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Piscean Orthoreovirus (PRV) in Processing Plant Wastewater: A Review of Risk Factors for Wild Pacific Salmon

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This report has been reviewed by staff of the Ministry of Environment and Climate Change Strategy, but the conclusions and recommendations expressed herein represent the views of the consultant authors, and these views may or may not be supported by the Ministry.

Executive Summary

Recent detection of Piscine Orthoreovirus (PRV) in seawater near a salmon processing plant increased concerns that wild salmon in BC are at risk of harm by being exposed to and infected by live PRV. The virus is recognized as a causative factor to the disease Heart and Skeletal Muscle Inflammation (HSMI) in Norwegian salmon. This report reviews scientific studies on PRV and HSMI that pertain specifically to the strain of PRV identified in BC salmon to a) assess the potential risk PRV poses to the health of exposed wild salmon in BC, b) identify significant data gaps in making such an assessment, and c) add context to the finding of PRV in seawater around fish processing plants.

PRV is the only marine virus among the Orthoreovirus genus. As a group, the Orthoreoviruses are non-enveloped viruses that are resilient in the environment compared to other marine viruses. After infection, PRV concentrates in the blood, where it replicates by forming viral factories inside the red blood cells. Real time based PCR tests are well established for detecting PRV RNA sequences in blood or other fish tissues. Even so, knowledge of PRV and methods to detect it are relatively new to science and do not extend back to the start of commercial salmon farming in BC.

The difficulty is that PRV-infected fish may not show disease signs, including HSMI. While studies on Norwegian salmon established that HSMI develops in salmon either deliberately injected with purified PRV, PRV-infected blood or cohabited with donor PRV-infected fish, not all fish develop HSMI. This is despite all fish becoming PRV positive and all fish with HSMI being PRV-positive.

In BC, PRV infections of Atlantic and Pacific salmon can similarly be produced with injection of infected red blood cells and cohabitation. In several studies, the development of HSMI has yet to be demonstrated for the BC strain of PRV.

Clearly, the linkage between a PRV infection and disease symptoms is not a foregone conclusion, and the situation is not well defined in BC.

This leads some scientists to suggest that either the genetics of PRV, the genetics of fish or the environmental conditions and aquaculture practices in BC are fundamentally different from Norway.

Infection studies in BC reveal no mortality of salmon resulting from a heavy PRV infection. Similarly, while the high PRV prevalence in BC's farmed salmon detected by molecular techniques, routine health monitoring reveals few deaths associated with symptoms of HSMI.

There have been no unusual spikes in mortality associated with PRV infection in BC. This situation contrasts dramatically with the situation in Norway where PRV infection rate is similarly reaching 100%, HSMI is evident and HSMI-related mortality is assessed around 20% industry-wide.

The situations in BC and Norway differ in terms of outcomes of a PRV infection. Furthermore, a PRV-positive test is a poor indicator of the long term mortality in BC's farmed salmon. By all accounts, PRV in BC acts in a benign fashion.

The difference between BC and Norway represents an important and major information gap world-wide with respect to PRV because scientific investigations are very limited.

Scientists predict biological impacts to respiratory function, swimming ability, cardiac function and even migratory ability of salmon because the target tissue of a PRV infection is the red blood cell, while heart and skeletal muscle inflammation are signs of the HSMI disease.

In contrast, numerous studies in BC have seen no effect of a heavy PRV infection on the concentration of red blood cells, the oxygen binding properties of infected red blood cells nor any major sustained changed in either the aerobic exercise capacity or hypoxia tolerance of the infected fish. Thus, the current weight of evidence suggest that salmon in BC are mostly indolent to infection of the PRV strain present in BC.

The cohabitation studies show that PRV can be transmitted horizontally among fish. Thus, there is an expectation that PRV can spread to wild fish in the vicinity. However, there is no strong evidence so far that PRV can pass from an infected parent to a fertilized fish egg – vertical transmission. This may explain why farmed Atlantic salmon smolts in BC enter seawater PRV-free and juvenile anadromous salmon sampled during their downstream migration are also PRV-free.

Fish therefore contract PRV from the environment after entry to seawater, but the source of the infection is not known. Again findings for PRV infections in juvenile salmon differ between BC and Norway. This difference needs a resolution, with further studies including with whole genome sequencing of PRV to compare the two genomes and collaborative studies to infect BC fish with the Norwegian strain of PRV and vice versa (a study already underway).

While PRV may be detectable in seawater around salmon processing plants, many questions remain concerning the viability of the detected virus; PCR detection does not imply live virus as only part of the

genetic material is assayed and wastewater is typically disinfected prior to discharge leaving genetic material that may not be viable.

This leads to recommendations to allow a rigorous and transparent risk assessment that has implications for all sectors and communities including First Nations.

Natural and endemic levels of PRV in BC's wild salmon and the waters in which they live need to be fully understood. PRV exists in wild salmon throughout their migratory range at varying levels of prevalence.

The marine reservoirs of PRV in BC and the transfer dynamic between farmed and wild fish populations needs to be scientifically assessed through a rigorous sampling design.

An association study claimed a higher prevalence in wild salmon sampled closer to salmon farming areas, especially those in the central coast. However, the authors acknowledged the weakness of their study design as it does not "constitute an extensive, structured surveillance of wild salmonids in BC" and the authors did not attempt to "construct precise estimates of PRV prevalence in wild salmon with tight confidence limits". Also the authors report large year to year changes in the PRV prevalence, being much lower in 2014 than in 2012. Similarly, another study in Alaska and Washington State reports high variability in PRV prevalence among species of salmon. These observations require further investigation.

So far, no diagnostic test can detect the live virus. The only test used for screening PRV is based on methods (qRT-PCR) that detect a small part of the PRV genetic materials. These tests on wastewater are insufficient to provide information on the viability or integrity of the virus after treatment and release. In summary, several key parameters are still unknown to better provide a reliable risk assessment of PRV and HSMI to BC's wild salmonid populations. Research gaps highlighted in this review need to be addressed as PRV is a recently discovered virus and its pathogenicity to salmonids is still a mystery.

Contents

Requirements:

Piscine Orthoreovirus (PRV), also known as Piscine Reovirus, is linked through its co-occurrence with diseases such as heart and skeletal muscle inflammation (HSMI). Recent media coverage has raised public concern about the presence of PRV and consequences for Pacific salmon.

The Ministry would like a report prepared that provides a summary of the current state of knowledge relevant to the risk posed to Pacific salmon by PRV presence in the effluent discharged from fish processing plants. We expect that this contract will include a review of the information provided by the Fisheries and Oceans Canada in May 2017 (http://www.dfo-mpo.gc.ca/science/aah-saa/species-especes/aq-health-sante/prv-rp-eng.html), and other studies relevant to Pacific salmon.

The report should characterize the risk that PRV and HSMI, or other diseases related to PRV, pose to wild Pacific salmon stocks. The analysis should also identify any other fish species that carry, or could carry, the PRV and are processed at fish processing plants operating in British Columbia.

Objectives

The objectives of this review are to:

- Supply information to help quantify the risk of Piscine Orthoreovirus (PRV) and understand the immediacy of actions necessary to deal with the impacts from fish processing plants
- Better understand the actions necessary to lessen the impacts from processing plants
- Increase the confidence by the public, government and all concerned stakeholders that relevant and current information has been assessed to understand the risk posed by processing plants.

This report summarizes the current state of knowledge relevant to the risk posed to Pacific salmon by PRV presence in effluent discharged from fish processing plants. This report includes:

- A review of the information [provided by Fisheries and Oceans Canada in May 2017 in addition to subsequent reports,
- Characterization of the risk that PRV and Heart and Skeletal Muscle Inflammation (HSMI) or other diseases related to PRV, pose to wild salmon stocks,
- Identification of all species known to carry or that could carry PRV and are processed at fish processing plants in British Columbia, and
- Other pertinent and available information that is relevant to understanding the impact of fish processing plants and reportable viruses on Pacific salmon.

Piscean Orthoreovirus (PRV) in Processing Plant Wastewater

A Review of Risk Factors for Wild Pacific Salmon

Preamble

The Ministry of the Environment produced a Request for Proposal to examine the *State of Science on PRV in BC waters*. Specifically, the Ministry requested a literature review to help assess the risk to wild salmon that may come into contact with Piscine Orthoreovirus (PRV) present in fish processing plant wastewater. Further, the Ministry sought clarity on the issue of Pacific salmon contracting Heart and Skeletal Muscle Inflammatory disease (HSMI) as a result of infection from PRV that could be present in wastewater. Finally, the Ministry sought guidance to identify mitigation procedures and knowledge gaps for further research. To provide information on the requested topics, this report presents summaries of the current scientific information on PRV in farmed and wild salmon, the implications of PRV in the development of diseases such as HSMI and provides recommendations on the risk that PRV can pose to Pacific salmon if released to the environment by sources such as processing plants.

A summary is provided to report the assessed risk to wild Pacific salmon posed by exposure to processing plant wastewater that contains PRV. Knowledge and research gaps are identified and reported.

Chapter 1 Background of PRV discovery and the association with HMSI

Piscine Orthoreovirus (PRV) was identified in this decade. In a scientific era that can trace pathogens by genetic fingerprint, it is rare for 'new' pathogens to be identified. The association of PRV as a causal agent of heart and skeletal muscle inflammatory disease (HSMI) has been a difficult issue to describe accurately for all salmon-farming regions: PRV is present in all salmon-farming areas, yet HSMI is not. Further, there is no known record of HSMI in wild Pacific salmon although PRV is endemic among all species. It is important first to understand the history of HSMI and second, the association with PRV. This association between HSMI and PRV is not universal and the suspected root cause of HSMI is unclear. The discovery of PRV is an important point in understanding the current situation regarding exposure of wild salmon to PRV in the environment.

PRV was identified as a novel reovirus in 2010 by Palacios et al., using next generation sequencing. The reason for the discovery of PRV was a result of an investigation into the causal agent of fish exhibiting the condition of HSMI. At the time (1999), HSMI was causing notable mortalities in farmed Atlantic salmon (*Salmo salar*) in Norway (Kongtorp et al. 2004a,b). Essential to understanding the association of PRV in the development of HSMI is the relationships of the virus to the host in the context of its environment. These relationships differ between BC and Norway; HSMI has been present in Norway for decades while in BC, HSMI-associated mortalities have not been witnessed despite detection of the virus in wild and farmed salmon.

The key uncertainty was understanding the causal relationship between PRV and HSMI and demonstrating that PRV caused the disease (Koch's Hypothesis, 1891). The classic method for determination of the direct cause of disease by a pathogen is to purify the agent in cell culture and apply this to target organisms. In this manner, the disease is, or is not induced by a single, purified agent. However, no methods of cell culture are known for PRV and in the case of HSMI, the unknown etiological agent could not be identified. In the lab, injection of homogenized tissue from diseased fish into healthy individuals caused HSMI, and naïve cohabiting fish were also affected (Kongtorp and Taksdal 2009). This primary method of inducing disease is a generally-accepted approach that does not single out the causative agent because many agents could be contained within the homogenate. A viral etiology was proposed for HSMI when antibiotic treatment had no effect on disease development (Kongtorp et al. 2004a, Kongtorp and Taksdal 2009) and viral particles were detected (Watanabe et al. 2006).

As purified PRV is not available, Wessel et al., (2017) took advantage of the high levels of replicating PRV in salmon host red blood cells and developed methods to extract and purify infectious PRV particles. Infected Atlantic salmon were bled at the peak of the infection and the particles purified from the blood cells and inoculated into naïve individuals. The virus propagated and infected cohabitant naïve fish. These infected fish developed signs consistent with HSMI behaviorally and by histopathological examination of tissues. The authors concluded that PRV was the causative agent for HSMI. This was later confirmed by other authors.

Confounding the issue is that not all Atlantic salmon in Norway that test positive for PRV will then develop HSMI despite the virus being ubiquitous in the marine environment (Finstad et al., 2012; Lovoll et al., 2012) and infection rates of above 50% exist with mortality of 2% on average (Garseth et al., 2013; Kongtorp et al., 2004a). Likewise, the ability of injection or cohabitation to cause HSMI in Atlantic

salmon is in contrast with experiments that show high infection transmission, but no onset of disease in Atlantic and sockeye salmon (*Onchorhyncus nerka*; Garver et al., 2016a,b). In all cases to date, the infection profile characteristically consists of a viral amplification phase about eight weeks post infection (WPI) and a reduction past that point (Finstad et al., 2014; Garver et al., 2016b) although levels of PRV remain elevated for a prolonged period (Garver et al., 2016a). Finstad et al., (2014) working with Norwegian Atlantic salmon and inoculant of Norwegian origin demonstrated that the signs of HSMI can receded in surviving PRV-infected fish as indicated by a decrease in cardiomyopathy over time. In contrast, no signs of HSMI were induced in BC origin Atlantic salmon or sockeye salmon (Garver et al., 2016a,b) despite high PRV loads in blood and kidney. Several authors speculate that a likely differentiation between Norwegian and BC strains of PRV could account for the observed instances of pathogen expression (Myers, 2017; DFO, 2018).

The research focus after cohabitation and injection studies in BC (Garver et al., 2016a,b) and Norway (Wessel et al., 2017) has not focused on what causes HSMI, but rather how PRV induces HSMI in Atlantic salmon. It is noteworthy that the loads of PRV injected into fish in BC are comparable to those in Norway, yet the development of HSMI could not be duplicated in Pacific or BC Atlantic salmon under controlled conditions. The possibilities to explain this conundrum may relate to strain differences, host differences and environmental factors required for disease development (Myers, 2017; DFO, 2018). Of the latter, stressful events such as seawater entry of smolts has been suggested as a primary stressor as smolts can develop HSMI weeks after entry into seawater (Kongtorp et al, 2007; Lovoll et al., 2012). However, to mimic a stressful event, Polinski et al., (2016) experimentally compromised the immune system of BC Atlantic salmon with another pathogenic virus (the infectious hematopoetic necrosis virus; IHNv) before exposure to PRV. Despite the immune challenges posed by IHNV, HSMI could not be induced. Current research is being undertaken at the Centre for Aquatic Technologies – Canada in Souriee, Prince Edward Island where both Norwegian and BC strains of PRV (amplified by the erythrocyte method) have been injected into both Atlantic salmon (east and west coast strains) and Pacific salmon. Preliminary results are not available (Polinski pers. comm.). These results are much anticipated to help delineate the future path of PRV research.

The commonly-accepted incidence of HSMI losses in Norway range up to 20% of the production volume and averages about 2% overall (Kongtorp et al., 2006). As with most farming practices, behavioural changes in the fish such as lethargy, cessation or reduction in feeding, and loss of condition, trigger fish health protocols to sample fish. Samples of the morbid fish are generally sent for examination by fish

health professionals and in the case of HSMI indications in Norway, the fish are examined for histopathological effects in heart and skeletal muscle (see below Chapter 3). This practice involves trained and certified professionals (in Canada accreditation is through the American Society for Clinical Pathology; ASCP) to distinguish HSMI from similar indications such as pancreatic disease or salmon rickettsia (Kongtorp et al., 2004a).

Di Cicco et al., (2017) examined samples from a routine fish health monitoring program at a BC salmon farm in the Broughton archipelago and described the progression of cardiac lesions consistent with histopathological signs described in Norway for HSMI cases. The period of sampling was also consistent with the time period for which Norwegian salmon would be expected to exhibit losses from HSMI. The occurrence of HSMI indications was observed in 20-40% of the sampled fish with others exhibiting some level of heart inflammation. The authors note that there was no elevation observed in mortalities during the period although lesions were apparent. Amongst the fish sampled with cardiomyopathy, the detection of PRV was statistically significant along with two other identifiable parasite pathogens. Although the study could not confirm a causal relationship between HSMI and PRV, the indications are that 'full blown' HSMI is not a production or fish health concern and that the relationship of the BC PRV is inconsistent with the effect of Norwegian PRV in relation to the induction of HSMI in Norway.

The study also demonstrated the source of PRV infection of the farmed salmon was from a marine reservoir as the freshwater smolts were screened for PRV which was not detected. This is in contrast with Lovoll et al., (2012) who noted that 36% of freshwater smolts tested positive for PRV, but not HSMI. Johansen et al., (2016) noted HSMI histopathological heart lesions in parr that were most prominent after 10 weeks of PRV-injected and cohabitant parr. It is unknown if current practices in Norway include screening smolts for PRV as in BC.

The reservoir of PRV infection has been postulated to be in wild salmon and is endemic to the eastern Pacific (Purcell et al., 2018) and in Norwegian waters (Wiik-Neilsen et al., 2012; Garesth et al., 2013). In BC, the origins of PRV putatively predate the introduction of Atlantic salmon to BC where transcripts of PRV segments were detected in steelhead trout (*Onchorynchus mykiss*) samples from embedded histology blocks and confirmed from archived samples of Pacific salmon from 1984 (Marty et al., 2015). In Norway, PRV has been detected in several marine species although not ubiquitous among those samples (Wiik-Neilsen et al., 2012; Garseth, et al., 2013). Further, wild salmon have not been diagnosed with HSMI in Norway or BC (Garseth et al., 2013; Purcell et al., 2018) although PRV is enzootic in farmed and wild salmonids on the Canada/US Pacific coast (Siah, et al., 2015).

Conclusions

The discovery and description of PRV and HSMI in farmed Atlantic salmon is relatively new with respect to the identification of cultured fish pathogens having been observed and identified over the last decade. In comparison to other fish diseases, HSMI is an emergent disease and awaits further discovery and mitigation measures. Because of the relatively recent emergence of the disease, there is much to be discovered in the etiology and presentation, especially as it pertains to the obvious regional differences in the relationship between PRV and HSMI. With the majority of information still to be discovered, there is consensus among researchers that:

- PRV is endemic in Pacific salmon species along the coast from Alaska to Washington State with variable levels of prevalence (Siah et al., 2015; Purcell et al., 2018)
- PRV likely existed before the commercial introduction of Atlantic salmon (Siah et al., 2015)
- PRV infection can cause HSMI at a lower incidence than that for PRV infection in Norway, and HSMI has never been reproduced in a Norwegian lab without the presence of PRV (Wessel et al., 2017)
- PRV has not yet been shown to cause HSMI in BC, and HSMI cannot be induce by introduction or exposure to PRV (Garver et al., 2016), even though there is evidence to suggest a PRV-infection was associated with inflammation of heart tissue moribund farmed salmon (; Di Cicco et al., 2017)
- PRV infections do not elevate mortality levels in farmed Atlantic salmon in BC, as it does in Norway (Kongtorp et al., 2009; Di Cicco et al., 2017)
- Farmed salmon contract PRV from endemic sources (Siah, et al., 2105; Di Cicco et al., 2017; Purcell et al., 2018).

Chapter 2 Description of PRV and how PRV infections (invasion and multiplication of the virus) are assessed by scientists and pathologists.

In order to understand why PRV is infectious, can exist in open seawater and is prolific inside a salmon after infection, it is necessary to understand the nature of the virus, how it behaves in the host and why no meaningful immune response is launched immediately after infection. Many of these reasons for PRV persistence and scientific interest reside at the molecular level, and are characteristics of a Reovirus in general. For example, Mammalian Orthoreovirus can reside in other adult host species

without expression of disease (cf. Guglielmi et al., 2006). Thus, it is essential to provide a background description of PRV in the context of how it is constructed, its genome and how infections in fish arise.

Physical description of PRV

The virions of Reoviridae family viruses measure 60-80 nm in diameter and possess two concentric capsid (enclosing) shells. No envelope is present meaning that the virus does not 'cloak' itself in an outer layer. The inner capsids of all genera display sharply defined subunits; the outer capsids of rotaviruses and orbiviruses lack well-defined subunit structures. The PRV genome consists of double-stranded (ds) RNA in 10-12 discrete segments, with a total genome size of 16-27 kilobase pair (kbp), depending on the genus. There are three lengths of ds RNA and classification is on this basis: four Short (S) strands, three Medium (M) strands and three Long (L) strands. Each length is sequentially numbered, for example Short 1 = S1 and so on (Palacious et al., 2010). The double-shelled particle containing the ds RNA strands is the complete infectious form of the virus[1].

The dsRNA genome is never completely unprotected during the replication process, which prevents activation of an antiviral state by the host cell in response to exposure of the RNA. Viral polymerase synthesizes mRNA from each of the dsRNA segments internally to the virus. These mRNAs are translocated to the cell cytoplasm where they are translated.

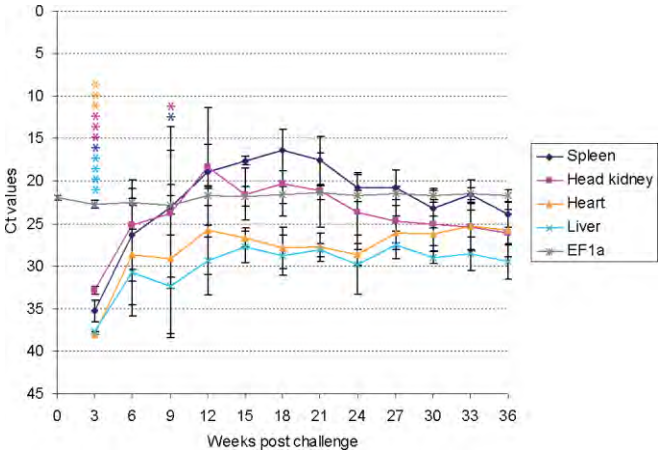
By never exposing the dsRNA genome to the host cell, it prevents activation of an antiviral state in response to the dsRNA. The viral polymerase lambda3 synthesizes a capped (protected) and non-polyadenylated monocistronic (not recognized) mRNA from each dsRNA segment. These capped mRNAs are translocated to the cell cytoplasm where they are translated and thereby made into new viral particles. Full-length plus-strand transcripts from each of the dsRNA segments are synthesized into viral proteins and genomic RNAs aggregate in cytoplasmic viral factories. The new capsid is then assembled on the sub-viral particle. Mature virions are released presumably following cell death and associated breakdown of host plasma membrane.

These descriptions of the PRV are important to understanding how the virus works during the infection process. It also leads to the identification and phylogenetic description of genetic subtypes that may differ with geographic region.

[1] S.C. Johnson: Exploring PRV and HSMI in Europe and B.C. DFO ACRDP Workshop, Campbell River, BC. November 27-28, 2017

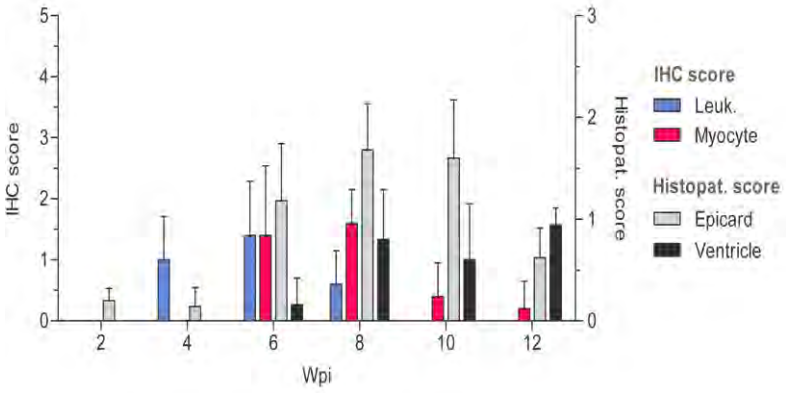
PRV Infections

PRV is a systemic virus whose sequences have been present in several organs of salmonids including head kidney, liver, spleen, blood cells and heart. PRV RNA sequences have been detected with high loads in spleen and head kidney in comparison to heart and liver (see Figure below; Lovoll et al., 2010), which are also well vascularized and contain red blood cells.



PRV RNA sequence detected in spleen, head kidney, heart and liver of Norwegian Atlantic Salmon.
Data shows Ct values of PRV partial segment L1 in spleen, head kidney, heart and liver after weeks of intraperitoneal injection challenge (Lovoll et al., 2010)

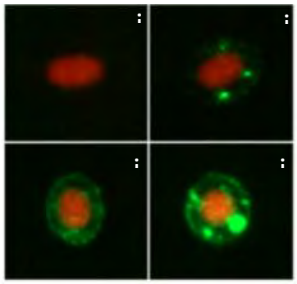
Targeting PRV capsid proteins, immunohistochemistry was used to investigate PRV localization and distribution in heart tissue of Norwegian Atlantic salmon during HSMI development. Both inoculated and cohabitant group of Atlantic salmon showed that PRV capsid proteins are first present in leukocyte-like cells and later in cardiomyocytes of the heart ventricle (Finstad et al., 2012). It takes two weeks for PRV to infect the cardiomyocytes once it has infected blood cells (Figure below).



Immunohistochemistry score of PRV capsid protein in leukocytes (blue) and myocytes (red) and histopathological changes of epicardial (gray) and ventricular (black) of inoculated Norwegian Atlantic Salmon (Finstad et al., 2012).

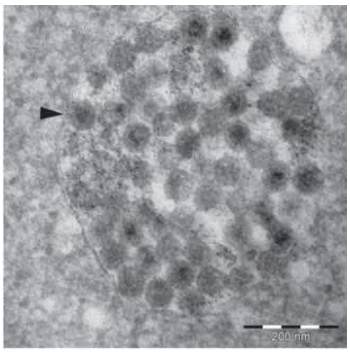
Blood and cardiac cell infection coincide with histopathological changes of epicardial and ventricular tissues. The authors concluded that these results corroborate the linkage between the presence of PRV in blood and myocytes and the development of HSMI (Finstad et al., 2012).

Cohabitation studies have shown that PRV will first infect erythrocytes four weeks post-challenge (Finstad et al., 2014) and these cells will be highly infected in comparison to all the other organs including spleen and head kidneys during that time. More than 50% of erythrocytes were PRV positive in the early phase of the infection (5-7 weeks post infection) and the number of infected blood cells decreased at 8 weeks post infection (Finstad et al., 2014). Using immunofluorescence and confocal microscopy, cytoplasmic inclusions have been observed at the perinuclear region or scattered in the cytoplasmic compartment of the blood cells (Figure below).



Localization in red blood cells by immunofluorescence microscopy
i) Negative blood cells, ii) inclusions at the perinuclear regions of blood cells, iii) inclusions scattered in the cytoplasm and iv) large cytoplasmic inclusions (Finstad et al., 2014)

The cytoplasmic inclusions correspond to viral factories which contain reovirus-like particles as shown by electron microscopy (Figure below).



Electron microscopy of cytoplasmic inclusions
Reovirus-like particles (arrow) present in cytoplasmic inclusions of red blood cell (Finstad et al., 2014).

These viral factories correspond to the location where the viral genomes are replicated and viral particles are formed during the infection. Because red blood cells are nucleated, they contain all the transcriptional and translational machineries needed for generation of generational viral particles (Finstad et al., 2012, 2014).

The level of viral genetic materials detected in blood cells in cohabitant Norwegian Atlantic salmon peaked at 4 weeks post challenge and plateaued during all the experiment period. However, the level of capsid proteins peaked at 4 weeks, remained high for 1-2 weeks and then decreased after 6 weeks post challenge. No protein is detectable after 6 weeks post challenge although the load of genetic materials remain high after 6 weeks post challenge (Haatveit et al., 2017) and up to 36 weeks post challenge
(Lovoll et al., 2010).

For Pacific salmon, a controlled challenge study was performed at the Pacific Biological Station in Nanaimo (BC). BC Atlantic and sockeye salmon were exposed intraperitoneally (ip) and by cohabitation to PRV (Garver et al., 2016). PRV sequence levels peaked in blood cells at two weeks post ip challenge and at 6 weeks for Atlantic salmon and 12 weeks for sockeye salmon during the cohabitation experiment. The PRV genetic material remain persistent during the 41 weeks experiment period in both Atlantic (100% prevalence) and sockeye (80% prevalence; Garver et al., 2016). Although the infection patterns of BC PRV strain in BC Atlantic salmon were similar to those of the Norwegian PRV strain that induces HSMI in Norwegian Atlantic salmon, BC Atlantic salmon did not develop changes in their hematocrit during infection, or develop histopathological changes related to HSMI and there were no increases in mortalitiy. In comparison to infected BC Atlantic salmon, infected sockeye sentinels had a lower viral load in both kidney and blood samples compared to Atlantic salmon sentinels. These data suggest that sockeye salmon could be less susceptible to PRV infection than Atlantic salmon. Similar to infected Atlantic salmon, PRV-infected sockeye salmon did not develop histopathological changes related to HSMI (Garver et al., 2016a,b).

PRV genetic diversity in BC and worldwide

Given the important differences in the outcomes of experimental PRV infection studies in Norway and BC, it is critical to know if the two viral strains of PRV differ.

Due to its variability between isolated sequences, PRV segment S1 has been widely used for phylogenetic analysis in several studies. Based on phylogenetic analyses of the available sequences of segment 1 (Seg-S1) of the virus, it is possible to group the strains into two genotypes I and II; at the same time, genotype I is subdivided into two sub-genotypes Ia and Ib. Takano et al. (2016), describes a new virus closely related to PRV based on phylogenetic analyses of segment S1 and λ3, but different enough to be designated as Piscine orthoreovirus 2 (PRV-2).

Kibenge et al. (2013) sequenced PRV segment S1 from 12 samples from British Columbia including: wild cutthroat trout (*Oncorhynchus clarkii*), farmed steelhead trout (*Oncorhynchus mykiss*), wild chum

salmon (*Oncorhynchus keta*) and farmed Atlantic salmon (*Salmo salar*) from Chile (Kibenge, et al. 2013). Their phylogenetic analysis grouped Norwegian PRV strains into a single genotype of two sub-genotypes (la and l b) with BC strains clustering with sub-genotype la and Chilean strains with sub-genotype Ib. A larger survey was performed by Marty et al., (2015) who tested salmonid tissues from Alaska and BC between 1974 and 2013 (Marty, et al. 2015). These RT-qPCR tests amplified PRV sequences from salmonids tissues collected from Alaska and BC in the 80's. Taking advantage of Marty et al., (2015) and the recent surveys in Washington State, Siah et al., (2015) pursued a phylogenetic analysis using a partial segment of PRV segment S1 (Siah, et al. 2015). The authors investigated both the occurrence and genetic diversity of PRV sequences isolated from wild and farmed fish collected in these regions. This latter study analyzed 71 sequences isolated from salmonids collected from 21 different locations spanning from Alaska to the Columbia River over a 13 year period (2001-2014). The results revealed 10 distinct sequence types over 71 sequences with 1.1% maximum nucleotide diversity. The phylogenetic analysis was performed using the methods of Garseth et al., (2013) as a reference for consistency. Results showed a high genetic homogeneity within western North America because all the sequence types were not statistically different and were grouped with some Norwegian sequence types that cluster within Group II. The Norwegian sequences including the two Chilean sequences (GenBank accession numbers KC782501 and KC795571) of Group I differ by more than 4% from Group II sequences.

This difference in genetic identity indicates a high variability of PRV segment S1 in the Norwegian PRV. In contrast, the western North American S1 sequences suggest a low level of PRV diversity in this area. For instance, the sequence type isolated from BC salmonid tissue archived in 2001 was identical to the sequence from samples collected in 2014 from Alaska, BC and Washington State.
Taken together, Myer (2017) concluded it is likely that genetic variants account for the 'relatively benign' nature of the BC PRV strain, which appears unable to produce a significant outbreak of HSMI unlike elsewhere.

Conclusions

The mechanism of PRV infection is similar between PRV strains found in Norway and in BC. The target of infection is the erythrocyte and many other tissues are infected. The BC PRV strain does not exhibit the same pathogenicity as the Norwegian PRV and this may be due to phylogenetic differences. Although widely used, segment S1 does not provide a high resolution to discriminate between the closely related PRV strains. Therefore, the analysis of the PRV genome is highly recommended. So far, only ten full genome

sequences are available in databases and include PRV sequences isolated from Norway (Palacios et al., 2010; Haatveit et al., 2016; Wessel et al., 2017), Chile (Kibenge et al., 2013) and Canada (Kibenge et al., 2013, Siah et al., 2015) farmed Atlantic salmon as well as wild coho salmon (*O. kisutch*) from BC and the Columbia River (Washington State; Siah et al., 2013). The analysis confirmed that PRV sequences isolated from salmonids in BC and Washington State are clustered in a different group compared with the Norwegian and Chilean strains. Importantly, the genome analysis was able to significantly discriminate between the PRV sequences isolated from wild coho and farmed Atlantic salmon sources.

This initial study provides evidence that PRV genomic analysis is an accurate tool to study the connectivity between PRV strains isolated from wild and farmed salmonids in BC and worldwide. By analogy, the same approach is used in Ebola epidemiological studies where genomic analysis unravelled the geographical dynamic pathway of the virus during the 2013-2016 epidemic (Holmes et al., 2016; Dudas et al., 2017). However, the number of PRV genomes currently available is still too small to be able to provide a more accurate assessment of the PRV evolution in BC aquatic environment and worldwide. This will need broader international research studies by sequencing several hundred to thousands of PRV sequences that could be isolated from different host species. Currently, efforts are underway for local, national and international institutions to collaborate in sequencing more PRV sequences.

Chapter 3 How HSMI is assessed by scientists and pathologists.

There are two concomitant ways to diagnose HSMI as caused by PRV. The first method developed was histopathology and the second, detection and quantification of the putative causal agent, PRV. In the former case, examination of the tissues is done by a qualified histopathologist. The method involves a visual determination and interpretation of the disease in order to diagnose. This method describes the effect of the pathogen and is an indirect measure. In the latter case, a quantitative measurement of the putative infectious agent is determined using a specific set of molecular probes and primers. This method is a direct measure of the target pathogen and does not demonstrate clinical pathology. Together, the combined methods assist with a more complete diagnosis than either one of the two methods.

Histopathology

The first description of the clinical and histopathological signs of HSMI were published by Kongtrop et al., (2004a). Here they describe the gross clinical characteristic signs of HSMI in Norwegian fish occurring 5–9 months after sea-transfer as abnormal swimming behaviour, anorexia and up to 20% mortality. Subsequent necropsy of the affected fish pointed towards circulatory failure with blood clots in the heart cavity being the most common finding (Kongtrop et al., 2014a,b). There may also be ascites, yellowish or blood-filled liver, splenomegaly and pin-prick haemorrhages (petecia) in the adipose tissue. The authors note that there were no external lesions present on the fish examined, however, muscular lesions have been reported subsequently (c.f. Finstad et al., 2012).

The germane histopathology descriptions are complicated to the lay person as they are precise and exact in their description of what is seen by the pathologist using light or confocal microscopy. This is not a practice that is common to the uncertified fish health professional and required certification by the ASCP Board in order to diagnose a condition such as HMSI.

Heart

According to Kongtorp et al., (20014a) and Ferguson et al., (2005) a range of histological changes are present in the hearts of affected fish. The most common lesions were ventricular, in which both the spongy and compact layers of the cardiac tissues are infiltrated by mononuclear cells, comprising macrophages, lymphocyte- and plasma-like cells signifying that an inflammatory response has been initiated. These inflammatory cells are localised within and around cardiac muscle cells in a diffuse or focal pattern, most evident in the compact layer. In severe cases, cellular infiltration can be extensive, and result in necrotic tissue. Affected myocytes exhibited signs of degeneration with condensation, eosinophilia, loss of muscle striation, vacuolation and central nuclei. Several cells were also undergoing pyknosis or karyolysis (decaying nuclei). Necrotic cells appeared in association with inflammatory infiltrates. Hypertrophic nuclei could be observed in a few myocytes within the spongy muscle layer. Endocardial cells were also multifocally hypertrophic, appearing most often adjacent to myocyte inflammation. (Cells that were trying to recover muscle mass.)

As an example of the exactness and complexity of the histopathological description of HSMI, Furgusson et al.,(2005) describe the heart inflammation thus:

> “Histopathological changes in myocardial spongy layer were characterized by widespread vacuolation, degeneration and subsequent cavitation of cardiac myocytes, with loss of striation, increased eosinophilia, karyorhexis of myocyte nuclei, and infiltration by a limited number of

> neutrophils and macrophages, some of the latter being ceroid-laden. In conjunction with these changes there was karyomegaly of myocyte nuclei, and in two nests of smaller nuclei were present within the substance of the myocyte (Ferguson et al., 2005)."

The key points here are to demonstrate that the diagnosis of HSMI is not a simple measure. Routine visual fish health monitoring of moribund, fresh silver (recently dead) fish or healthy fish cannot be diagnosed pen-side by non-histopathologists. The process is complex and requires specialized equipment to preserve, stain, embed (in paraffin blocks), slice with a microtome and analyse. The language used to describe the pathology is complex and precise so as not to confuse the diagnosis with other causes.

For example, Yousaf et al. (2013) describe the immunohistochemical (IHC) profile of the other three diseases that can cause heart inflammations: Cardiac Myopathy Syndrome (CMS) and Pancreatic Disease (PD) cases in Norwegian Atlantic salmon. While many IHC indicators were common for all three diseases, and specifically the inflammation of the heart tissue and the aggregation of white blood cells in the damaged areas, the authors describe that the HSMI IHC profile is consistent with Major Histocompatability Complex (MHC) II and CD+3 presence, indicating that infection and response of white blood cells is occurring. Indeed, Ferguson et al. (2005) note that histopathological diagnosis of HMSI bears much resemblance to CMS and PD. The interpretation of the results from IHC required expert interpretation as indicated that the rankings of infection and damage range from 'strong' to 'moderate' to 'low'. Noteworthy is that the identification and characterisation of HSMI by IHC is precise because the presentation of the disease is very similar to CMS and PD.

In more common terms, the heart tissue responds to an insult of infection by launching an immune response that involves macrophages and neutrophils – white blood cells. Damage is noted in the heart tissue by the presence of spaces (vacuoles), loss of muscle integrity, the destructive fragmentation of the nucleus (karyohexis). Noteworthy is that these are secondary signs of infections and do not detect the root cause of the infection. The impact on heart tissue is that there is an immune response to an infectious agent. This response is indicated by infiltration of white blood cells presumably to attack the infectious agent and to repair/eliminate damaged tissue. Indeed, Knogkorp et al., (2004b) and Ferguson et al., (2005) and more recently, several other researchers (c.f.: Wiik-Neilsen et al., 2012b) note that cellular repair of heart tissue is apparent and common in a number of clinical cases of HSMI.

Skeletal muscle

Skeletal muscle appears to be less affected than cardiac muscle (Kongtorp 2004a, Ferguson et al., 2005); fish exhibiting cardiac inflammation or lesions may not have any skeletal muscle signs of infection, depending on the severity of the infection (Kongkorp et al., 2004a). In those samples where infection was apparent, the clinical signs were increase in the muscle strands with the presence of white blood cells infiltration and some necrosis of the tissues. Both white and red muscle is affected (Ferguson et al., 2005). The observations of variable infection and immune response of striated muscle in affected fish leads to the notion that the muscle is a secondary clinical sign of infection.

Liver

In Kongtorp et al., (2004a) a subset of fish displaying heart inflammation also displayed cellular necrosis of liver tissue. The signs of infection paralleled those of heart and skeletal muscle with no prominent cellular response to the hepatocytic lesions.

Detection of HSMI in Chile: Coho and Atlantic salmon

Godoy et al., (2016) collected coho and Atlantic tissue samples over the period of 2012 to 2015. The researchers used gross pathology observations, RT-qPCR for PRV (segments L1 and S1) and conducted histopathology on tissues. Table 1 summarizes the frequency of the significant gross pathology findings noted from Atlantic salmon affected with HSMI and coho salmon affected with HSMI-like disease. Both salmon species tested positive for PRV by qPCR. Atlantic salmon displayed histopathology consistent with other methods of diagnosis for HSMI (Kongtorp et al., 2004a). Although the histopathology for Atlantic salmon indicated HSMI, the observations for coho salmon were less confirmatory and the authors indicate HSMI-like infection.

Table 1. Histopathological lesions appearing in heart and skeletal muscle inflammation (HSMI), pancreas disease (PD) and cardiomyopathy syndrome (CMS) in Atlantic salmon

Lesions	HSMI	PD	CMS
Epicarditis	+	+	+
Myocarditis and degeneration of compact myocardium	+	+	–
Myocarditis and degeneration of spongy myocardium	+	+	+
Skeletal muscle inflammation and degeneration	+	+	–
Multifocal necrosis of hepatocytes	+	–	+/–
Necrosis of exocrine pancreas	–	+	–

On sequencing the PCR product, the authors note that: “phylogenetic analysis of PRV segment S1 sequences, all the Chilean PRV strains from Atlantic salmon grouped as sub-genotype Ib, whereas the Chilean PRV strains from coho salmon were more diversified, grouping in both sub-genotypes Ia and Ib and others forming a distinct new phylogenetic cluster, designated Genotype II that included the Norwegian PRV-related virus”. This observed difference in PRV sequences obtained from coho furthers supports that PRV of Pacific salmon origin may differ from the putatively ‘Norwegian’ strain and that coho salmon do not exhibit HSMI signs consistent with Atlantic salmon. This ‘side by side’ example of the differences may also indicate that Pacific salmon have a differential response to infection.

Cross-diagnosis with other infections

Kongtorp et al., (2004a) first described the clinical signs of HSMI as anorexia and abnormal swimming behaviour followed by a decrease in feeding behaviour. These similar conditions were noted by other researchers for Atlantic salmon (Ferguson, et al., 2005; Haugland et al., 2012; Yousaf et al., 2013; Godoy, et al., 2016). Further, efforts were made to distinguish HMSI infections from other infections that effect the heart such as Cardiac Myopathy Syndrome (CMS; Ferguson, et al., 2005; Lovoll et al., 2012; Haugland et al., 2012), Infectious Hepatic Necrosis (IPN; Ferguson, et al., 2005), Pancreatic Disease (PD: Yousaf et al., 2013) on the basis of histopathology and immunohistochemistry (Yousaf et al., 2013). While there are similar signs of disease that are common to pathogens, Fergusson et al. (2005) and Godoy et al. (2016) define the subtle differences in histopathology description that may be presented in a determining way. However, the interpretations are subtle and involved skilled interpretation. Further, co infection by other viruses that cause changes to heart cellular integrity also confuse diagnosis (Wiik-Neilsen et al., 2106). On the basis of viewing slide preparations alone, the cause of HSMI cannot be determined easily (Ferguson et al., 2005; Di Cicco et al., 2016) and diagnosis is usually supported by the presence of the PRV virus (Godoy et al., 2016).

Conclusions

The diagnosis of HSMI is complex. The disease is characterized by signs of lethargy, anorexia, wasting and loss of normal schooling and feeding behaviours. These behavioural signs are typical of several diseases and therefore a definitive diagnosis of HSMI cannot be made based solely on observing these behaviours. On examination of the internal organs and tissues, even the experts agree that a clear diagnosis can be confounded by cellular changes seen in other cases. These changes may be due to other pathogens or indeed, by multiple pathogens with the same target organs. To be certain, histopathology to discern the subtle differences must conducted by board-certified histopathologists.

In the case of HSMI in Norway and Chile, diagnosis of HSMI can be done with the dual histopathology and RT-qPCR methods to detect PRV. However, detection of PRV is not indicative of future or impending losses to HSMI in Atlantic salmon (Feguson et al., 2005; Lovoll et al., 2012), especially in Pacific salmonids (Garver, et al., 2016a) as infected tissue can repair without overt signs of disease and mortality.

Chapter 4 PRV and HSMI as a threat to wild salmon.

As reported in several publications (c.f.: Kongtorp 2004a,b), HSMI is of production and conservation concern in Norway (Garseth et al., 2013). As HSMI can cause significant mortality in farmed salmon, it is important to understand how a pathogen reservoir can affect wild or enhancement stocks (Garseth et al., 2013). To help evaluate the threat that viral loads would place on wild Pacific salmon via net pen culture or through release in processing plant wastewater, it is noteworthy to examine the efforts of Norwegian scientists when assessing the extent of pathogen spread among Northern Atlantic salmon stocks.

PRV in Non-Salmonid Species

Salmonids have been the target of non-cultured and hatchery fish in Norway surveyed to detect reservoirs of PRV (Garseth et al., 2012). The seminal publication regarding other, non-salmonids species is by Wiik-Nielsen et al., (2012). In this work, the authors conducted two trawls in Norwegian waters. The location of the trawls in relation to salmon farms was not disclosed.

In all, 1,627 fish were sampled for tissues and placed into pools of 2-5 fish per species. The pooled tissue was processed and tested for PRV. Of the 37 species tested, four pools tested positive for PRV, however the Ct values were high indicating a weak detection or low viral load. No similar studies have been undertaken in BC, however one such study was recently approved for funding and will commence in the summer of 2018 (S.C Johnson, pers. Comm.).

Fish species	PCR
Ammodytidae	
Lesser sand eel *Ammodytes tobianus* (L.)	0/2 (10)
Raitt's sand eel *Ammodytes marinus* (Raitt)	0/2 (6)
Anarhichadidae	
Atlantic wolffish *Anarhichas lupus* (L.)	0/5 (22)
Argentinidae	
Great silver smelt *Argentina silus* (Ascanius)	1/28 (176)
Carangidae	
Atlantic horse mackerel *Trachurus trachurus* (L.)	1/1 (1)
Clupeidae	
Atlantic herring *Clupea harengus* (L.)	1/29 (190)
Cyclopteridae	
Lumpsucker *Cyclopterus lumpus* (L.)	0/1 (1)
Gadidae	
Atlantic cod *Gadus morhua* (L.)	0/18 (98)
Blue whiting *Micromesistius poutassou* (Risso)	0/22 (104)
Greater forkbeard *Phycis blennoides* (Brünnich)	0/2 (6)
Four-bearded rockling *Rhinonemus cimbrius* (L.)	0/1 (1)
Haddock *Melanogrammus aeglefinus* (L.)	0/28 (121)
Common ling *Molva molva* (L.)	0/4 (6)
Norway pout *Trisopterus esmarkii* (Nilsson)	0/64 (226)
Poor cod *Trisopterus minutus* (L.)	0/9 (29)
Pollock *Pollachius virens* (L.)	0/21 (81)
Silvery cod *Gadiculus argenteus* (Guichenot)	0/12 (60)
Whiting *Merlangius merlangus* (L.)	0/12 (54)
Atlantic pollock *Pollachius pollachius* (L.)	0/1 (2)
Blue ling *Molva dypterygia* (Pennant)	0/2 (2)
Lophiidae	
Anglerfish *Lophius piscatorius* (L.)	0/8 (14)
Lotidae	
Tusk *Brosme brosme* (Ascanius)	0/10 (12)
Merlucciidae	
European hake *Merluccius merluccius* (L.)	0/9 (25)
Osmeridae	
Capelin *Mallotus villosus* (Müller)	1/16 (90)
Phycidae	
Atlantic halibut *Hippoglossus hippoglossus* (L.)	0/1 (1)
Lemon sole *Microstomus kitt* (Walbaum)	0/3 (11)
American plaice *Hippoglossoides platessoides* (Fabricius)	0/2 (10)
European plaice *Pleuronectes platessa* (L.)	0/6 (24)
Witch flounder *Glyptocephalus cynoglossus* (L.)	0/5 (15)
Scophthalmidae	
Megrim *Lepidorhombus whiffiagonis* (Walbaum)	0/3 (4)
Sebastinae	
Norway redfish *Sebastes viviparus* (Kroyer)	0/19 (83)
Rosefish *Sebastes marinus* (L.)	0/6 (20)
Sebastes sp. (Cuvier)	0/11 (52)
Squalidae	
Spiny dogfish *Squalus acanthias* (L.)	0/1 (5)
Sternoptychidae	
Pearlsides *Maurolicus muelleri* (Gmelin)	0/1 (2)
Triglidae	
Grey gurnard *Eutrigla gurnardus* (L.)	0/2 (4)
Zoarcidae	
Checker eelpout *Lycodes vahlii gracilis* (Sars)	0/1 (1)

Hawley and Garver (2008) examined the viability of VHSV in seawater to test potential transmissibility of the virus in Pacific scenarios. They found that VHSV persisted for up to four days under simulated conditions. VHSV is common in temperate Pacific coastal waters and does show up in farmed salmon. Garver et al., (2013) examined several parameters affecting the persistence and potential transmission of IHNV in BC waters. They conclude that environmental conditions have the greatest effect on potential spread of the virus with IHNV being 'inactivated' in as little as 3 hours in non-turbid conditions (plankton) and full sunlight. Ocean currents also play a role in dispersion and dilution to levels not considered sufficient to induce infection.

In order for a virus to be transmitted from 'free living' to infections to a host, such as during exposure to processing plant wastewater that contains PRV or other reservoir sources, the period of dispersion or 'shedding' has to be determined. The survival of the virus in seawater, hence viability and ability to infect, has to be determined (Johansen, et al., 2011). In the case of PRV, the reservoir of PRV in host species is not determined, nor is the viability of the virus in wastewater. What is known is that the virus is endemic and ubiquitous in BC waters.

Conclusions

The natural reservoirs of PRV have yet to be defined in Norway, BC or elsewhere. As the virus was identified and described in the last decade, there are several components to PRV research that have yet to mobilize. In relation to the threat that processing non-salmonid species and the potential for these species to release PRV virons to the environment, the risk cannot be described.

Chapter 5 Current scientific knowledge of PRV, HSMI and other related pathologies prevalent in salmonids from West Pacific Coast of North America; focus on the situation in British Columbia (BC).

It is understood that there is a relationship between PRV and HSMI in Norway. Surveys of farmed and wild fish in Norway have elucidated that the virus is endemic and ubiquitous. The losses to HSMI are notable and prevalent. In BC, PRV is likewise prevalent and present in both wild and farmed salmonids, yet does not cause mortality. The remaining challenge is determining the extent of PRV in BC and why there has not been observed mortality as in other areas.

PRV prevalence in BC

The first study on prevalence of PRV in BC was performed by Saksida et al., (2012). In this study, 44 pooled samples of 200 juvenile pink salmon (*Oncorhynchus gorbuscha*) were collected in May 2008 in the Broughton Archipelago. These tested negative for PRV (Saksida et al., 2012). A year later, Kibenge et al.,
(2013) published on the genetic diversity of PRV detected in samples from BC. In this study, a total of 14 samples including 10 farmed Atlantic salmon were collected at harvest, two wild cutthroat trout (*Oncorhynchus clarkii*), one wild chum salmon (*O. keta*) and one farmed steelhead trout (*O. mykiss*) from BC were tested by RT-qPCR for PRV. All the samples were collected between February and July 2012, however, the locations was not reported. All the tissue samples (Gill, heart and kidney) were positive for PRV and were classed between the middle to lower levels of detection (Ct value).

Following this study, Marty et al., (2015) extended the Pacific salmon surveillance work by screening archived samples between the years 1974 to 2008 (n=363) and fresh frozen hearts from 2013 (n=916) using RT-qPCR. In addition, 404 samples were analyzed by histopathology to diagnose for HSMI (Marty et al., 2015). All the farmed fish collected in 2013 were positive for PRV with Ct values ranging from 25.5 to 37.9 (mid to lower range of detection). However, only 4.5% of the 626 wild salmon sampled in the same year were positive for PRV. Chinook salmon had the highest proportion of PRV positives with 21% of the total fish, Ct values ranging from 26.5 to 35.5 followed by coho salmon (*O. kisutch*) with 5% of positive PRV samples from 60 total fish (range of Ct values = 14.7–37.1), one sockeye salmon was PRV positive among 180 and no pink salmon were PRV positive. No wild salmon collected between 2007 and 2011 were screened positive for PRV. However, 85% (134 out of 168 total fish) of archived farmed chinook and Atlantic salmon sampled from 2000 to 2008 were PRV positive (Ct values ranged from 19.6 to 39.9).

Paraffin blocks containing embedded tissues taken from wild and farmed salmonids captured between 1974 and 1994 were screened for PRV by Marty et al., (2015). PRV was detected in 49 out of 195 samples (25%; Ct ranged from 27.9 to 39.9). The earliest detection was from a wild Steelhead collected in 1977 with a Ct value 38.3-38.4 (lower limit of detection). As the Ct value was high, it was not possible to confirm the detection by sequencing the amplified qPCR samples. The earliest confirmed sample by sequencing was from a chinook sampled in February 1992. The source of these sample was not specified from either a farmed or wild origin.

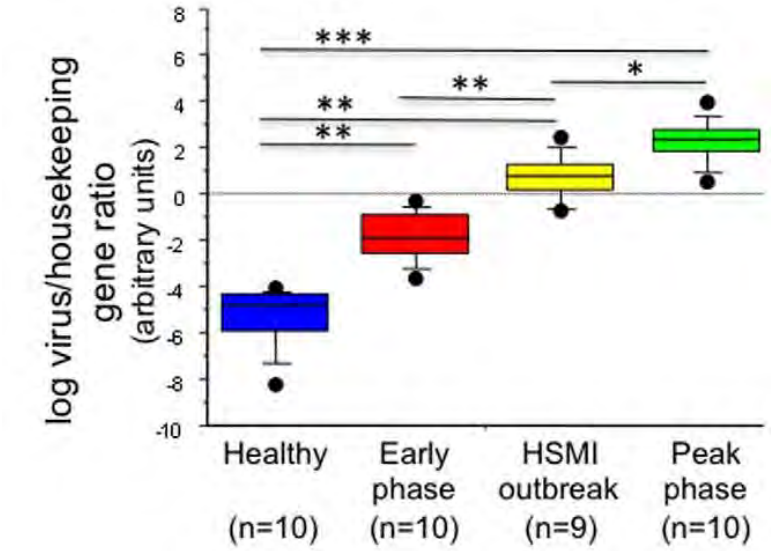
Recently, Morton et al. (2017) performed a geographical surveillance study by screening wild and farmed salmonids for PRV. The sampling protocol used market fillets purchased from 10 BC market chains located in southwestern BC in 2012-2013 and included 262 Atlantic salmon and 35 steelhead, but no information was provided on the exact location of the farms from which these fish were harvested. PRV was detected in 95% of farmed salmon and 69% of farmed steelhead. In addition, wild salmonids (n=601) including chinook (*O. tshawytcha*), chum (*O. keta*), pink, sockeye (*O. nerka*), coho, steelhead (*O. mykiss*), Kokanee (O. kisutch) and trout (O. mykiss/clarkii) were sampled from the south of BC in 2012-2013 as well as 344 sockeye, 10 Dolly Varden (*Salvelinus malma*), 41 chinook and 19 cutthroat trout collected in 2014-2016 from Oweekeno Lake, BC. The sampling plan included nine geographical regions with 2 regions distant from aquaculture areas, two regions close to the fish farming but with low exposure (n=369 fish), five regions very close to farmed Atlantic salmon (n=233 fish). PRV was detected in 95% of farmed salmon and 69% of farmed steelhead. Based on their anaylsis, 76% of Cultus Lake trout were positive to PRV and only 3% of the Oweekeno Lake were PRV positive. While Morton et al. (2017) concludes there are differences in PRV prevalence between areas with and without salmon farms, the number of samples are few and the collection location of the samples is undefined, which makes the conclusion unclear. Regardless, the study does demonstrate and confirm the presence of PRV in both wild and farmed salmon. Further work on sequence identification of the PCR product would help define the origin of the infections.

Purcell et al., (2017) tested PRV from 2,252 returning wild salmonids from 121 stocks of Alaska and Washington State in 2012-2013. Globally, the prevalence was 3.4% with variation within stocks ranging from 2 to 73%. Consistent with the reports by Marty et al., (2015), PRV was mainly prevalent in coho (11.8%) and chinook (4%) salmon. Neither sockeye nor chum salmon tested positive in either Alaska or Washington State. Only one pink and one steelhead collected from Puget Sound/Salish Sea and Columbia River respectively tested positive for PRV.

In summary, PRV has been detected in all marine BC salmonids, sockeye, chinook, coho, pink, steelhead, cutthroat trout and Atlantic salmon. PRV is more prevalent in Atlantic salmon than in coho, chinook, pink, chum, cutthroat trout and steelhead. The earliest confirmed PRV RNA detection was in a in chinook sampled in February 1992. Although the source of this sample was not reported, this confirms that PRV was present in BC for at least 26 years. The questions remains as to the source/origin of PRV in BC waters.

Relationship between PRV RNA levels and HSMI

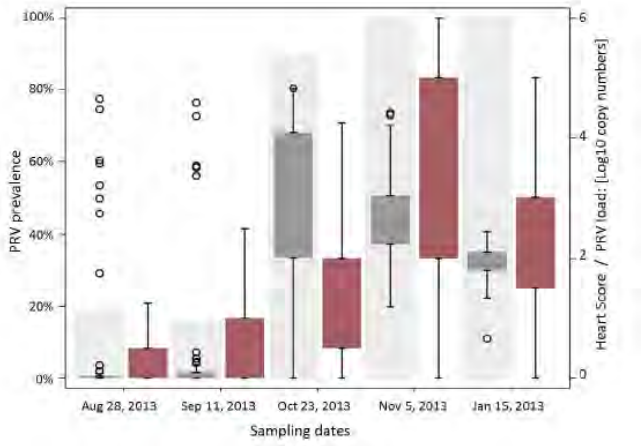
PRV was identified in Norwegian Atlantic salmon in 2010 (Palacios et al., 2010) a decade after the first reported case of HSMI in Atlantic salmon on the west coast of Norway (Kongtorp et al., 2004a). Once discovered, it remained to be confirmed that PRV was the main etiological agent for HSMI. Therefore, Palacios et al., (2010) quantified PRV RNA levels in 29 HSMI Atlantic salmon cases and 10 healthy fish. The authors used the log ratio of L1 partial sequence relatively to the Elongation Factor 1 α(EF1 .)αThe Log ratio of L1/ EF1 0.5> αwas used at a threshold for positive and negative samples. Twenty eight out of 29 HSMI fish had Log ratio L1/ EF1 0.5> αand all the healthy fish had Log ratio L1/ EF1 0.5< α(Palacios et al., 2010) indicating that the fish were positive specifically for PRV. The Log ratio was correlated with the different stages of HSMI severity in farmed fish (see Figure below).



Graph representing log ratio L1/EF1α of healthy and fish at different stage of HSMI (from Palacios et al., 2010).

However, this method had some bias because the mRNA level of the host housekeeping gene EF1α can be influenced by the size of the tissue at different fish life stages and the integrity of the host cell in comparison to the virus (Lovoll et al., 2012). Therefore it was suggested that a method with less bias was to normalize to the total RNA input (Lovoll et al., 2012). This latter method was used by the Norwegian Veterinary Institute to quantify the relative level of PRV partial L1 in fish with HSMI.

HSMI was reported in BC for the first time only recently by a longitudinal study on one BC salmon farm during a full marine production cycle from May 2013 to October 2014 (Di Cicco et al., 2017). This study showed that PRV prevalence and load (Log_{10} PRV copy numbers) were associated with the severity of HSMI (see Figure below).



Plots of PRV prevalence, heart score and PRV load from August 2013 to January 2014. Light grey plots are PRV prevalence, red box plots are heart scores and dark box plots are PRV load as Log_{10} PRV copy numbers (from Di Cicco et al., 2017).

A laboratory experiment of intraperitoneal injection of PRV in Atlantic salmon as well as through cohabitation of both Atlantic and sockeye salmon failed to induce HSMI in the infected fish (Garver et al., 2015). This discrepancy between field association of PRV to HSMI and the failure to induce HSMI by injecting PRV in healthy Atlantic salmon remain to be elucidated.

Conclusions

The information on prevalence and abundance of PRV in Pacific and farmed salmonids is beginning to grow. In many ways the accomplishments of researchers in Norway are beginning to carry over to researchers in BC. The complexity and depth of the work is beginning to elucidate the risk of viral load on farms in relation to wild salmon. Research gaps remain to determine the relationship between BC PRV and the potential to cause HSMI.

Chapter 6 Considerations on the risk-related PRV presence in blood water; IHNv as a Model Approach.

Risk assessments are a common tool to assist in regulatory and cultural practice for salmon farming and other food production industries where livestock are concerned. This is particularly important when

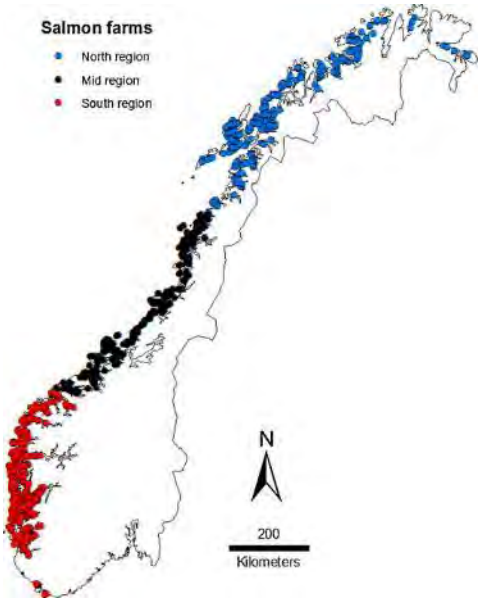
cultured animals will come into contact with wild animals that may carry pathogens or exhibit overt signs of disease and are infectious. In the case of salmon farming, fish undergo health checks prior to transfer to seawater net pens. During their seawater residency, they can come in contact with a variety of pathogens, some of which have been vaccinated against. However, in the case of PRV, no vaccine has been developed and salmon may be exposed to PRV from wild or cohort sources.

Identifying the risks associated with infection in the context of disease is an important exercise. Developing a model for the waters of coastal BC is challenging because there are external factors that have not been evaluated fully, such as changing climate conditions, variation in wild salmon abundance (potential reservoirs), and variation in currents or freshwater inputs from coastal freshwater waterways.

In regard to PRV, there have been risk assessment studies undertaken in Norway and in Washington State. As well, the Fisheries and Oceans, Ecosystems and Oceans Sciences has begun a series of risk assessments undertaken as recommended by the *Cohen Commission on the Inquiry into the Decline of Sockeye Salmon in the Fraser River*. The Canadian Science Advisory Secretariat (CSAS) has begun the process by publishing a Science Advisory Report (2017/048) to assess the risk of IHNV transfer from Atlantic salmon farms to wild fish in the Discovery Islands area. This assessment is of value to the discussion here because of the study design and evaluation process.

Norway

The seminal review of PRV in Norway was conducted just after the identification of PRV as a putative causal agent (Kristoffersen, et al., 2013). In the assessment, the authors included over 1,200 cohort controls and cases of HSMI in all geographic seawater salmon farming locations in Norway (North, Mid and South Regions; see Figure below from Kristoffersen et al., 2013). The data set included the years 2002 – 2010 which spans the research genesis into HSMI and PRV. The authors note that HSMI was not well described to all salmon fish health professionals and was not a reportable disease in the beginning of the study which may have affected reporting. As well, PRV was not identified as a causal agent until 2010 (Palacious, et al., 2010) and the detection of PRV was not considered in the assessment. Another consideration was the post-analysis observation that the data does not include PRV-infected fish that did not develop disease. The authors evaluated the risk of an outbreak occurring in response to risk factors irrespective of the presence of PRV.



The risk factors considered were:

- Infection pressure (the number of infective agents in environment),
- Number of fish on site
- Smolt characteristics (spring/fall; large/small)
- Months at sea
- Geographic location (North, Mid or South regions)

The risk of an outbreak occurring was predicted to be increased with increased stocking size (number of fish) in the cohort, the time from smolt to harvest and the weight of the fish (smaller was higher risk than larger). There was also a geographic effect with the Mid Regions the highest and reducing risk northward and southward.

The odds of contracting HSMI increased with the number of fish on a farm but not the absolute stocking density. That is, bigger farms with more fish rather than the number of fish per cubic meter of water represented a higher potential reservoir of pathogen hence, infection pressure. Farms with over 1.5 million fish had an odds ratio near 2, while farms with 500,000 fish had an odds ratio below one, or lower. If smaller fish were on a site and the infection pressure was high, the risk of contracting HSMI was higher than with older fish. This corresponds with the smolt entry date because S0 smolts are smaller in size that S1, or even a 'large' smolt of 1 kg and is consistent with other findings (Kongtorp et al., 2004a,b; Lovoll et al., 2012). The time-to-harvest corresponded to a lower likelihood of HSMI which follows that a larger smolt and reduced exposure to infection pressure. Noteworthy is that the authors observe that the presence of PRV is not a measure of exposure as measured against the intensity of exposure to the disease agent. This plays a role in determining whether disease is induced, rather than merely a result of exposure versus non-exposure to PRV; fish can be PRV positive and not express disease signs
(Wiik-Neilsen et al., 2012; Garseth et al., 2013). Further, the study does not take into consideration the presence of PRV in wild fish that may be located close to farms (Kristoffersen et al., 2013).

In summary, the authors note: "Even though PRV seems to be widely distributed in the environment, the finding that infection pressure has a large influence on the risk of developing HSMI suggests that it

might be possible to reduce clinical outbreaks if measures are taken to reduce infection pressure. This could be achieved by removing diseased sites or by vaccination."

Given that HSMI is a smaller concern to production in Norway (Kristoffersen et al., 2013) and that losses in BC to HSMI have not been observed in Provincial Fish Health Surveys (Marty et al., 2015) yet are suspected in association with PRV infections (Di Cicco et al., 2016), mitigation strategies for BC are likewise undefined as there is no vaccine for PRV. The infection pressure from processing plant wastewater remains undetermined and the Kristoffersen et al., (2013) study does not address the issue directly other than to infer that exposure to the virus per se is not infection pressure because it may not result in disease.

Washington State

The Pacific Northwest Fish Health Protection (PNFHP) committee is a collection of technical and policy representatives from Washington stakeholders in salmon conservation, tribal and commercial interests. They discuss and resolve issues of common concern on all matters concerning fish health. The PNFHP produced a technical Information Report No. 10 (Myers, 2017) on the risk of PRV to wild Pacific salmonids. The PNFHP considered available data in relation of the risk of PRV from salmon farms and published their conclusion that PRV is a low risk regarding HSMI in Pacific salmonids based on the following observations:

1. The disease "heart and skeletal muscle inflammation" (HSMI) has not been reported in wild salmon populations in Norway or elsewhere and appears to only be a threat to farmed fish

2. While PRV causes HSMI in farmed Norwegian Atlantic salmon, high levels of PRV genetic material have been detected in asymptomatic wild and cultured salmonids with no evidence of HSMI disease

3. Histopathological lesions of HSMI were recently described as statistically correlated with the presence of PRV at one Atlantic salmon farm in British Columbia, Canada (BC) while other studies have detected the presence of PRV genetic material in wild and cultured chinook, coho and pink salmon and steelhead trout from Washington State, BC and Alaska where years of surveillance have reported no presence of HSMI

4. Molecular testing of archived fish tissues in BC has shown that PRV was present in asymptomatic wild and farmed Pacific salmon since 1987 and may have been present as early as 1977 before Atlantic salmon were imported for aquaculture

5. HSMI has not been reported in Pacific salmon or steelhead in North America to date

6. Laboratory studies with chinook and sockeye salmon have demonstrated that PRV is infectious and will persist for quite some time but does not cause fish mortality, HSMI, or any other apparent disease

7. Development of HSMI and HSMI-like diseases of farmed salmonids (Atlantic and coho salmon; rainbow trout) infected by PRV may be a result of different viral strains, host specific antiviral responses and environmental stressors that do not appear to be present or active for indigenous salmon on the Pacific Coast

8. The presence of PRV genetic material in Pacific salmon tissues is not sufficient evidence for a diagnosis of HSMI disease. (Myers, 2017).

The PNFHP relies heavily on information that has been presented earlier in this report that demonstrates the BC strain of PRV does not infer disease (Garver et al. 2016 a,b) and that PRV predates and is ubiquitous in the waters of the Eastern Pacific (Marty et al., 2015; Purcell et al., 2018). The experts do not consider processing plant wastewater in their assessment as it pertains to infection potential of un-infected stocks and imply that is not of concern as in their statement, the virus in the Pacific is 'relatively benign'.

Canadian Science Advisory Secretariat (CSAS) Science Advisory Report 2017/048 on IHNV

By publishing a Science Advisory Report (2017/048), the Canadian Science Advisory Secretariat (CSAS) has begun the process to assess the risk of IHNV transfer from Atlantic salmon farms to wild fish in the Discovery Islands area. This assessment is of value to discussion here because of the study design and evaluation process may be transferrable to PRV.

CSAS held meetings of stakeholders and scientists to assess the risk of Infectious Hematopoietic Necrosis Virus (IHNV) to sockeye salmon in their migratory corridor through the Discovery Islands as it relates to the presence of salmon farms. While the assessment did not cover risks to wild salmon posed by the presence of PRV, the similarities are evident:

- Both viruses are contagious,
- Both viruses are present in farmed and wild salmon,
- Both viruses are ubiquitous in the marine environment and
- Both viruses are of public concern as it relates to wild fish health.

In addition, the CSAS IHNV report described oceanographic conditions where major fish processing plants are located. The information also described residency time of outbound migratory fish as they make passage through the areas of both salmon farms and processing plants. In addition, the report

covers viral persistence in the environment as it relates to mixing and exposure to UV radiation that results in viral degradation.

The CSAS risk assessment findings were that the overall risk to migratory salmon as posed by IHNV from salmon farms was minimal. There are extenuating circumstances, not least of which is the complete vaccination of the smolts entering the area, that deflect the relevance of the situation comparing IHNV in culture situations to processing plant wastewater that contains PRV. However, the study detailed how oceanographic conditions cause near immediate dispersion of point-source pathogens during high tidal flux. Ocean mixing varies with location and along Discovery Passage where two major processing plants are located (See figure 23 from Chandler et al., 2017) where current speeds in Johnstone Straight and Discovery Passage range between 0.5 and 1.5 m/s. There are no data to indicate what level

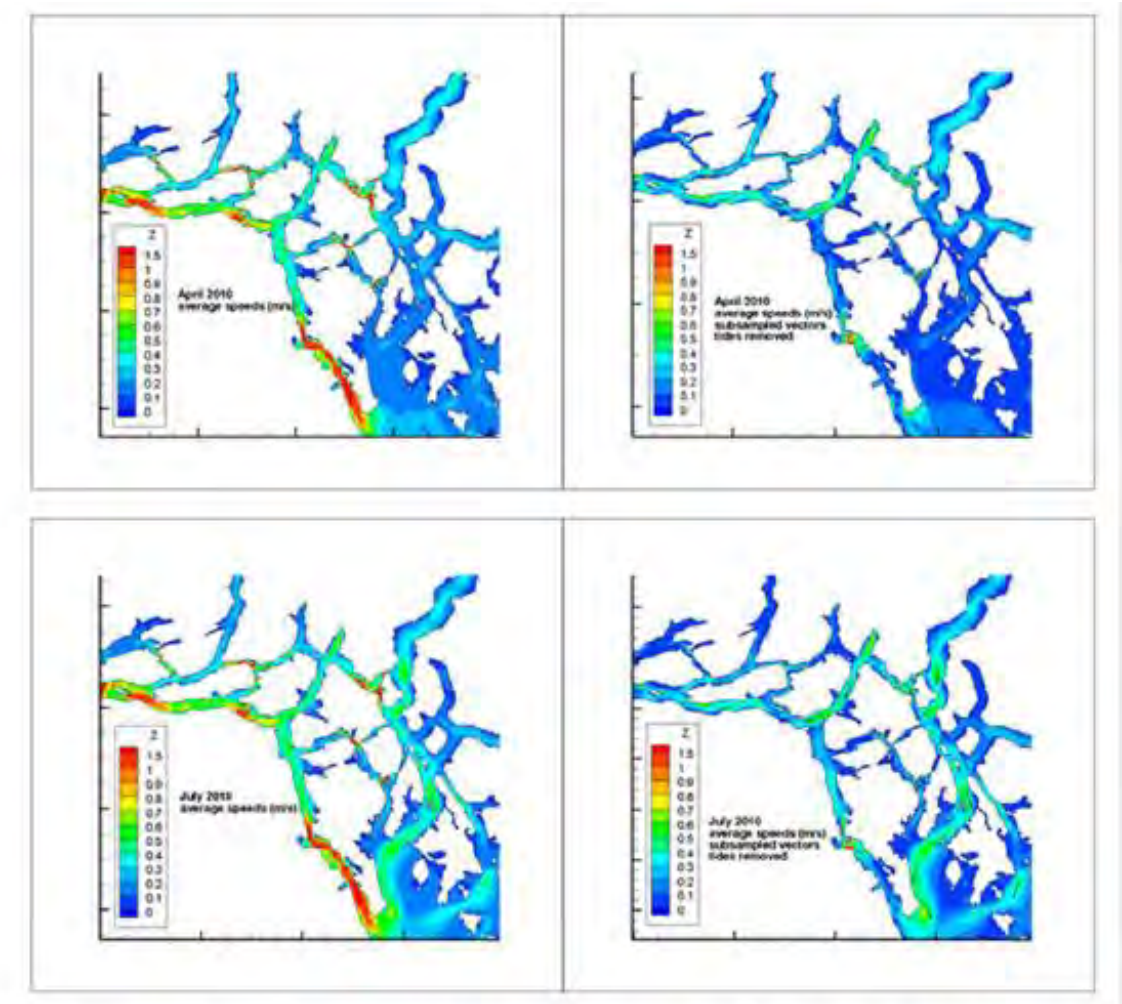


Figure 23. Average FVCOM current speeds at one meter depth for April and July 2010. Left panels include tides whereas right panels have the primary tidal contributions removed and include a few current vectors to show primary flow directions.

of viral particles are required to demonstrate infection pressure to migratory salmon. Miller (2018; unpublished) has estimated the release of viral particles to be 13 to 1,769 viral particles per ml from processing plan wastewater[2]. Two oceanographic unknowns exist in relation to establishing infection potential: 1) the required concentration of PRV to constitute an infection threat and 2) the dispersion characteristics that would reduce viral counts to below that level. However, as the oceanographic dynamics for the area have been studied for at least the areas around three processing plants, the exercise of modelling the dispersion exists and awaits attention.

CSAS (2017) stated a residency time for returning adult sockeye salmon to be an average of 3 days depending on the route taken through the Discovery Islands, the currents and the stock of salmon. Juvenile salmon gain outbound passage through the area in 5-14 days with the same conditions of passage. If measuring the passage of a smolt past a fixed point is applicable to encountering and being exposed to wastewater from a processing plant, Rechisky (2018, Kintama Research, pers. comm.) presented data showing radio-telemetry tagged sockeye smolts made transit past a salmon farm in just 4 min. As described above, unknown are the factors of infection pressure and the duration of exposure at know viral loads required to induce infection.

CSAS (2017) also brings forward other factors that are considered important to assess point source risks from potential sources viral load. These include freshwater inputs, changes in salinity, temperature and UV exposure. Ocean mixing via wind and water as well as overall turbidity play a role in exposing viral particles to degrading elements. These parameters are not yet defined for PRV and should be a point of research effort to enable a more accurate assessment of environmental risk.

Other viruses in wastewater and the threat to exposure; IHNV as an example

While research on the effect of PRV from processing plant wastewater is in its infancy, some measures to inactivate other viruses has been done. These studies are for well-known viruses where cell culture of the pathogen is possible, unlike PRV. Processes that are effective exist and can be brought on line in BC, but the correct procedures require investigation and testing.

[2] This article is not peer reviewed. Independent evaluation of the raw data noted that parameters of the assay need to be defined further to accurately estimate viral copy number.

In BC, fish processing plants use a variety of mechanical, chemical and physical processes to treat their effluent prior to discharging into the aquatic environment. The most common methods of effluent treatment are coagulation, flocculation, filtration, sedimentation and disinfection. These processes aim to physically remove particles and then disinfect the remaining pathogens from the effluent. Particles present in effluent are removed by coagulation and chemical flocculation. The generated flocculates coagulate and form heavier particles that settle to the bottom of treatment chambers. The remaining effluent is passed through filters with pore diameters ranging between 500 to 100 microns which is then disinfected which is the process of deactivating the pathogens present in the effluent.

The common chemical disinfectants used in the treatment of effluent are chlorine (Cl_2) and ozone. For viruses, chlorination is one the most effective disinfectants. Sodium hypochlorite (bleach; NaOCl) is used to generate the hypochlorite ion (OCl^-). The hypochlorite ion easily penetrates the cell membrane or wall of the pathogen present in effluent (Skall and Olesen, 2011). Although chlorine is widely used in waste treatment, the uncontrollable chemical composition of the effluent with organic materials influences the efficacy of the chlorine available for disinfection, but also alters contact time required to inactivate the pathogen (Torgersen & Hastein, 1995).

The amount of chlorine needed for pathogen disinfection will depend on several parameters including temperature, pH, organic materials and pathogen concentration. In general, a higher chlorine level will be required at low temperature, high pH and high concentration of organic materials and pathogen (Skall and Olesen, 2011). In Norway, 50 mg/L of free chlorine was acceptable to induce 3 log reduction of aquatic viral pathogen such as VHSV, IHNV and bacteria. However, this dose was determined under clean conditions which is not the case in most of the processing plant effluent (Skall and Olesen, 2011). Therefore, more efficient and stringent treatment processes to remove dissolved solids are needed to allow a more efficient chlorine treatment as a disinfectant.

UV disinfectant is also used as a physical disinfection system by its effects on the genetic materials of the pathogens (Kurth et al., 1999). There are three types of UV irradiations (A, B, C) based on their emission wavelengths. UVC is the more commonly used irradiation in processing plants for disinfection due to its efficient effects on many aquatic pathogens where it can induce mutation or breaks the genome of the pathogen. At high irradiance, UVC can form links between RNA to protein which can inactivate the viruses (Smirnov et al. 1991). UVC efficacy was tested against fish pathogenic viruses such VHSV, IPNV

and ISAV (Øye and Rimstad, 2001). The efficacy of the UVC will depend of the dose and the time of exposure. One study showed that UVC irradiation at the dose of 33 ± 3.5 $J.m^{-2}$ and 7.9 ± 1.5 $J.m^{-2}$ can achieve a 3 log level of reduction of both VHSV and ISAV in freshwater respectively. However, a UVC dose of 1,188 ± 57 J m^{-2} was needed to deactivate IPNV in freshwater solution with 40 to 140 times higher dose than ISAV and VHSV, respectively. These differences in resistance to UVC are probably due to the Rhabdoviridae viruses such ISAV and VHSV being single stranded RNA viruses whereas the Birnaviridae are double stranded RNA viruses (Øye and Rimstad, 2001). Noteworthy is that PRV is also double stranded.

When using processing plant wastewater, the UVC dose needed to achieve a 3 log reduction increased to 72 ± 16, 31 ± 1.8 and 3367 ± 275 J m^{-2} for ISAV, VHSV and IPNV respectively. The high particulate matter content in wastewater would explain the increase in the dose of UVC needed to inactivate the fish pathogens. It is documented that viruses can be adsorbed in particulates and therefore interfere with the efficacy of the UVC treatment (Øye and Rimstad, 2001; Jolis et al., 2001). This is confirmed by Miller 2018, (unpublished) who measured PRV in centrifuged effluent pellets and filtrate.

A similar laboratory experiment for IHNV and VHSV was performed at the BC Centre for Aquatic Health Sciences (Afonso et al., 2012). In this study, both IHNV and VHSV inoculated in cell culture and wastewater samples were exposed at 4°C and 15°C to different UVC doses up to 10 mJ cm^{-2}. Interestingly, the authors showed that both viruses were completely deactivated after 48 hours in effluent blood water at 15°C. However they can be detected in effluent after 48 hours when incubated at 4°C. Therefore, temperature plays a role in the deactivation of the viruses in processing plant effluent. A 3 log reduction of VHSV in effluent was achieved at a dose of 3.82 mJ cm^{-2} VHSV whereas 4 mJ cm^{-2} is needed to induce a 2.26 log reduction for IHNV. However, 1.46 and 1.16 fold lower dose were needed to deactivate INHV and VHSV inoculated in cell culture in comparison to effluent. Similar to previous experiments, it was suggested that the turbidity and the content of particulate materials present in the effluent could explain the high dose needed to inactivate fish pathogens in effluent.

Work is ongoing to determine the correct procedure to filter and disinfect PRV from processing plant effluent. Hampering the progress is the detection of viable virus. This is not possible by current methods. RT-qPCR is very effective at detecting RNA/DNA, but it does not indicate if the target is viable.

Coupled with the lack of available cell cultures for PRV and the lack of adequate wet lab capacity in BC, testing PRV viability in effluent is in a holding pattern.

Conclusions

Norwegian studies indicate that smaller autumn smolts on farms with high numbers of fish and in areas where HSMI is more common affect the risk of witnessing losses to HSMI. Washington State fish health professionals note that HSMI is a problem in Norway, but the strain of virus in BC does not pose the same threat. Infection with PRV in BC Atlantic or Pacific salmon is apparent and the benchmarks of infection are established, but there is no observed mortality. Heart inflammation occurs with the BC strain, but it does not cause apparent morbidity or mortality. If the CSAS work is taken into account, a more accurate risk scenario can be developed. Inactivation of viral particles is possible through effluent treatment. The procedures differ between virus types and require research and development. Work is underway to establish protocols for PRV disinfection and a serious impediment is detecting live PRV virus. Increasing information from ongoing research will greatly assist our understanding of the risks of wild salmon being adversely affected by wastewater exposure from processing plants.

Chapter 7 Current scientific knowledge of the health risk of PRV and HSMI to wild Pacific salmon in BC.

It is understood that PRV is contagious and is transferred between marine reservoirs and net-pen cultured salmon. There are observable effects of HSMI signs and detection of changes within affected tissues. However, in subclinical infection, or in less susceptible species, PRV infection may not affect the overall health of the fish. Current research addresses the issue.

Biological impact of PRV infection: How to measure biological impact in fishes

The biological impact of any infection can be difficult to determine, even in humans who have access to a well-funded health care system. Ultimately a concept of a continuum of care exists between infections that have cause death and a healthy individual. In between these two extremes a variety of states exist for an infected animal that is for intents and purposes healthy but infected, through to the infection causing serious harm to the animal – collectively these might be called sub-lethal biological effects. When it comes to fishes, while the sub-lethal effects of toxicants have been (and continue to be)

studied for several decades, the study of sub-lethal effects of infections is in the early days of study. Indeed, most studies focus on pathological effects of a disease and as a result primarily use pathological testing – a fish is either already dead or is sacrificed to harvest tissues for microscopic examination that would reveal tissue damage. Such is certainly the status for the study of the biological effects of a PRV infection both in BC, (Di Cicco et al. 2017; Morton and Routledge, 2017) and globally (Godoy et al., 2016).

At the outset it must be accepted that, as in humans, some infections can be benign or indolent in their biological impact. In speaking recently about a diagnostic panel that could detect early stages of viral disease development, DFO scientist Dr. Miller-Saunders said: "Any organism at any point in time will carry in their bodies an array of pathogens including viruses that exist in background levels but not necessarily causing disease. Disease occurs when they start causing damage to the host cells." (*Aquaculture North America* interview, 28 Jan 2018). So we can detect PRV infections used a qPCR molecular test for the virus, but this in and of itself does not mean there is either a disease state, or a biological impact of concern. For example, about 50% of known prostate cancers are characterized as indolent, and treating for the cancer (radiation therapy, chemotherapy and surgery) could have worse biological outcomes for the patient that doing nothing because the cancers are slow growing. The challenge is to identify aggressive forms of prostate cancer that have lethal consequences from those that do not. However, a PSA test alone cannot make this important distinction. Likewise, a qPCR test cannot yet predict the outcome of a PRV viral infection. A good example of an indolent infection is already known for wild Pacific salmon. When millions of wild sockeye salmon return to the Fraser River they invariably become infected with the parasite *Parvicapsula*, which leaves spores in the fish's kidneys - a classic pathological symptom. Nevertheless, most sockeye salmon will normally successfully spawn and naturally die before the disease progresses to a lethal stage. As the fish has succeeded in contributing to future generations, it could be argued that there was no biological impact in the salmon of the *Parvicapsula* infection.

Given that it is almost impossible to experimentally test the reproductive success of wild Pacific salmon infected with PRV, we must resort to measuring sensible biological endpoints; ones that are typically related to the known pathologies. For PRV, red blood cells (RBCs) are considered as virus factories – this is where the PRV virus resides and replicates itself, borrowing the hosts DNA/RNA machinery (Finstad et al., 2014; Wessel et al., 2015). Thus is it reasonable to propose that PRV could impair the role of RBCs in transporting oxygen to tissues, either by impairing the RBCs' ability to

bind with oxygen, or by reducing the number of RBCs in the blood. Furthermore, if PRV is the sole causative agent for HSMI, then PRV will cause inflammation and necrosis of the heart and skeletal muscle. Skeletal muscle is essential for locomotion and the heart the central part of the system that supplies oxygen to tissues. It is reasonable to propose that PRV could impair both the role of the heart in transporting oxygen to tissues and the ability of skeletal muscle to use oxygen for swimming activity.

It is this type of reasoning that has led scientists to investigate the respiratory physiology of PRV-infected salmon. Such studies must be done in a controlled manner, i.e., naive fish are deliberately infected with PRV and the responses and characteristics of these infected fish are compared directly with a suitable group of control fish. Because disease and the biological impacts of disease take time to develop, these groups of fish then must be tested over time post-infection, typically while measuring the level of infection in the fish. The remainder of this section of the report documents the results of such studies (both those published in the refereed scientific literature and unpublished results know to the authors).

Controlled infection studies with salmon in BC: published studies

Garver et al., (2016) used an infection trial to try to disentangle the consequences of a PRV infection and the Jaundice Syndrome reported in wild Pacific salmon in BC. Jaundice Syndrome is an acute to peracute systemic disease, and the time from first clinical signs to death is likely less than 48 hours; renal tubular epithelial cell necrosis is the most consistent lesion. Jaundice Syndrome was thought at the time to be caused by PRV.

Chinook, sockeye and Atlantic salmon were intraperitoneally inoculated with a PRV- positive organ homogenate from jaundiced chinook (10 moribund or dead chinook salmon with clinical signs of jaundice from a seawater net-pen farm in Clayoquot Sound, BC). The injected fish were adult chinook salmon maintained in sea water, and sockeye salmon and Atlantic salmon smolts moved to sea water on the day of the injection challenge, i.e., a double challenge. Injected fish were monitored for 22 weeks and the presence of PRV was tested using qPCR.

No gross or microscopic evidence of jaundice was reproduced in any of the three species over the 5-month monitoring period. In contrast, all fish of all three species became PRV-positive, with high PCR Ct values (30-40) for PRV-L1 PCR in liver and brain samples. Despite this sustained PRV infection, there was no related morbidity or signs of disease. The authors did not sample heart and skeletal muscle to test whether or not the PRV infections resulted in HSMI.

The authors conclude that:

- Jaundice Syndrome was not transmissible by injection of material from infected fish, and therefore is of low transmission potential.
- PRV was not the sole aetiological factor for the jaundice condition.
- The Pacific coast strain of PRV is transmissible by injection
- Ddespite a high and persistent PCR infection level for PRV, evidence of PRV-related morbidity or disease was absent.
- The Pacific coast strain of PRV was of low pathogenicity for Atlantic salmon, chinook and sockeye salmon under the test conditions that were used.

Garver et al. (2017) sought to specifically explore the effect from injection of PRV-infected red blood cells into juvenile Atlantic salmon and the cohabitation of sentinel (PRV-free) juvenile Atlantic and sockeye salmon with PRV-infected donor Atlantic salmon. The objective of the study was to measure the establishment and replication of PRV (via qPCR) in the erythrocytes of the host, and the manifestation of HSMI symptoms (via histology) in kidney samples. The authors found that PRV-injected fish universally became heavily infected with PRV (using qPCR assessment) for 24 weeks, the duration of the experiment. The infection dynamics and the genetic makeup of PRV from this study were very similar to previous reports for Norwegian PRV in association with HSMI. However, the BC fish with a heavy PRV infection state maintained hematocrit, did not develop HSMI or other signs of PRV-related disease and did not show an increase in mortality.

When viral loads in both blood and anterior kidney were highest at 1-2 weeks post-injection, gene expression analysis of Mx mRNA transcripts showed only modest up-regulation in infected blood samples relative to naïve blood-injected or media-injected controls. Mx expression is regarded as a gene involved in the classic antiviral interferon response pathway and has been previously shown to be up-regulated in association with HSMI. Blood, spleen and liver were ahead of heart tissue in having higher loads of PRV transcripts, while skeletal muscle had still lower PRV transcripts.

All Atlantic salmon sentinels became infected with PRV following four weeks of cohabitation with PRV-infected donor Atlantic salmon. They remained infected throughout the 41-week study period. High viral loads in both the blood and anterior kidney of sentinel fish were comparable to, if not higher than, the loads reached by i.p. PRV injection in Atlantic salmon.

Most, but not all, sockeye salmon sentinels became infected by eight weeks of cohabitation with PRV-infected donor Atlantic salmon and PRV infections persisted throughout the 41-week study period. However, infected sockeye sentinels had lower PRV loads in both kidney and blood samples compared to Atlantic salmon sentinels and PRV infections appeared to take longer to develop. Thus, some individual sockeye appeared refractory or were able to clear PRV infections, all of which suggests that sockeye were slightly less susceptible to PRV than Atlantic salmon.

Similar to i.p. injection challenge of Atlantic salmon, sentinel Atlantic or sockeye salmon with a heavy PRV infection state maintained hematocrit, did not develop HSMI or other signs of PRV-related disease and did not show increased mortality. Nine fish did die during the experiment; none had inflammation in the heart or skeletal muscle.

These experimental findings provide further support to previous reports purporting the avirulent nature of PRV in western North America despite high viral loads as determined by qPCR. Furthermore, high loads of PRV, as determined by qPCR, clearly can exist in the absence of disease symptoms and a change in hematocrit. Thus, the fundamental infectious processes of western North American PRV resemble those of PRV found associated with HSMI. Unanswered questions remain regarding the harm produced by the PRV infection.

Polinski et al. (2016) challenged juvenile sockeye salmon with injection of PRV-infected erythrocytes resulting in high quantities of viral transcripts becoming present in the blood and kidney of infected fish. This peaked at 34 days post-injection and remained lower but elevated through to 62 days post-injection. Despite the high PRV load, no morbidity or other signs of clinical disease occurred over a 62-day period. In general, histopathological lesions were few, mild and unspecific for heart and kidney samples taken on days 21 and 62. PRV caused almost no kidney-specific gene expression changes at either 2 or 3 weeks in contrast to the well-developed responses generated against IHNV.

In summary, the studies described here failed to demonstrate HSMI signs in a manner that was effective for Norwegian research. Further, there is compelling evidence to suggest that Pacific salmon have an inherent mechanism to reduce the effect of PRV infection that is not present or apparent in Atlantic salmon. The western North American PRV is able to evade or block host responses during primary infection, amplification and hematogenous dissemination within salmonids of western North America during non-pathogenic infection (Polinski et al., 2016; Purcell et al., 2018). Polinski et al., (2016) suggest that the general absence of cellular responses and apoptosis signalling in blood compared to tissues

following mammalian reoviral infection is suggestive of a fundamental difference in the way blood cells and target tissue cells interact with this virus. They also hypothesize PRV interacts differently with blood cells compared to target tissue cells, and that PRV can evade antiviral responses during erythrocyte entry and replication in vivo.

Controlled infection studies with salmon in BC: unpublished studies

An as yet unpublished study combined researchers from UBC and DFO, Nanaimo using domesticated Atlantic salmon smolts. This research was presented in full at the Workshop held in Campbell River in 2017. In the study, the respiratory physiology of PRV-infected fish (RBCs infected with PRV were injected into each fish) was compared with a blood-injected control group and followed for 21 weeks post-injection. Testing was performed at targeted times 0, 3, 9, 18 and 21 weeks. PCV confirmed that peak infection around 3 weeks and persistence of the infection throughout. Thus, the infected fish had replicating PRV virus and the levels of PRV were similar to those common to known PRV infections in salmon.

When compared with control salmon, PRV-infected RBCs had hemoglobin with the same affinity to bind oxygen and the same capacity to maximally bind oxygen. The percentage of circulating RBCs in PRV-infected fish was unchanged as well.

Eleven respiratory indices were also measured in each fish – a suite of tests that monitored fish for a 4-day period at each of the specified target times post-injection. These indices were compared between control and PRV-infected fish to test whether:

- Fish used a higher minimum metabolic rate to fight the PRV infection
- Their maximum ability to use oxygen was impaired by the PRV infection
- Fish were more agitated and using more oxygen to fight the PRV infection
- Their energy reserves used for burst exercise were depleted by the PRV infection
- Their ability to tolerate hypoxic water was impaired by the PRV infection

The indices revealed no consistent and systematic effect of the PRV on any of the respiratory indices over time. A modest, but significant decrease in maximum performance was seen at week 21 in PRV-infected fish, but there was an equally modest increase in maximum performance at week 9 in PRV-infected fish. These subtle differences are most likely explained by biological variability between the different groups of fish that were sampled at each time period.

Consistent with the near absence of physiological differences between the control and PRV-injected fish for all respiratory indices, very few PRV-infected fish developed cardiac lesions (heart samples had been taken for pathology after the respiratory tests were concluded) and for those where a lesion was seen, the lesion was ranked either mild or moderate.

The research group has also used a similar experimental design to examine the same respiratory indices in sockeye salmon smolts, but the analysis of these data is incomplete at the time.

Controlled infection studies with salmon elsewhere in the world: published studies

Lund et al. (2017) used a similar line of reasoning as described above to independently examine the respiratory impacts of infection with the Norwegian PRV strain on Norwegian Atlantic salmon smolts. They followed the PRV infection using PCR for 15 weeks and obtained similar infection levels. While they injected erythrocytes infected with PRV (similar to the BC study) to produce a PRV infection, the control fish may not have been handled similarly (it is not stated that control fish were sham-injected with anything; there is no statement that they used uninfected donor fish for the control injection of uninfected erythrocytes). Omission of a proper blood-injected control would be a serious experimental design flaw that could affect the interpretation of the results for PRV-infection.

A second group of PRV-infected fish were periodically challenged with hypoxic water (starting after the testing at week 4) because current thinking is that PRV infections can get worse in stressful environments; hypoxia was intended to promote this stress.

Compared with control fish, hypoxia tolerance was significantly reduced at week 7 in PRV-infected fish, which led the authors to conclude that experimental PRV infection weakens the fish's tolerance of hypoxia. By week 10, PRV-infected fish still had a lower hypoxia tolerance, but the hypoxia-stressed fish were no different in their hypoxia tolerance compared with control fish. This led the authors to conclude that periodic hypoxic exposure can offsets the effect of PRV-infection on hypoxia tolerance.

PRV-infection significantly increased cardiac inflammation, starting around week 7 and persisting through to week 15, when the study ended. However, periodic hypoxia did not influence infection levels or histopathological changes in the heart at any time when compared with the other PRV-infected fish. This finding has the implication that neither PRV infection levels nor histopathological changes were linked to the physiological change that was measured. The authors note that the hypoxia tolerance of the control fish was not very high compared with previous studies on the same species, and suggest that

challenging the fish so soon after the stress of moving salmon into seawater may have been a confounding issue.

The response of maximum heart rate to acute warming as measured at week 10. Between 12C and 20C there were no significant differences in maximum heart rate among the control, PRV-infected and hypoxia-stressed PRV-infected groups except at 19C when maximum heart rate was about 5% lower than the other two groups. This significant difference existed despite no histological difference in the levels of cardiac inflammation, and the authors suggested that PRV infection reduced aerobic scope (which was not directly measured in this study), cardiac performance and thermal performance. The changes reported were transient and modest.

Hemoglobin concentrations were also measured. Control fish significantly increased hemoglobin concentration at week 4, compared with other time points. Both groups of PRV-infected fish had a significantly lower hemoglobin concentration at week 7, a negative impact not apparent at either weeks 3 and 4 in PRV-infected fish and at week 10 in both PRV-infected fish and hypoxia-stressed PRV-infected fish.

The oxygen binding affinity of RBCs was measured in control fish and hypoxia-stressed PRV-infected fish at week 10 (PRV-infected fish were not tested). Compared with control fish, the erythrocytes of hypoxia-stressed PRV-infected fish had a higher affinity for oxygen, which may have been related to their almost significantly higher ATP concentration in the erythrocytes, but their maximum oxygen saturation was lower than the control fish.

Conclusions

It is clear that HSMI can be induce by PRV exposure in Atlantic salmon in Norwegian studies using either PRV injection or cohabitation with PRV-infected salmon. However, the same pattern of HSMI development has not been seen in BC with studies of cohabitation or injection of PRV, despite the virus showing near identical patterns of increased copy and viral load. Another apparent difference between the research carried out in the two countries is the difference in viral response in wild fish. Atlantic salmon in Norway appear more resistant to PRV than their farmed counterparts although tracing mortality is difficult (Garseth et al., 2015), yet hatchery fish have a higher incidence of infection compared to wild. In BC, rates of infection are lower in Pacific salmon compared to farmed Atlantic and the Pacific salmon do not develop morbidity or mortality when monitored for some weeks after infection. It becomes apparent that Pacific salmon do not respond to PRV infection in a life-threatening way and that the BC PRV strain is somehow less virulent or more benign.

Chapter 8 Research gaps and future directions: the risk of PRV to Pacific Salmon.

Research Gaps and Needs

It is clear that in BC, PRV does not cause mortality by HSMI as witnessed and demonstrated in Norway. This is despite high viral loads in wild (Purcell et al., 2018) and farmed fish (Finstad et al., 2014; Wessel et al., 2015; Garver et al., 2016a,b). There is heart inflammation and repair in fish with PRV in BC and Norway that varies with the species of fish affected (Kongtorp et al., 2004a; Ferguson et al., 2005; Wiik-Neilsen et al., 2012; Olsen et al., 2015). Koch's Postulate is a definitive measure to determine the mechanism of infection, transmission and presentation of the disease. To fulfill the postulate, there needs to be a pure supply of viable and infectious pathogen, healthy test fish and controlled conditions under which to conduct the investigation.

1) Gap: Researchers are unable to produce a pure culture to test if HSMI can be induced using BC PRV.
Need: a method of viral culture or construct to conduct infection studies.
2) Gap: There is a lack of wet lab capacity to work on fish diseases in BC.
Need: Increase the capacity.
3) Gap: demonstrate a cause-and-effect relationship of PRV infection and HSMI in BC Need: An effective challenge model.

Infection with PRV does not equal disease. The weight of evidence supports the idea that in BC, different Pacific species have different mechanisms of immunocompetency to limit PRV effects. Mortality is not caused by PRV in BC salmon.

1) Gap: qPCR for PRV is not a reliable indicator of the health outcome for a salmon infected with PRV

Need: A non-lethal assessment tool to evaluate the health outcome for PRV-infected salmon in BC

2) Gap: Explain why the PRV strain in BC does not induce HSMI as demonstrated in Norwegian PRV and define the virulence factors.

Need: Whole Genome Sequencing of the PRV genome and bioinformatics analysis of the sequences.

3) Gap: why do strains of Pacific salmon differ in response to PRV?

Need: Access to pure strains of PRV and wet lab capacity to test the effects.

4) Gap: The presence of PRV in wild salmon populations to complement Purcell et al., (2018).

Need: Dedicated and cooperative methods to sample salmon with adequate support.

5) Gap: Identify other reservoirs of infection.

Need: Survey non-salmonids.

Commercial, recreational and ceremonial fishers, as well as salmon processing plants, all need water to process fish. Bleeding the stocks is required for food safety and product quality. The process requires dispersion of wastewater in compliance with regulations.

1) Gap: Determine if PRV in wastewater is active or inactive.

Need: Definitive testing protocol to test viability of PRV isolated from wastewater.

2) Gap: Methods of disinfection that meet the parameters of viral destruction.

Need: More accurate test methods.

Risk Assessment

In determining the risk of PRV infection from the zone of influence of processing plant waste water we had to put aside unknown factors that include:

- Quantification of a range for viral particles that would be infectious,
- If viable virus leaves the pipe,
- Current average levels of PRV in wild salmon species and their susceptibility to infection,
- Residency time in the effluent plume,
- Attraction or avoidance behaviour of wild salmon with respect to the effluent plume,
- Likelihood of wild fish in the vicinity of the processing plant during processing,
- Volumes of effluent, and
- Viability of PRV in the marine environment.

In the worst case scenario, wild salmon would be in proximity to the effluent pipe, be naïve to the virus, and have sufficient contact time with the effluent plume and virulent particles in order to become infected. While possible, it is not likely that negative health effects to wild salmon would be predicted on the basis of transient or casual proximity to an effluent pipe. Placing caged fish in the proximity of wastewater could be used to test these ideas.

In consideration of the literature reviewed, we conclude that the risk of wild salmon contracting HSMI as a result of exposure to viral PRV particles from processing plant effluent is low. We base this assessment on the following observations:

1. Salmon in BC can have high levels of PRV and remain asymptomatic without compromise of physiological fitness,
2. Surveys of Pacific salmon positive for PRV have not disclosed impacts on fish health; the presence of PRV in salmon is not definitive of disease,
3. PRV predates the introduction of Atlantic salmon to BC and decades of fish health surveys have not reported HSMI in wild salmon, and
4. The threat posed to salmon by Norwegian strains strongly indicates that the BC strain is different and does not induce HSMI like the Norwegian strain.

This assessment is based on review of the current literature and information available. There are research gaps and needs that are identified that will further refine the risk assessment when complete.

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Appendix One: Credentials

BC Centre for Aquatic Health Sciences

The BC Centre for Aquatic Health Sciences (BC CAHS) is a non-profit research and diagnostic facility that serves the fisheries and aquaculture sectors. Through our 13 year history BC CAHS has assisted the aquaculture industry with innovative and developmental strategies to increase high quality production. BC CAHS is a fee-for-services, stand-alone research and diagnostic facility that operates independently from foundations or government operations and maintenance financial assistance. BC CAHS assists in research programs to identify fish and shellfish pathogens and develop assays for routine detection and screening and will conduct the assay development and validation. BC CAHS mission is to improve the health of aquatic animals and ecosystems through applied scientific knowledge.

BC CAHS' members have served as expert scientists on government inquiries, commissions and reviews related to fish since 2005 to provide unbiased and scientific input on the effects of salmon farming and the impacts on wild salmon stocks. This includes the Cohen Commission and the Salmon Aquaculture Review. We work with all levels of government and industry on research projects to ascertain the impacts and mitigation of industrial farming in BC waters. We are recognized worldwide for third-party advice, unbiased opinion and scientifically factual reporting. Current participation on interdisciplinary boards and panels such as the Organic Standards Board of Canada, Technical Advisory Panel on Aquaculture, the DFO Canadian Science Advisory Secretariat, Genome BC Working Group on Fisheries and Aquaculture and Vancouver Island University Aquaculture Program Advisory Council exemplify the respect and position the BC CAHS holds in the scientific and regulatory community.

Dr. Ahmed Siah

Dr. Siah is a well-published researcher in the field of fish and shellfish pathogens, their isolation, genomic sequencing and detection. Dr. Siah is involved with numerous research projects, developing new technologies in the field of aquatic health diagnostics & implementing & validating molecular biology technologies for diagnostics. His germane work in the area of Piscine orthoreovirus (PRV) has led to several advancements in the diagnostic capability in farmed and wild salmonids stocks. Dr. Siah has extensive experience in developing & implementing diagnostic methods for emerging pathogens of interest in British Columbia waters including PRV. Recently, Dr. Siah authored and co-authored two publications on PRV prevalence and phylogenetic analysis in West Pacific Coast of North America. His research studies were presented at national conferences and workshops.

Dr. Jim Powell

Dr. Powell has over 30 years' experience in the areas of fisheries and aquaculture sciences and is an established authority on fish reproduction and broodstock management both locally and internationally. His primary work has been in the area of fish physiology and adaptive management strategies of fish culture for a range of fresh- and salt-water species. He has extensive experience in aquaculture drug development, testing, registration and implementation in addition to work in fish health. Most recently, Dr. Powell was involved in fisheries and aquaculture management in the recreational and conservation sector with involvement in threatened and endangered species recovery. Dr. Powell is a founding Board member of the BC CAHS and has served on the board since inception. Dr. Powell was the initiator and organiser of several scientific workshops including the meeting on "Exploring PRV and HSMI in Europe and British Columbia" in Campbell River November 27 -28 2017.

Dr. Anthony Farrell, University of British Columbia

Dr. Farrell is a professor of Zoology at the University of British Columbia and a Fellow of the Royal Society of Canada. With over 400 research publications in scientific journals, Tony's research emphasis is to understand fish cardiorespiratory systems and apply this knowledge to salmon migratory passage, handling stress and recovery, sustainable aquaculture and aquatic toxicology. He has received multiple awards, including the Fry Medal, which is the Canadian Society of Zoologists highest honour to a scientist, the Beverton Medal, which is the Fisheries Society of the British Isles highest honour to a scientist, and the Medal of Excellence, which is the highest honour of the American Fisheries Society. He is a former President of the Society of Experimental Biologists and is the incoming Editor-in-Chief for the Journal of Fish Biology.

Signed:

Ahmed Siah Jim Powell Tony Farrell