Findings from the Strategic Salmon Health Initiative (SSHI) related to Piscine orthoreovirus in British Columbia

Participating authors: Kristi Miller, Andrew Bateman, Gideon Mordecai, Amy Teffer, Art Bass, Emiliano Di Cicco, & Brian Riddell

Overview

Over the past several years, there have been increasing public concerns over the role of infectious disease in BC salmon declines, and specifically whether open-net salmon farming may increase pathogen exposure and disease risks to wild Pacific salmon. One pathogen, piscine orthoreovirus (PRV), has been of particular public concern, as it is the causative agent of an emerging disease. In Norway, PRV is recognized as the causative agent of heart and skeletal muscle inflammation (HSMI); a disease among the most impactful to the country's industry[1]. In BC, on the other hand, there has been substantial controversy surrounding PRV and its ability to cause disease.

SSHI research has shown that the developmental pathways of HSMI, and related jaundice/anemia, found in BC salmon are highly consistent with PRV-caused diseases in other parts of the world. We have shown that affected fish show activation of genes indicative of a viral disease, and that PRV specifically occurs within the cells and tissue regions where disease-related lesions exist. We have also shown that there are no viruses, other than PRV, consistently present in HSMI and jaundice/anemia disease outbreaks we have studied on farms. Importantly, similar analytical approaches applied to wild Chinook salmon are revealing the same pattern of PRV-related disease development as observed on farms. Presently, there are no vaccines or other effective treatments for PRV.

Below, experts within the SSHI respond in detail to four questions submitted to PSF and pertaining to our state of knowledge on PRV in British Columbia.

A note on terminology

To understand the discussions around PRV, it is necessary to be clear on terminology. To start, the terms "disease" and "infection" can generate confusion. Infection with a potential pathogen (virus, bacterium, fungus, or parasite) can, but need not, lead to disease. In general, disease refers to a set of symptoms and/or tissue lesions (damage) associated with a specific pathogen affecting individuals. Disease may result in physiological impairment, behavioural changes, and - in extreme cases - death, but it can also be present with no outward signs. It is important to note that an infected individual that does not express disease can still be a source of transmission of the pathogen for an extended period, as exemplified by SARS-CoV-2 currently impacting human populations around the world.

The term "endemic" can also cause confusion. Ecologists and the general public use the term to indicate that a species is native (and strictly unique) to a geographic region. Epidemiologists use the term to indicate an infectious agent's consistent presence and transmission within a host population, *without implying the origin of the infective agent*. Endemic in the ecological usage contrasts with "exotic," i.e., a species not native to a region. The contrast with the epidemiological usage is an "epidemic," during which the prevalence of an infectious agent proliferates beyond its usual background level within a host population. Classifying an infectious agent as endemic (or epidemic) is independent of the severity of any associated disease[2]. For instance, malaria is endemic to certain regions of the world that are home to its mosquito vector, but remains a very serious disease.

1. How do we know if PRV in BC came from Norway, and is the common BC variant "endemic" to BC?

The source of PRV in BC can be inferred by analyzing the genetic sequence of many samples of the virus and comparing each sample to all others. The PRV genome is comprised of ten segments: long molecules of RNA[i] that instruct an infected host cell how to make more of the virus. Presently, three types, or "strains," of PRV (PRV-1, PRV-2, and PRV-3) have been determined by genome similarities and differences among strains. The evolutionary relatedness, or genetic similarity, of samples are determined by comparison of the viral genome sequence and construction of a "phylogenetic" (i.e. family) tree.

Analyses of such phylogenetic trees have recently found that there are currently two major branches (sub-strains) of PRV-1, named PRV-1a and PRV-1b[3], which can be differentiated based on two of the ten genome segments ("S1" and "M2"). *Only PRV-1a has been detected in BC.* The SSHI team has used all of the PRV-1 sequences on the public record, many in BC coming recently from the SSHI itself, to construct the most comprehensive phylogenetic tree to-date for the PRV-1 strain [Mordecai et al. 2020, *in prep*,4].

We can estimate the geographic origin of PRV in BC by comparing overall similarity and specific changes in the genome sequence of the virus found in different regions of the globe. The similarity of PRV-1a sequences in Norway, Chile, and Canada suggest a recent, shared transmission history of the virus (Figure 1). The small amount of variation in PRV-1 within BC contrasts with the diversity of PRV-1 in Norway (both PRV-1a and PRV-1b are present in Norway). Importantly, our analysis places the Norwegian PRV-1a sequences as ancestral to the lineage in BC [4]. Together, these findings suggest an evolutionary "bottle-neck" event, which could indicate a single introduction to the north Pacific. The simplest explanation for the higher diversity of PRV-1 in the Atlantic than in the north Pacific is that the virus originates from the Atlantic. Also important is the relationship of PRV-1 from escaped farmed salmon in Washington State [5]. These sequences appear to represent a secondary, more recent introduction of PRV-1a to BC, more closely related to a lineage of PRV from Iceland (reported to be the source of brood stock for Washington farms[5]). This newly introduced lineage of PRV-1a demonstrates

i *An RNA virus is a virus that has RNA (ribonucleic acid) as its genetic material.*

how aquaculture activities can introduce viruses across continents. As circumstantial evidence in support of this finding, there are other examples of infectious agents that likely have been introduced to BC from Norway. For instance, a virus recently discovered in Norway (Atlantic salmon calicivirus) is extremely common in the Atlantic salmon industry in BC [6].

In reference to the specific question, whether or not PRV is endemic in BC depends on one's perspective and the definition used (see introduction). The various definitions of "endemic" all relate to one or more of three concepts: whether PRV is native to BC, whether the variant in BC is unique, and whether PRV is widely circulating in BC. Some scientists have argued that PRV originates from BC [7], in-part based on a weak detection of PRV in an archived steelhead sample from 1977 [8], which would predate Atlantic-salmon aquaculture in BC. This detection, however, was unrepeatable and never confirmed by genetic sequencing (standard practice when screening for novel or exotic infectious agents). The earliest confirmed PRV detection comes from a Chinook salmon sampled in 1992, after the establishment of Atlantic-salmon farming in BC [8]. As discussed above, updated genetic evidence points to an Atlantic origin of PRV-1, strongly suggesting that the virus does not originate from BC. The majority of PRV-1 found in BC (aside from that in escaped Washington fish) does appear to come from a single lineage, suggesting a single introduction event. Differences between sequences of PRV-1a in BC and Europe are consistent with an Atlantic origin followed by spread in the east Pacific during recent decades.

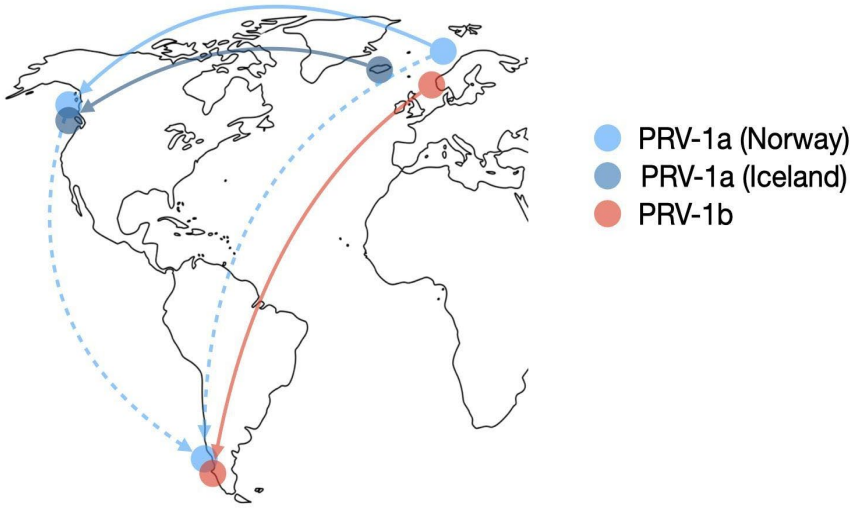

Figure 1. Major global transmission events for PRV-1. Circles represent the lineage of PRV in each location. Lines show estimated transmission of each lineage according to a phylogenetic analysis (Mordecai et al. 2020 *in prep*). Note: PRV-1a closely related to that found in market fish from Iceland was detected in escaped Atlantic salmon from a farm in Washington [5], but not in wild fish.

PRV-1 is common in farmed salmon in the Pacific and also present in wild salmon, but in a much lower overall proportion than farmed fish. Mounting genomic evidence supports

widespread transmission of PRV among wild and farmed fish (Mordecai et al. 2020 *in prep*), but associated mortality appears low (on farms) or unknown (in wild salmon). Whether PRV is stable or increasing in BC remains unclear, such that either "endemic" or "epidemic" may be the right epidemiological description, *but not in reference to a species native to the north Pacific*. To avoid confusion, we suggest avoiding the term "endemic," as it can be interpreted in very different ways.

2. How do we know that HSMI occurs in BC net pens?

Globally, PRV has proved challenging to study, which has contributed to disagreements surrounding its effects. In particular, it has resisted attempts at continuous culture that would facilitate experiments with pure virus. Using virus filtered from infected tissue, laboratory challenge studies have failed to recreate any of the behavioural symptoms of disease, let alone mortality that are often, but not always, associated with HSMI on salmon farms. As a result, it took several years to establish a cause-and-effect relationship between PRV and HSMI in Norway [13], where the disease was first described. Moreover, the fact that HSMI generally causes low overall mortality on farms (usually <5% and at most 20%) [9] , sometimes spread over several months, has meant that the disease often may be missed, perhaps only detected when present during diagnosis of a more acute disease, or other concurrent conditions.

In 2017, a team of scientists and veterinarians from Canada and Europe reported a 2013 clinical case of HSMI in an Atlantic salmon farm in BC and described the full development of the disease over eighteen months [10]. The single affected farm was part of a SSHI study, in which four groups of Atlantic salmon from two companies were monitored over their entire ocean production cycles. For almost a year, PRV was common in samples from the affected farm, occurring in combination with characteristic inflammatory lesions in the heart and skeletal muscle of the majority of fish. Specific staining of the virus in prepared tissue confirmed that the heart inflammation surrounded PRV-infected cells at the microscopic level [11] and therefore that the inflammation in the heart and skeletal muscle seen in HSMI represents the host's response to an actively replicating viral infection. Neither systemic inflammation nor relevant co-occurring infections were present. The affected farm also displayed increased mortality in some of the net pens, enough to trigger the company to twice submit fish samples to BC's provincial Animal Health Centre for testing. The independently submitted samples had the same histological lesions as those described by the SSHI [10]. High throughput infectious agent monitoring and high throughput sequencing revealed that PRV was the only agent correlated with the disease. Taken together, this combination of observations would be considered diagnostic of HSMI globally, except in Canada. Indeed, this case study from BC included three European experts on HSMI (Rimstad, Wessell and Ferguson) and has been widely cited as an example of HSMI by the international scientific community (32 citations to date).

The HSMI case reported in 2017 [10] represents the only fully characterized HSMI outbreak to have occurred in BC, but findings by DFO and BC's provincial testing centre provide supporting evidence that this was not an isolated case. Notably, DFO's aquaculture surveillance program (the audit program) collected samples between 2011 and 2013 from nearby farms that showed

similar lesions in association with high levels of PRV [11]. One nearby farm, in particular, showed the same pattern of lesions in two consecutive production cycles, in 2011 and 2013. The 2013 audit was, in fact, performed at the peak of the HSMI outbreak described in the SSHI study.

DFO's audit program has observed lesions consistent with HSMI in Atlantic salmon since at least 2008 [12]. While PRV has been established as the causative agent of HSMI [13], some Canadian authorities question the ability of the specific form of PRV-1a in BC to cause disease, despite the results of the SSHI study [10]. The skepticism relates to a lack of clinical signs or mortality seen in experimental laboratory challenges in BC and relatively weaker inflammatory reactions in challenged fish than observed in Norway [14, 15]. However, as stated previously, PRV infection trials worldwide have failed to reproduce the behavioral changes or increased mortality associated with the disease on farms [13,16–19]. Moreover, the field outbreak study by Di Cicco [10] demonstrated a similar severity of inflammatory lesions as observed in Norway could occur in the field. However, with only one farm under intense study, it is hard to know how much the inflammatory lesions impact behaviour and survival of farmed salmon in BC. In Norway, clinical signs and mortality can vary greatly, which is why they are not required for the diagnosis of HSMI [20]. As with many diseases, the impact of PRV on disease manifestation depends on the virus, the environment, and the health of the host. At present, the Department of Fisheries and Oceans Aquaculture Management Division requires that the disease be fully demonstrated in the laboratory and on farms, and that measurable population-level mortality occurs on farms before considering the diagnosis of HSMI. Based on these standards, DFO has, to date, failed to diagnose HSMI in BC, despite the evidence documented by the SSHI.

What is important, based on what we have documented in farmed salmon, is that the strain of PRV in BC is pathogenic [10,11,13] (i.e. it can cause disease). From the perspective of wild fish, salmon farms can function as incubator populations that can become sources of PRV, whether that transmission originates from HSMI-affected fish or asymptomatic carriers. Transmission becomes the starting point for potential disease development in wild salmon.

3. How do we know that PRV causes jaundice/anemia in Chinook and likely affects other Pacific Salmon species?

The assertion that PRV causes jaundice/anemia in Chinook salmon comes from a combination of sources: analysis of DFO audit data from Chinook farms [11,21] , analyses of wild Chinook salmon [22], a challenge study [23] and - critically - supporting comparative analysis of PRV-related disease development in Pacific salmon worldwide.

All known strains of PRV have been shown to cause disease in salmonids. In general, the effect of a virus in one species cannot predict the effect of the same virus in another species; however, all PRV-related diseases in Pacific salmon (documented in Norwegian and Chilean Rainbow trout, Japanese and Chilean coho salmon, and BC Chinook salmon) share common features [11,24–27]. All include anemia as a consequence of the rupture of red blood cells infected with PRV; all can inflame the heart; and, most importantly, all can severely damage the liver and kidney, inducing jaundice (yellow) discoloration during the later stages of the most extreme

cases. While anemia and jaundice discoloration can result from several different conditions, strong associations between PRV-related diseases and these symptoms in Pacific salmon have been shown globally. This worldwide perspective is the strongest demonstration that PRV can cause diseases in Pacific salmon including jaundice/anemia in BC Chinook salmon as reported in one SSHI study [11].

The latter study mapped out the developmental pathways of disease related to PRV-1a, and showed evidence that the same virus sub-strain of PRV can cause different diseases in Atlantic salmon (HSMI) and Chinook salmon (jaundice/anemia). The key difference appears to be due to a lower tolerance to heavy PRV infection in Chinook red blood cells. This also suggests that the threshold of PRV infection to instigate disease may be lower in Chinook than Atlantic salmon.

The sole study in which Chinook salmon were challenged with PRV-1a [23] found early jaundice/anemia lesions exclusively in infected fish. Ignoring these signs of disease due to limitations in their study design and lack of sufficient controls, the authors concluded that PRV-1a *did not* cause disease, since the fish neither turned yellow nor died from the challenge. However, in field outbreaks, yellow discolouration and mortality due to jaundice/anemia are extreme clinical symptoms that only occur in a portion of affected fish ([11]; KM Miller, unpublished observations). Since such outward clinical signs have never been recreated in PRV challenge studies worldwide, the conclusions of this study should be considered inconclusive. Further, DFO audit data showed that jaundice/anemia has occurred repeatedly in farmed Chinook salmon during the winter months in association with PRV. The industry also acknowledges this disease can be the largest source of mortality during the winter, although rates of mortality for “yellow” fish remain low overall. More research could be done to establish what percentage of fish that die from this disease show external yellow coloration, and hence how many individuals may have been missed by this one criterion.

4. What do we know about PRV infection rates in wild Pacific salmon, and how does infection affect survival?

Over the course of the Strategic Salmon Health Initiative, we have identified over 50 infectious agents in wild Pacific salmon, found fifteen previously uncharacterized viruses [28,29], elucidated connections between infections and environmental features, drawn connections between infection and migratory survival in juveniles and adults, and uncovered relationships between certain infectious agents and the performance of individual salmon stocks. Here, we focus on patterns of infection and early indications of impacts of PRV on wild Pacific salmon populations.

PRV has variable prevalence (the proportion of individuals affected) among wild Pacific salmon species and life stages (Figure 2), and displays a range of prevalence across different salmon populations in the northeast Pacific. We note that prevalence estimates are merely a snapshot in time, and wild fish impacted by disease can be rapidly lost from the population, often due to predation. Unless samples are taken at the height of an infectious outbreak and before associated mortality occurs, we therefore might not expect to see high prevalence for a pathogen that causes even moderate illness. It is also important to note that PRV prevalence in

juvenile BC Chinook salmon increases from spring to winter, with the highest levels of infection observed on the west coast of Vancouver Island over the winter - the same temporal period when PRV-related diseases in farmed Atlantic and Chinook salmon have been reported to occur. As most juvenile BC sockeye salmon migrate northward to Alaska by mid-summer, and hence are not available for sampling during fall/winter, it is unclear whether the limited positive test results in our samples indicates inadequate post-infection sampling, low susceptibility to PRV infection, and/or elevated PRV-related mortality in sockeye salmon.

We would expect wild fish to be more sensitive than cultured fish to disease impacts, as any degree of physiological compromise could elevate the risk of indirect death by starvation or predation. The SSHI has found that in juvenile wild-caught Chinook salmon in the fall and winter period, PRV viral load is strongly correlated with the expression of a set of genes predictive of viral disease [11]. Some of the same fish showed early pathological (disease) signs consistent with jaundice/anemia in farmed Chinook salmon, suggesting the development of disease in the presence of high loads of PRV [11]. Among fish showing activation of genes associated with viral disease, virtually all showed at least mild evidence of pathological disease, and several fish contained multiple lesions. The majority of these affected fish were sampled overwinter in Quatsino Sound off the West Coast of Vancouver Island, where PRV prevalence and loads were also highest.

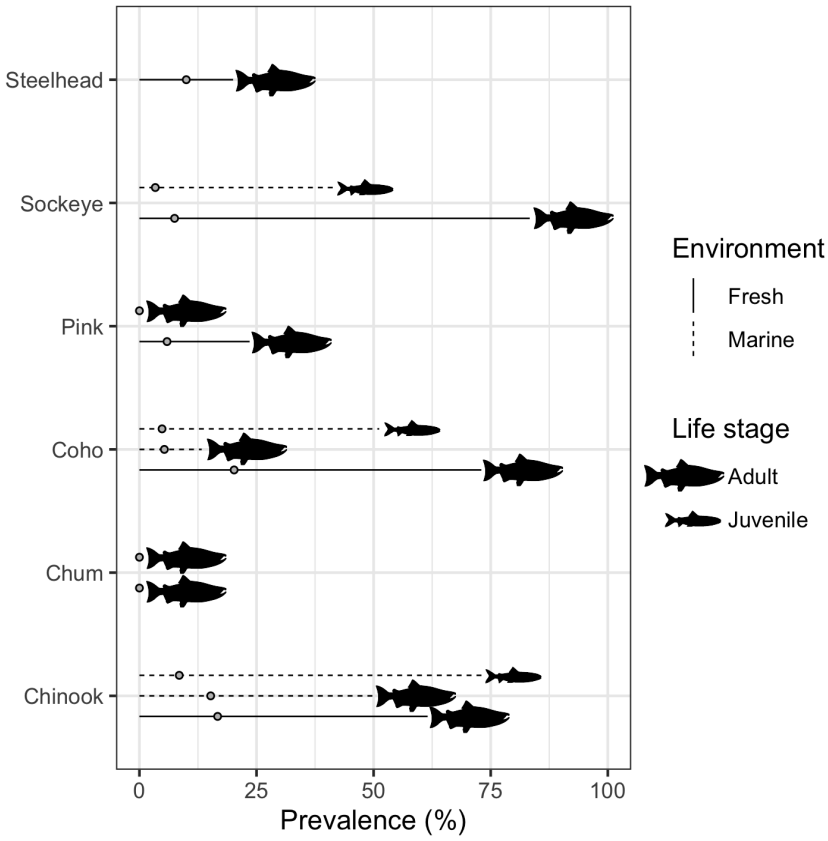

Figure 2. Prevalence of PRV in the northeast Pacific Ocean for wild-caught (wild- and hatchery-origin) salmon. Adult [8,30] and juvenile (SSHI data [22,31,32]) ranges show variation among populations and seasons; prevalence in juveniles generally increases from summer to the first winter at sea. Solid lines indicate returning adults in freshwater. Line segments indicate data ranges (salmon images solely indicate classification), and circles show mean values.

Several researchers have suggested a spatial association between PRV prevalence in wild fish and salmon aquaculture facilities. Morton and colleagues [33] suggested that this occurs along the east coast of Vancouver Island, and researchers in Europe have found a higher incidence of PRV in countries and regions with salmon aquaculture [34]. Using the SSHI dataset, we are currently examining this relationship in Chinook salmon using new statistical analyses.

A note on sources of evidence presented by the SSHI on PRV and disease in BC salmon

In relating PRV to HSMI and jaundice/anemia in BC salmon, the SSHI has drawn on the expertise of two veterinary pathologists (Di Cicco and Ferguson) to make the specific diagnoses. Dr. Di Cicco also serves as a consultant to scientists in Chile who are dealing with large-scale outbreaks of PRV-related diseases in Coho, Atlantic salmon, and Rainbow trout. The SSHI has also consulted and co-published with two Norwegian virologists (Rimstad and Wessel), who have dedicated much of the past 10 years to studying PRV diseases in farmed Atlantic salmon and Rainbow trout. The SSHI team has applied a range of specific tools to study the role of PRV in natural outbreaks of HSMI and jaundice/anemia in BC salmon. First, SSHI scientists not only conducted genetic tests for PRV and showed that affected individuals do, in fact, carry high loads of the virus, but they also performed "*in situ* hybridization," staining specific genes found in the virus to microscopically confirm that PRV is co-localized within the areas where lesions are developing. This approach has led to a better mechanistic understanding of the two diseases linked to PRV in BC, and it is now being applied by our Norwegian colleagues [35]. These relatively novel approaches have been in addition to traditional microscopic histopathological investigations of disease lesions.

Second, to confirm that each disease is, in fact, virally mediated rather than bacterially, environmentally, or otherwise induced, the SSHI team applied a novel viral disease development (VDD) "panel" that determines whether specific genes in a fish have been activated. The panel reveals when salmon experience a viral disease, but not when they merely carry virus, and it can differentiate fish experiencing viral versus bacterial diseases. Applications of the VDD panel to studies of HSMI and jaundice/anemia have revealed that the VDD viral-response genes are only activated in tissues when PRV viral particles are found outside of the red blood cells. This is the point at which PRV begins to infect other tissues and cause corresponding damage. We acknowledge that the VDD panel has yet to undergo the level of validation required for application as a diagnostic tool, but research using the tool has stood up to the rigors of peer review, and has yielded substantial insight.

Third, SSHI researchers have applied modern genetic pathogen monitoring to thousands of fish, both farm and wild. Screening for dozens of infective agents, this technique characterizes known infective agents present in affected fish. Importantly, for a subset of fish, the SSHI has also used modern genetic sequencing techniques to show that PRV is the only virus consistently detected in individuals with HSMI and jaundice/anemia symptoms.

Next Steps

Researchers in the SSHI have directed significant effort to study PRV, but there are many other infectious agents that may contribute to declining returns of Pacific salmon. Before the SSHI program wraps up in March 2021, we intend to:

1) put PRV findings into perspective with ~60 other infective agents assessed within the SSHI;
2) establish a ranking of impact potential for all of the agents under study and identify additional research on potentially impactful agents;
3) examine the potential for cumulative effects derived from co-infection; and
4) evaluate the evidence that amplification of infective agents around farms poses a risk to wild salmon infection and survival.

To assess the risks of these novel agents, there is a serious need for greater availability of level-2 bio-containment facilities to support controlled infection studies required to establish cause and effect relationships between novel agents and any effects in wild salmon.

Building on developments during the SSHI, future research could:

1) examine the role of cumulative effects of stress and disease on early marine survival, using a novel salmon Fit-Chip technology developed in Dr. Miller's laboratory;
2) apply new genomic technologies to improve effectiveness of BC hatcheries;
3) apply environmental (e)DNA methods to non-invasively study a range of species important to salmon survival (pathogens, harmful algae, prey, predators, competitors) and to establish indices of ecosystem health; and to
4) conduct infection challenge trials as noted above.

These research topics will contribute to understanding the roles of climate change and human-derived impacts on health and condition of Pacific salmon and the ecosystems they inhabit, and inform mitigation strategies to support the health and sustainability of BC's wild Pacific salmon.

References

[1] "Mowi Annual Report 2019," https://corpsite.azureedge.net/corpsite/wp-content/uploads/2020/03/Mowi_Annual_Report_2019.pdf.
[2] "Principles of Epidemiology in Public Health Practice, Third Edition: An Introduction," https://www.cdc.gov/csels/dsepd/ss1978/SS1978.pdf.
[3] M. J. T. Kibenge, et al., *Virol. J.* **2013**, *10*, 230.
[4] A. K. Teffer, et al., *FACETS* **2020**, *5*, 234.
[5] M. J. T. Kibenge, et al., *Virol. J.* **2019**, *16*, 41.
[6] G. J. Mordecai, et al., *bioRxiv* **2020**, 2020.02.13.948026.
[7] A. Siah, et al., *PLoS One* **2015**, *10*, e0141475.
[8] G. D. Marty, et al., *Journal of Fish Diseases* **2015**, *38*, 713.
[9] R. T. Kongtorp, et al., *J. Fish Dis.* **2004**, *27*, 351.
[10] E. Di Cicco, et al., *PLoS One* **2017**, *12*, e0171471.
[11] E. Di Cicco, et al., *FACETS* **2018**, *3*, 599.
[12] "Update: BC fish pathologist consulted with government and industry vets," https://www.ctvnews.ca/w5/update-b-c-fish-pathologist-consulted-with-government-and-industry-vets-1.3690863.
[13] Ø. Wessel, et al., *PLoS One* **2017**, *12*, e0183781.
[14] K. A. Garver, et al., *PLoS One* **2016**, *11*, e0146229.
[15] M. P. Polinski, et al., *Sci. Rep.* **2019**, *9*, 3297.
[16] A. B. Mikalsen, et al., *PLoS One* **2012**, *7*, e37269.
[17] M. Lund, et al., *PLoS One* **2017**, *12*, e0181109.
[18] O. W. Finstad, et al., *Vet. Res.* **2014**, *45*, 35.
[19] O. W. Finstad, et al., *Vet. Res.* **2012**, *43*, 27.
[20] R. T. Kongtorp, et al., *J. Fish Dis.* **2006**, *29*, 233.
[21] K. M. Miller, et al., **2017**.
[22] Y. Wang, The Physiological Associations between Infectious Agents and Migrating Juvenile Chinook Salmon (Oncorhynchus Tshawytscha), University of British Columbia, **2018**.
[23] K. A. Garver, et al., *J. Fish Dis.* **2016**, *39*, 117.
[24] N. Vendramin, et al., *Vet. Res.* **2019**, *50*, 14.
[25] J. Cartagena, et al., *Aquaculture* **2018**, *493*, 79.
[26] T. Takano, et al., *PLoS One* **2016**, *11*, e0165424.
[27] M. G. Godoy, et al., *Virol. J.* **2016**, *13*, 98.
[28] G. Mordecai, et al., *eLife* **2019**, *8:e47615.*
[29] G. Mordecai, et al., bioRxiv (pre-print) **2020,** https://doi.org/10.1101/2020.02.13.948026.
[30] M. K. Purcell, et al., *J. Fish Dis.* **2018**, *41*, 347.
[31] O. Nekouei, et al., *Front. Microbiol.* **2018**, *9*, 3221.
[32] O. Nekouei, et al. *PLoS One* **2019**, *14*, e0221956.
[33] A. Morton, et al., *PLoS One* **2017**, *12*, e0188793.
[34] N. Vendramin, et al., *J. Fish Dis.* **2019**, *42*, 1107.
[35] M. Malik et al., *Viruses* **2019.** *11*, 824.