome steel beads. Cell-culture medium was added to each sample to achieve a 10% W/V suspension. Tubes were placed in the bead-beating machine and pulsed for 30-60 seconds, chilled for 2 minutes on ice, and returned to the machine for another 30-60 second pulse. Samples were incubated on ice for 5 minutes, then visually examined to verify cell lysis. Samples were centrifuged at 2000-4000 *x* g for 5 minutes at 2-8°C. Sample homogenate supernatants were removed from the debris pellet and processed for RNA. Samples not immediately used for RNA extraction were stored in an ultralow freezer (≤-60°C).

RNA extraction

Sample homogenate supernatants (50 µL) were extracted using the MagMAX™-96 Viral RNA Isolation Kit (Ambion® kit AM 1836, Life Technologies) using an automated system. Extraction Protocol AM1836 DW 50 V2 Aqua (ABI, Life Technologies) was followed by all testing laboratories. Laboratories used ISAV reference virus stock as the positive extraction control (PEC). Alaska and Idaho used ISAV reference virus stock that was inactivated by beta-propiolactone treatment. The PEC was diluted in each testing laboratory to achieve a cycle threshold (Ct) value within the range of 25-30. The negative extraction control (NEC) was the diluent (cell culture medium) utilized at each testing laboratory. VetMax Xeno™ internal positive control (Life Technologies) RNA was added to the extraction buffer for quality control of RNA recovery and real-time RT-PCR process.

Real-time reverse-transcription polymerase chain reaction assay

The OIE-recommended segment 8 TaqMan® probe-based ISAV detection method (OIEa, 2017; Snow, McKay, McBeath, Black, Doig, Kerr, Cunningham, Nylund, & Devold 2006) was used in a one-step (Workenhe, Kibenge, Iwamoto & Kibenge 2008), real-time RT-PCR multiplex assay that utilized an internal Xeno™ RNA control (VetMAX™-Plus Multiplex One-Step RT-PCR kit, ABI, Life Technologies). Negative amplification control (NAC) was RNA elution buffer or water. Positive amplification controls (PAC) included Cobscook Bay ISAV RNA standards at three different concentrations provided by the NVSL. Standard thermal cycling (ramp rates) was used. Thermal cycling conditions were reverse transcription (RT) at 48°C for 10 minutes, inactivation of RT enzyme and denaturation of cDNA at 95°C for 10 minutes, followed by 40 amplification cycles with denaturation at 95°C for 15 seconds and annealing and amplification at 55°C for 45 seconds (collection of fluorescence signal). Testing was conducted on either a 7500 Fast Real-Time PCR system (WADDL and NVSL) or the StepOne Plus™ (USFWS and ADFG).

Test analysis, acceptance criteria, and interpretation

Baseline and Ct settings impact the accuracy of test interpretations of real-time results and subsequent user assessment of sample status. The auto algorithm is most often used to set the baseline and Ct parameters. The auto algorithm calculates baseline and Ct parameters based on the assumption that all samples on a plate will have the typical or expected amplification curve (geometric, linear, and plateau). In this study, the auto setting was used and reviewed for each run. The component or multicomponent plots were reviewed to verify that true amplification, as evidenced by a sigmoidal curve, was occurring. In cases of high background, auto threshold with manual baseline setting (3-15) was applied. One testing laboratory experienced high background on some samples, which were extracted a second time and evaluated at the NVSL and/or the original testing laboratory.

To consider a test valid, all positive PCR and extraction controls had to fall within the expected Ct range, and NEC and NAC had to yield no amplification. The PAC set contained three replicates of a high, a mid, and a low concentration of ISAV RNA for a total of 9 PAC controls on each plate that were within the limit of detection of the assay. This was determined using serial tenfold dilutions of viral stocks and a synthetic RNA in the range of 1 copy to 1 X 10^7 copies of segment 8 target. The last serial dilution where all replicates of the synthetic RNA tested positive contained 1 X 10^3 copies of the segment 8 target. Analytical sensitivity of the assay is between 100 to 1000 copies in a 5 microliter volume.

Each sample was provided with a constant amount of Xeno™ RNA that was subsequently amplified, thus the Ct range for the Xeno™ RNA should be consistent (i.e., no more than 2.0 Ct shift for samples from the average of the controls on the same extraction). Data collected from the different machines/software and operators utilized during the proficiency testing of laboratories were used to establish the Ct range. The acceptable Xeno™ RNA internal control Ct value range established across the different machines and software used in this study was 27.5 – 34.0. Samples reported as not detected (negative) yielded no Ct value for ISAV. Any sample with an ISAV Ct value < 37 would have been considered screening test positive. Any sample yielding a Ct values of 37- 40 with a sigmoidal curve for ISAV would have been classified as suspect and required re-testing.

Any sample with a Xeno™ control value outside the acceptable range was extracted a second time (if remaining sample was available) and both the original RNA and the freshly extracted RNA were evaluated on the same run. Results from samples with both Xeno™ control values outside the acceptable range were considered indeterminate. Any sample deemed screening test positive or suspect would have resulted in confirmatory molecular testing on the sample, as well as additional sampling of the source population for testing by both virus isolation and molecular methods. Confirmatory molecular testing would have included sequence analysis of PCR amplicons generated by RT-PCR for a different region of segment eight and the HPR of the hemagglutinin esterase gene found on segment six.

Data Analysis

Results were tallied to summarize sampling effort across species, region, season, and time. Further analyses aggregated results and assessed sampling effort in terms of probability of detecting ISAV, if present, (1) by region, accumulated over species and time, and (2) by target populations with demonstrated susceptibility to ISA viruses (Atlantic salmon and steelheads), accumulated over time. As Atlantic salmon are farmed in Washington but not in other areas of the U.S. Pacific Northwest, and as the lethal collection of wild steelhead (an esteemed sport fish) would have met public resistance in Alaska[1], results from these two target populations were available only for Washington. We used an EpiTools epidemiological calculator (http://epitools.ausvet.com.au) to estimate a sample size of 332 fish necessary to achieve 0.95 probability of detecting ISAV, presuming 90% sensitivity and perfect specificity, present at a threshold prevalence of 1% or greater in any of the aggregate populations. This 1% detection threshold exceeds (is lower than) levels expected for HPR-deleted prevalence in Atlantic salmon in outbreak settings (McClure, Hammell, Dohoo, Nerette, & Hawkins 2004; Plarre, Devold, Snow & Nylund 2005; Gustafson, Ellis, Merrill, Robinson & MacPhee 2005) and, to address less susceptible species, also exceeds (is lower than) the 2% default described in the OIE Aquatic Code (Plarre, Devold, Snow & Nylund 2005; OIE 2017b). We also repeated analyses for a range of test sensitivities to explore the impact to conclusions should actual sensitivities vary across HPR-deleted genotypes or due to method modifications to the Snow assay. Diagnostic test sensitivity estimates are not available for HPR0, as gold standards or independent tests are not available for their quantification. Rather, this (1%) detection threshold simply approximates the proportion of kidney samples found positive in Atlantic salmon from endemic regions in the Faroe Islands (0.9% to 2.2%), though gill samples for this same population were 5-10 times higher (Christiansen, Østergaard, Snow, Dale & Falk 2011). Sampling in Pacific salmon and trout included gill tissue so should exceed this target. Targeted selection of moribund or compromised fish may further improve probability of pathogen detection, though most reliably for virulent strains (Gustafson, Gardner & Remmenga 2016a).

Results

[1] Steelhead sampled in WA were collected following lethal spawning from hatchery-origin adults returning to freshwater hatcheries after at least one year in marine waters.

Descriptive Statistics

A total of 4,962 free-ranging Pacific salmon (Chinook, chum, coho, pink, and sockeye) and steelhead trout, as well as farmed Atlantic salmon, representing seven marine regions (Figure 1, 2) were sampled and successfully tested over the three and one half year surveillance study, providing broad coverage of species inhabiting the U.S. Pacific Northwest states of Alaska (Figure 3) and Washington (Figure 4). Results are described both annually and in aggregate across the sampled species and periods. Results are also described separately for target species aggregated over the full study period and regions. All samples represent fish exposed to the marine environment, as all fish were collected from marine net pens or settings, or from rivers or hatcheries following their return from marine waters for spawning. All 4,962 successfully tested samples were negative for ISAV RNA; no RNA samples were screening test positive or suspect. Six additional samples were collected in 2013 from remote locations in two Alaska marine regions (five chum salmon from Arctic-Yukon-Kuskokwim and one coho salmon from Westward) and tested, but results were indeterminate and no remaining tissues were available for re-extraction and re-testing. These six samples were excluded from descriptive statistics and analyses.

In Alaska 2,984 marine-exposed salmon samples were collected and tested between April 2012 and November 2013. Forty-nine percent of the Alaska samples were collected in 2012 and 51% in 2013. In Washington, 1,978 marine-exposed salmon or trout samples were collected and tested between August 2012 and September 2015. Forty-one percent of the samples from Washington were collected in 2012, 38% in 2013, 17% in 2014, and 4% in 2015. In both states, sampling of free-ranging fish peaked in late summer/fall months (Figure 5) when most stocks of Pacific salmon spawn. Farmed Atlantic salmon were sampled by WDFW quarterly (from January 2013 to July 2015). At the Atlantic salmon farms, divers targeting moribund fish or fresh mortalities collected fish and WDFW staff selected fish samples for testing. Pacific salmon and trout species native to the regions comprised 92% of the sample total; farmed salmon comprised 8%. All sampled Atlantic salmon were categorized as moribund or recently deceased. Pacific salmon and trout free-ranging species were collected either as adults during their return to freshwater for spawning (>99%) or as sub-adults collected from the marine environment (< 1%).

In addition, 15 herring from an unrecorded location in Washington and 60 rainbow trout from a freshwater hatchery in the central region of Alaska were collected but excluded from the sample totals described above, based on unknown location or lack of saltwater exposure, respectively. These 75 fish, however, also tested negative for ISAV.

ISAV Status Assessment

Surveillance results support the absence of ISAV in the sampled regions and populations. Surveillance was designed to provide greater than 0.95 probability of detecting ISAV HPR-deleted during the sampling period if

present in 1% or more of a marine region's salmonid and trout population. Presuming mixing of stocks with equal opportunity for pathogen exposure, and 90% test sensitivity, this target is met with 332 or more samples per spatial or temporal group. Individual region-year surveys achieved this target in six of eighteen cases (Table 1). Results from all but one region exceed the sampling target when aggregated over the full study period (Table 1). Test sensitivity may differ from the 90% estimate, which was derived from a study using different extraction procedures (Abaneyh, Toft, Mikalsen, Brun & Sandberg, 2010). Consequently, we conducted the same analysis with differing sensitivity levels to show that results were largely robust at test sensitivities greater than 80%: up to two fewer region-year surveys met the target, but regional (aggregated) conclusions remained the same.

Atlantic salmon and steelhead trout exhibit the greatest susceptibility to ISAV of the tested species. The current study tested 393 Atlantic salmon from marine net pens in Washington (PSS region) and 565 steelhead trout distributed across all three regions in Washington. Accumulated results (across regions and years) for each key species also met or exceeded sampling targets for detecting virus in the sampled populations. Targeting fish in compromised conditions intentionally biased our sampling efforts toward detection and further strengthens conclusions of the absence of HPR-deleted virus.

Surveillance also found no evidence of ISAV HPR0 occurrence. However, without estimates of test sensitivity for this strain and without testing of gill tissues in the Atlantic salmon, the degree of confidence is more difficult to quantify. Rather, we simply conclude no evidence of HPR0 in the region, despite surveillance designed to detect HPR0 if it were present at a prevalence in Atlantic salmon (0.9 – 2.2% in kidney samples, 5-10x higher in gills) akin to that described in Christiansen et al (2011), or at a lower prevalence (given inclusion of gills) in Pacific salmon and trout.

Discussion

Results of this study provide sound evidence of the absence of ISAV in the regions, time periods, and species sampled. Although there are isolated marine populations and freshwater segments that were not sampled, it is reasonable to assume that salmonid populations of the U.S. Pacific Northwest share overlaps in range and thus opportunities for shared pathogen exposure in the marine setting. For example, decades of tagging studies of Pacific salmon demonstrate extensive geographic overlap among stocks from the U.S. west coast and Alaska (Myers, Aydin, Walker, Fowler, & Dahlberg 1996), and trophic ecology shows that chum, pink, and sockeye salmon use related food resources (Johnson & Schindler 2009). Consequently, the geographic and temporal breadth and depth of sampling, coupled with a focus on compromised fish and life stages, provide further indication that if ISAV does occur in this region of the world, it is with an uncharacteristic epidemiology and unusual (limited) pattern of spread.

ISAV, as an RNA virus lacking polymerase proof reading capability, is prone to genetic shift and drift, and as a segmented genome is also capable of re-assortment (Markussen, Sindre, Jonassen, Tens, Kristoffersen, Ramsell, Numanovic, Hjortaas, Christiansen, Dale & Falk 2013). Consequently, ISAV exists as a number of different

genotypes, the HPR0 grouping of which is thought to be both non-virulent and a putative progenitor of virulent forms (Cook-Versloot, Griffiths, Cusack, McGeachy & Ritchie 2004; Godoy, Kibenge, Suarez, Lazo, Heisinger, Aguinaga, Bravo, Mendoza, Llegues, Avendano-Herrera, Vera, Mardones, & Kibenge 2013). Though virulence is impacted by multiple segments of the ISAV genome, the HPR of segment six is commonly tracked and referenced in control programs (OIE, 2016b). Though non-cultivable and non-pathogenic, and thus more difficult to detect than HPR-deleted strains, HPR0 strains are found with some regularity in regions with previous experience with ISA disease (Christiansen, Østergaard, Snow, Dale & Falk 2011; Lyngstad, Kristoffersen, Hjortaas, Devold, Aspehaug, Larssen & Jansen 2012; Gustafson, Remmenga, Sandoval el Valle, Ibarra, Antognoli, Gallardo, Rosenfeld, Doddis, Enriquez Sais, Bell & Lara Fica et al., 2016b).

Epidemiologic study of HPR0 distribution and occurrence, however, is hindered by the lack of science-based estimates of diagnostic test sensitivity and specificity for this variant. While HPR0 appears with much higher frequency in gill than internal tissues (Christiansen et al., 2011), the inability to readily rule out surface contamination makes gill tissue a difficult choice in settings, such as farms, where it is important to differentiate between the animal and its environment. We addressed this concern by sampling internal organs of farmed Atlantics, but at levels sufficient to detect HPR0 should it exist at the apparent prevalence previously described for Atlantic salmon kidney samples from HPR0 endemic regions (Christiansen et al., 2011). We also included gill tissues in sampling of Pacific salmon and trout. Consequently, we expect that the existing sampling effort, using choice testing protocols (real-time RT-PCR) and tissues including gill in Pacific salmon (n=4569), covering multiple years and seasons, would eventually allow for detection of HPR0 should it or other known genotypes exist sub-clinically in these populations.

Experiences from other countries and regions suggest that virulent ISAV genotypes would be even more likely to be detected via strong surveillance (Ellis et al., 2006; Mardones, Perez, Valdes-Donoso & Carpenter 2011). Additionally, while this report focuses on results from three and one half years of systematic testing, the U.S. Pacific Northwest has been conducting routine testing for viral pathogens by cell culture on returning salmonids for decades (Amos et al. 2014) using cell lines (e.g., CHSE-214) suitable for detection of many North American, though less so European (Kibenge et al., 2000), genotypes of ISAV. Those efforts, combined with an industry, public, fisheries, and veterinary profession educated on the signs and significance of ISA through the experiences of Maine and Canadian Maritimes, provide further assurance that virulent forms of ISAV would quickly be discovered, whether by this background infrastructure or the here-described enhanced surveillance.

Conclusion

This study was a collaborative effort by fish health professionals and authorities of the U.S. federal, state, and tribal governments (Amos et al., 2014). Testing was conducted using OIE-recommended assays (OIE, 2017a) and following proficiency testing of all participating laboratories. Sampling protocols assured identification of fish and sampling locations and proper sample handling. Pre-set algorithms and contingency plans ensured reliable results and transparent standards for interpretation and response. Results for ISAV RNA were negative. We end,

therefore, by recommending that the U.S. Pacific Northwest continue to operate as free from ISAV, with continued vigilance and biosecurity practices to support a strong health status.

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Table 1: Sample sizes accumulated annually and over the full study period (total) by surveillance region. All samples were negative for ISAV RNA (See figures 1 and 2 for key to the acronyms)

Year	PSS	WAC	CR	AYK	CTR	SE	WR
2012	338	305	165	58	358	718	297
2013	496	120	145	55	479	720	299
2014	242	25	60	n/a	n/a	n/a	n/a
2015	82	n/a	n/a	n/a	n/a	n/a	n/a
Total	**1158**	**450**	**370**	**113**	**837**	**1438**	**596**

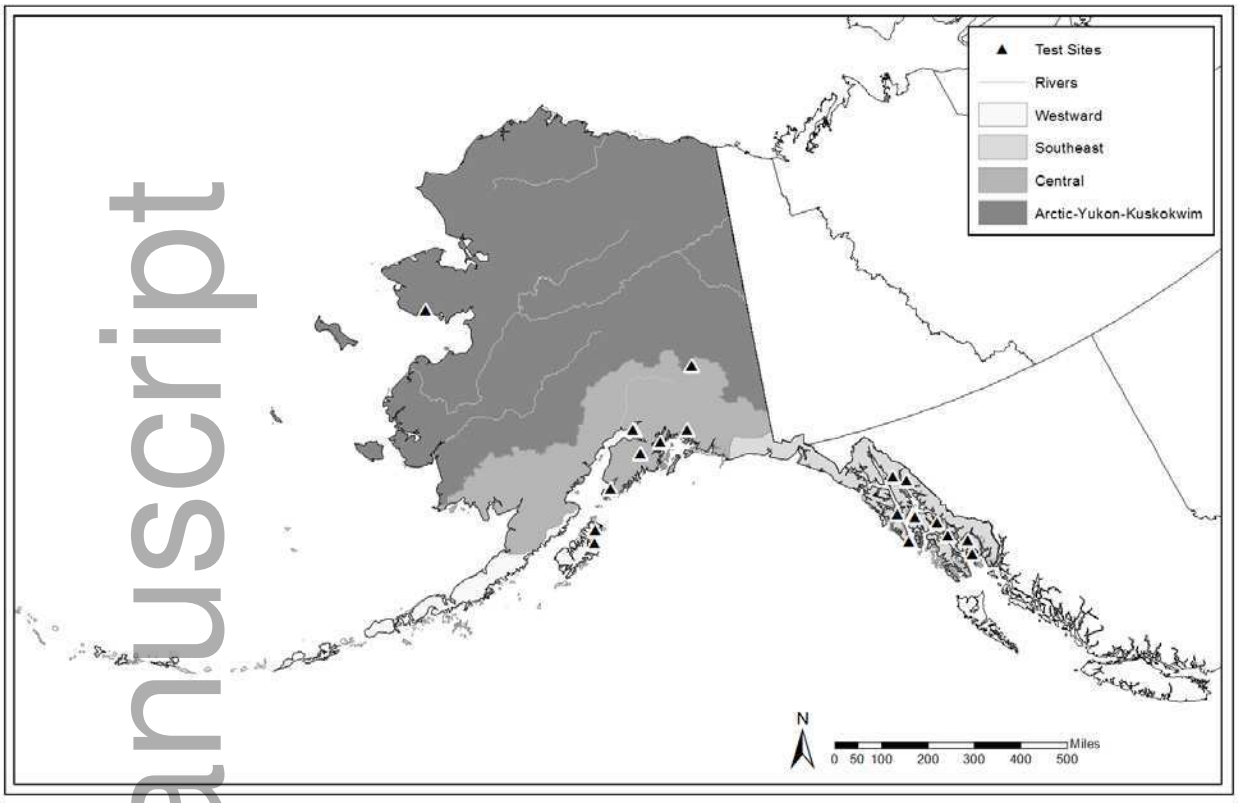

Figure 1: Map of the four marine regions in Alaska and locations of sample collection sites (test sites). Marine regions include Southeast (SE), Central (CTR - Copper River, Prince William Sound, Upper Cook Inlet, Lower Cook Inlet, Bristol Bay), Arctic-Yukon-Kuskokwim (AYK - Kuskokwim, Norton Sound and Kotzebue, Yukon), and Westward (WR - Kodiak Island, Alaska Peninsula, Chignik, Bering Sea/Aleutian Islands).

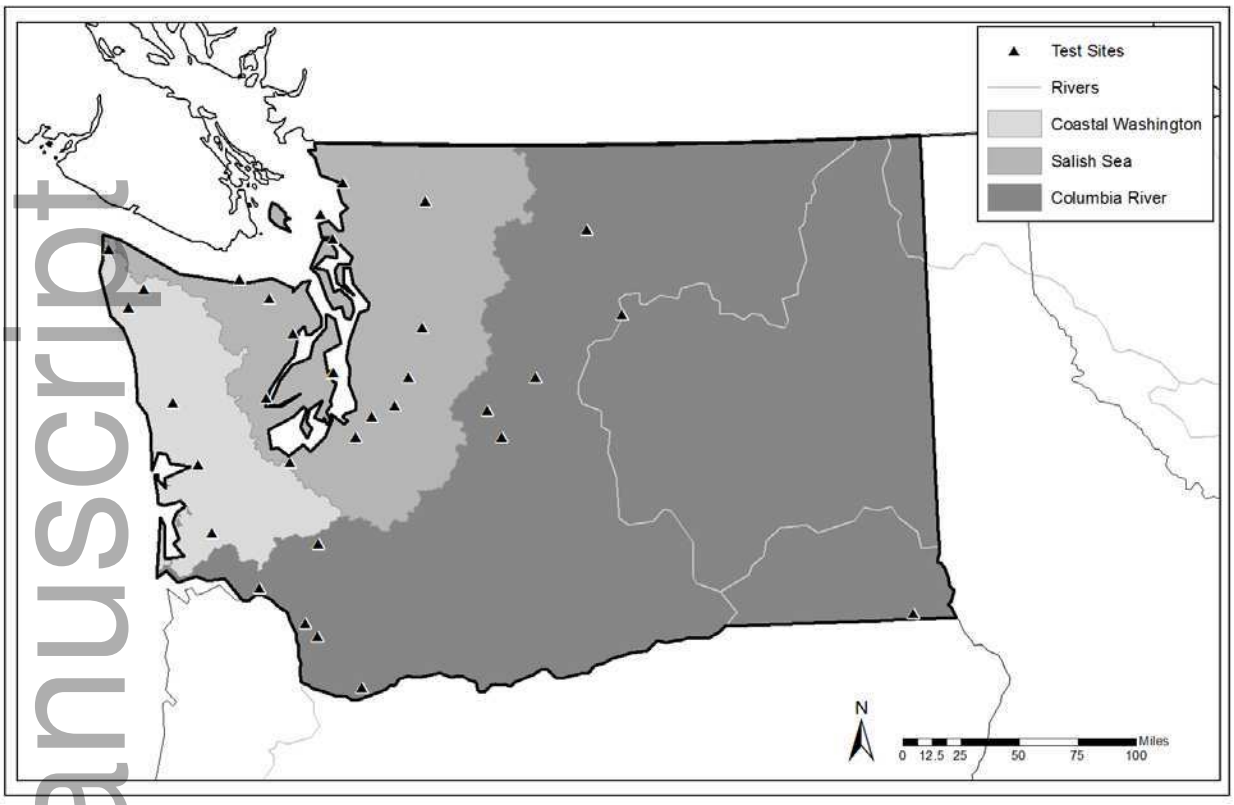

Figure 2: Map of the three marine regions in Washington and locations of sample collection sites (test sites). Marine regions include Salish Sea (PSS - all samples limited to Puget Sound and the Strait of Juan de Fuca), Coastal Washington (WAC), and Columbia River (CR). Atlantic salmon are farmed in the PSS region.

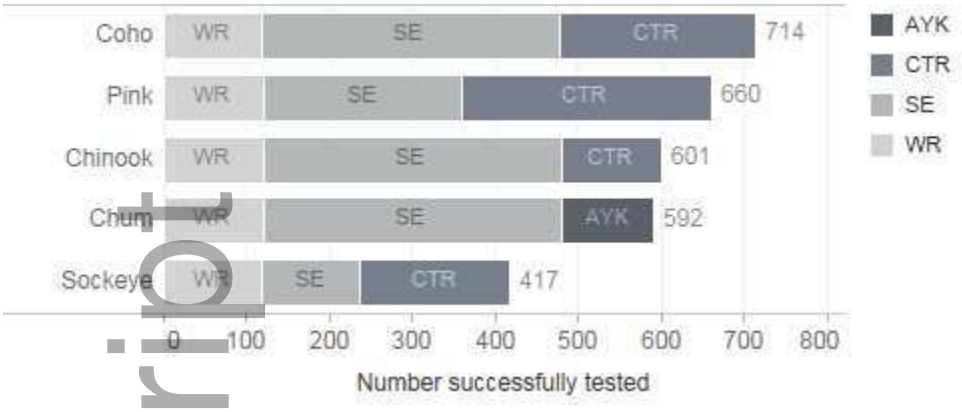

Figure 3: Number of salmon sampled and successfully tested for ISAV RNA in Alaska, by species and region. Marine regions include Southeast (SE), Central (CTR - Copper River, Prince William Sound, Upper Cook Inlet, Lower Cook Inlet, Bristol Bay), Arctic-Yukon-Kuskokwim (AYK - Kuskokwim, Norton Sound and Kotzebue, Yukon), and Westward (WR - Kodiak Island, Alaska Peninsula, Chignik, Bering Sea/Aleutian Islands).

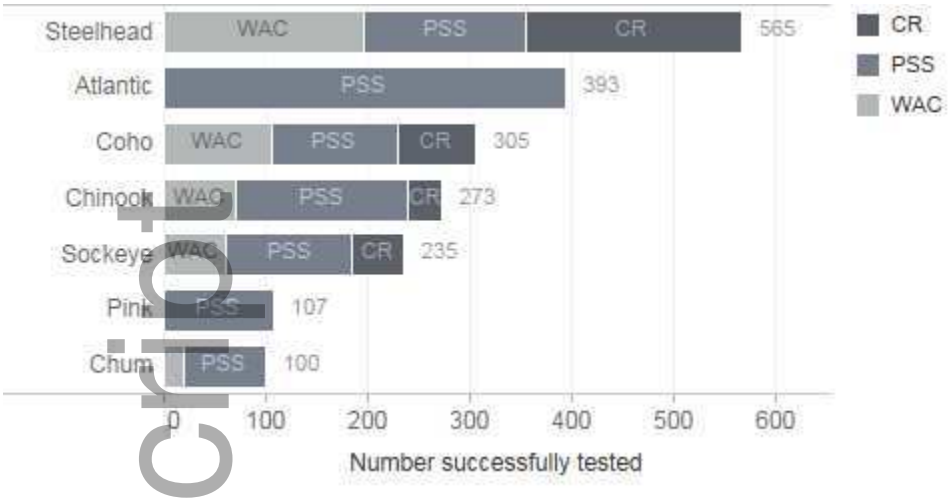

Figure 4: Number of salmon or trout sampled and successfully tested for ISAV RNA in Washington, by species and region. Marine regions include Salish Sea (PSS - all samples limited to Puget Sound and the Strait of Juan de Fuca), Coastal Washington (WAC), and Columbia River (CR).

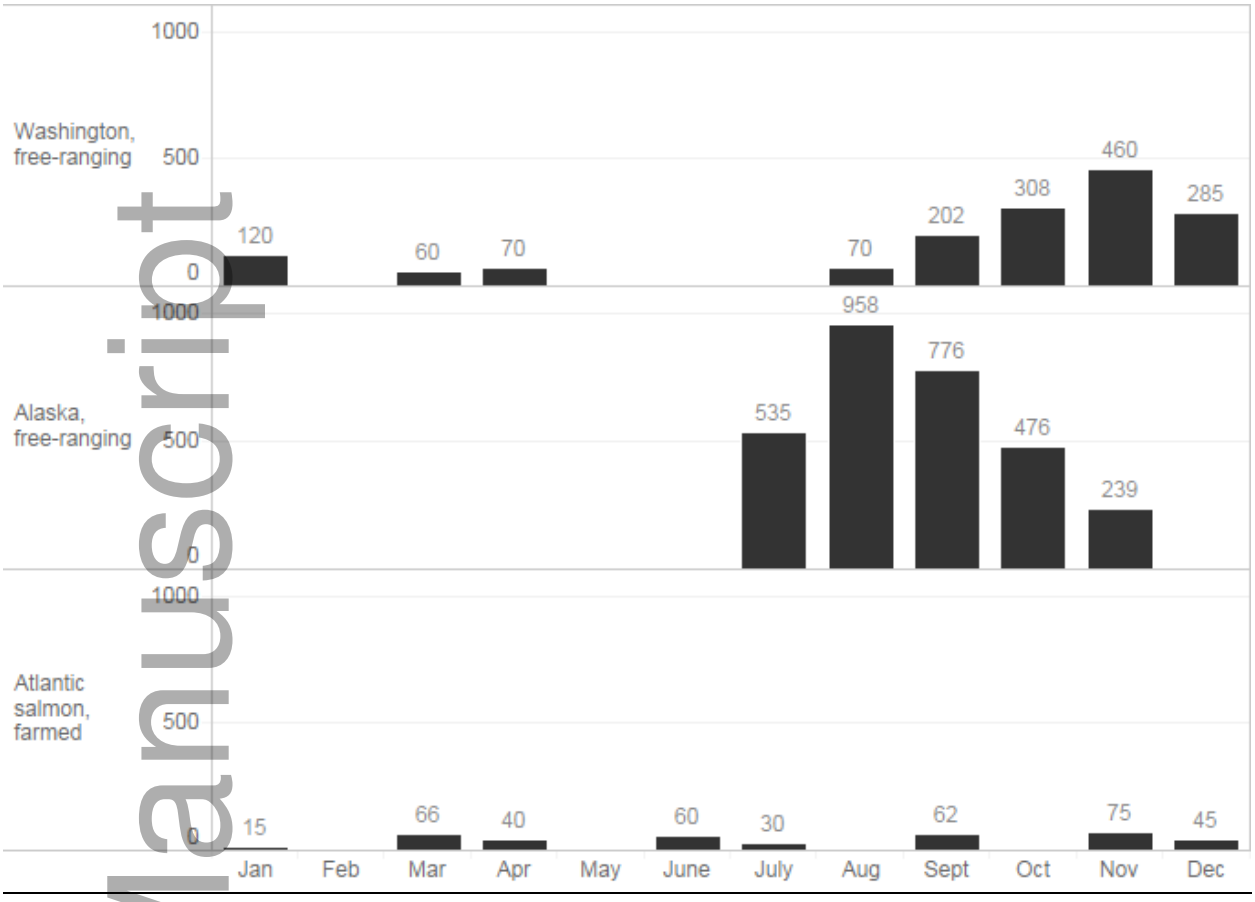

Figure 5: Seasonal distribution of ISAV sampling effort. Free-ranging species include the Pacific salmon and trout species. Atlantic salmon are farmed in Washington State. Sampling extended over 3 ½ years. Ten Chinook salmon from Washington coast were excluded here as their collection date was recorded by season (Oct-Dec) rather than month.