PSC Southern Boundary Restoration and Enhancement Fund 2010

Final Report

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PROJECT IDENTIFICATION

Project Title: Potential impacts of novel Parvovirus on declining Chinook salmon stocks

Project Type:	(*Check one*)
Development of improved information for resource management, including stock assessment; data acquisition & scientific understanding of limiting factors.	☒
Habitat restoration; rehabilitation or improvement.	☐
Enhancement of wild stock production through low technology techniques.	☐

Project Location:

Chinook salmon populations from the Fraser River, East and West Coast Vancouver Island, Puget Sound, Snake River, Upper Columbia, as well as southern and northern mainland areas, central coastal regions and SE Alaska.

Start Date:	May 1 2012	End Date:	June 30 2013	Duration:	14 months

	(Specify currency)
Total PSC Funding Requested:	$CAN/US $96,250 Can
Total Other Funding & In-Kind Contributions:	$CAN/US $118,111Can
Total Project Cost:	$CAN/US $214,361Can
Total PSC Funding Provided:	$CAN/US $86,570 Can

EXECUTIVE SUMMARY

The project aimed to quantitatively assess the prevalence of a novel salmon parvovirus in BC Chinook salmon smolts and assess whether this virus may be a differential that could explain the variability in performance among stocks. The deliverables included: 1) Genetic stock ID on 500 smolts, 2) resolution of sequence variants of parvovirus carried by BC Chinook salmon and development of a quantitative assay to assess all known variants, 3) Molecular assessments of viral load and prevalence in 2,600 Chinook salmon smolts, 4) Data analysis contrasting prevalence and intensity of infection between stocks with relatively low and high productivity levels, testing of three key hypotheses relating parvovirus to productivity, and 5) Determination of whether osmoregulatory performance is impacted under high parvoviral loads.

- Overall prevalence and load of Parvovirus in Chinook is low (<2% in smolts, no substantial loads observed), which suggests that this species may show a low susceptibility to this virus.
- Alternately, in sockeye, prevalence varies among stocks, life cycle and environment (range among smolt stocks 0 to >90%), and high loads are observed as smolts leave freshwater and in the early marine environment.
- The full viral genome sequence was obtained; the virus exhibits strain variation (3 dominant strains), indicating a high complexity in the genome of salmonid Parvovirus in BC. One of these strains was sequenced in both Chinook and sockeye salmon.
- We successfully developed TaqMan assays spanning the genome of the Parvovirus to increase the chances that all strain variants in Chinook salmon would be revealed; application of additional assays did not substantially enhance the number of positives obtained in Chinook salmon, suggesting that the low prevalence rate observed is accurate.
- Given the low prevalence and load, we did not run the full 2,600 Chinook smolts, nor did we complete objectives 4 and 5. However, we did complete the extractions for the 2,600 smolts and utilized 440 of these fish to run a preliminary assessment of the presence and load of 33 salmon microbes associated with salmon diseases worldwide on a platform that we have been developing with co-funding from Genome BC. The remaining 2,200 Chinook will be among the first samples run on this platform once its development and performance evaluation is complete.
- We present preliminary analyses of the prevalence and loads observed among the 33 microbes assessed on the Fluidigm BioMark platform (added to the project). While 20 of the 33 microbes were detected among the 440 fish surveyed, 11 microbes were present at high enough loads to potentially warrant further research focus. Preliminary statistical analyses suggest that some microbes show variation between years, life-history-types, environments, and/or seasons.

Recommendations on next steps

1. Full microbe surveillance utilizing BioMark platform of 45 microbes in Chinook salmon to determine which microbes associated with salmon diseases worldwide are carried by Chinook salmon, when and where they are first detected, and which are associated with loads, prevalence, and histological changes that indicate a potential for population-level impacts. This work will be undertaken under the new Strategic Salmon Health Initiative (SSHI) project co-funded by Genome BC, Pacific Salmon Foundation, and Fisheries and Oceans. We may seek funding from SEF to enhance our ability to assess associations between microbe loads and physiological consequences in migrating salmon.

Identify any invention or innovation that may have resulted from this Project, including any new process or technique.

This project in part spurred the development of the SSHI project and the high throughput pathogen screening on the Fluidigm BioMark system being developed. Preliminary application of this platform on Chinook salmon validated the low prevalence of the Parvovirus in Chinook salmon, but showed that Chinook smolts carry several microbes, some in considerable loads in the early marine environment. A large baseline of nucleic acids from multiple tissues was generated from this project and serves as a resource for future microbiome study in Chinook salmon.

INTRODUCTION

Productivity of many BC and Washington Chinook salmon stocks began declining 20 years ago, followed by sockeye stocks less than a decade later. There are clear indications that reduced marine survival, most notably in the early marine phase, is to blame. During this critical period, research by Beamish and others has shown that high but variable losses occur, the levels of which may be major determinants of year-class strength. Delineating factors that undermine performance during early marine life may therefore enhance our ability to manage fisheries by reducing uncertainty in pre-season forecasts and providing a greater mechanistic understanding of factors that may lead to multi-species declines. Indices of salmon fitness (e.g. growth and feeding) and oceanographic variables (SST, plankton) are presently being considered as potential predictors of year-class strength (Beamish et al. 2004, Chittenden et al. 2010, Beamish and Mahnken 2001, Johannes et al. 2011).

Through the Cohen Inquiry, it became abundantly clear to managers, stakeholders and the public at large how little we understand what pathogens and diseases may be impacting wild salmon. We are faced with multi-stock declines in Chinook, coho and sockeye salmon, and pathogens and diseases are considered to be high risk factors in these declines, but we don't know which ones.

Our genomic research has revealed considerable variation in signatures associated with strong differential immune stimulation among stocks, years, and environments where smolts are sampled, and we speculate that some of these signatures could be associated with the activity of infectious agents of disease (termed microbes). One such signature of interest was identified originally in a previous SEF project (SF-2007-I-37) and associated premature in-river mortality of returning adult sockeye salmon (published in *Science*; Miller et al. 2011). This signature, dubbed the "mortality related signature" or MRS, was later shown to occur across multiple tissues and in highest prevalence in smolts leaving the Fraser River, with prevalence declining during the first few months of ocean residence. This pattern was suggestive of a *potential* association with early marine mortality. In pursuing a mechanistic cause of this signature, we identified the sequence of a novel salmon Parvovirus. At the onset of this project, we hypothesized that this virus could be a contributing factor in this variation in early marine mortality of multiple salmon species, and sought to determine its prevalence, load and spatial distribution in Chinook salmon stocks that were in various states of decline.

Methods and Results

Objective 1: Genetic stock identification

The genetic structure of Chinook salmon is generally regionally based, with populations in the same geographic area being more similar to each other than to populations in more distant areas (Waples et al. 2004; Beacham et al. 2006). Our ability to assess stock-specific differences in migrating Chinook salmon is highly dependent upon the application of individual GSI to ocean-caught smolts. Beacham et al. (2012) has shown that using existing baselines, we can achieve an accuracy of individual assignment to region of greater than 90% for regions in southern BC. Our current coast-wide baseline (SE Alaska to California) has ~300 stocks with ~60 K individuals, and utilizes 13 microsatellite loci baseline.

We had already performed individual GSI on Chinook salmon collections from 2008-2010. We proposed to complete GSI analysis on the remaining 500 Chinook salmon smolts collected in 2011. These fish would then add another year upon which we could assess variability in prevalence and intensity of infection across stocks.

GSI was completed within the Molecular Genetic Laboratory at the Pacific Biological Station in Nanaimo, BC on 778 samples collected in 2011 and 2012; hence more samples than originally intended. There was a good representation from low productivity stocks on the east coast of Vancouver Island (ECVI) and Lower Thompson (LWTH), and more moderate numbers from Puget Sound, Snake, Skeena River, and the Columbia (Figure 1). Also present were large numbers of fish from high productivity stocks. This completed the GSI for all Chinook salmon

collected from 2008 through the summer of 2012 that had tissue samples from gill, brain, liver, heart, and spleen.

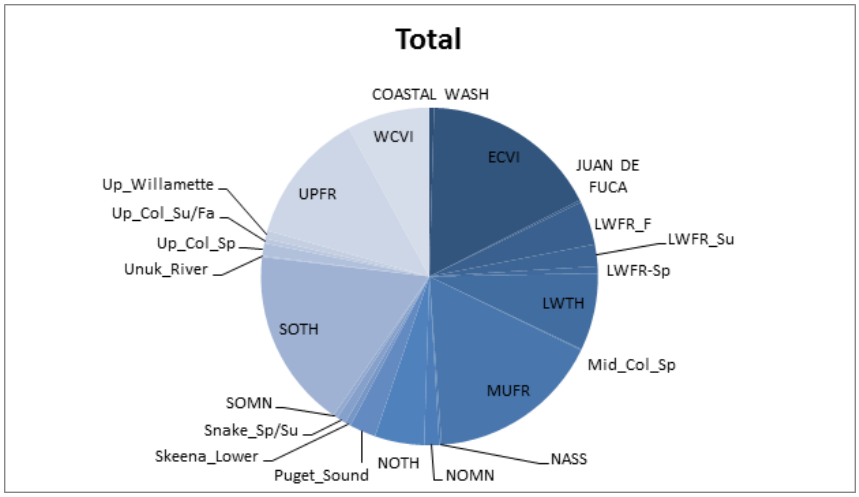


Figure 1. Stock representation from 2011/2012 GSI.

Objective 2: Resolution of sequence variants of the Salmon Parvovirus and Development of quantitative PCR tests

In sockeye salmon, previous to this project we had identified a number of sequence variants of the parvovirus (PSPV) that cluster into two distinct strains/lineages, and had developed a quantitative PCR test that amplified all known variants in sockeye salmon. We had also developed a number of non-quantitative tests. When applied to Chinook salmon, the parvovirus was not universally amplified using all primer sets, potentially suggesting that there may be divergent sequence variants in Chinook salmon that we had not accounted for. Hence, before we began a coast-wide survey of viral prevalence and load in Chinook salmon, we needed to ensure that the assays used were capable of detecting all potential strains of the virus in Chinook salmon. To do this, additional sequence information was required.

Parallel to this project, a PSPV challenge study was conducted in collaboration with the Aquatic Animal Health Section in 2012. Challenge material was obtained from PSPV screened sockeye smolt kidneys collected in 2011 from the Strait of Georgia. Post-challenge screening by quantitative (Q) PCR identified samples with very high loads of the PSPV DNA using our original QRT-PCR primer set (Rep68-F2-Rep68-R2) applied on an ABI 7900HT platform with an artificial probe-containing construct used to produce the standard curve. DNA extracts from two of these samples (Table I) were then sent to collaborators at the University of Oregon (Dr. Mike Miller) and University of San Francisco (Dr. Graham Ruby and Dr. Joe DeRisi) for next generation sequencing on an Illumina Hi-Seq and analysis using a pipeline developed in the DeRisi laboratory. Through application of the bioinformatic pipeline and seeding with the

original 454-based sequencing assembly that contained roughly half of the Parvovirus genome, the DeRisi team was able to extract and align the full genome sequence of the virus from both of our samples (barcoded TAGCTT and GGCTAC), which comprised 5,395 nucleotides.

Sample	Species	QPCR 7900 Liver (ct)	QPCR 7900 Kidney (ct)	QPCR 7900 Gill (ct)
18	Sockeye	17.7	23.8	34.1
111	Sockeye	16.8	23.8	28.7

Table I. Threshold Cycles (CT's) of the two samples provided from the challenge study for full genome sequencing. Note that the lower the CT, the higher the load of the virus.

The two isolates showed 99.6% homology to each other, with only 23 nucleotides showing variation between sequences. Most variation was within the hairpin structures at the ends of the virus, a region important for the efficiency of viral DNA amplification and other aspects of the viral life cycle (Li et al. 2012). In comparison with the previously identified 454 sequence contig, the isolates were 98.2% homologous, with 39 single nucleotide polymorphisms (SNPs) over the 2,075 overlapping sequence.

From this information, we designed a variety of new PCR primers to obtain long sequence reads to full genome sequences on more samples. Because initial screening indicated that the highest prevalence of the virus may be in sockeye salmon, initial testing and screening of all Parvovirus primers was conducted across a broad span of sockeye salmon stocks. Initially, 96 sockeye livers were amplified with 14 primer sets. The PCR conditions were as follows: 0.3µM each primer, 0.3µM dNTP, 1X Qiagen PCR buffer (15mM MgCl2), 0.75U Qiagen HotStar Taq, 63ng genomic DNA. 35 cycles of 95º for 30 seconds, 55º for 30 seconds, and 72 º for 3 minutes were performed. PCR was conducted with primer sets spanning the entire known length of the PSPV genome. All positive PCR products were identified for Sanger sequencing with the PCR primers as the sequence primers. Note that not all samples produced positive PCR products with all primer combinations, yet all amplicons were sequenced in an attempt to capture as many variant sequences as possible. This again suggested that there was sequence variation that we had not accounted for in the design of PCR primers (which at the time were placed in invariant positions). Sequencing was conducted using Life Technologies' Big Dye Terminator 3.1 sequencing kit and run on an ABI3730XL capillary sequencer. Sequences were aligned using Sequencher 4.6, and any gaps in coverage were re-amplified and re-sequenced as necessary. Four additional full length PSPV sequences were obtained via gene walking and aligned with the two Illumina sequences received from the Derisi lab; two were identified in 2011 samples and two in 2012 samples. Sequence diversity was calculated using MEGA 5 using number of base

differences per site. Codon positions included were 1st+2nd+3rd+Noncoding. All ambiguous positions were removed for each sequence pair. There were a total of 4,978 positions in the final dataset.

The phylogenetic tree built in MEGA included full length sequences obtain from the Illumina platform (labelled ParvoTAGCTT and ParvoGGCTAC) and the four new sequences derived from gene walking (Figure 2). The full length genewalking sequences from sockeye smolts included: 4901 (Chilko) sampled in 2010, 7353 (Chilko) sampled in 2011, B1342 (Mitchell-Quesnel system), and B1540 (Middle Shuswap-Shuswap system) sampled in 2012. Three divergent strains (lineages) were obtained, one which contained the two Illumina sequenced samples obtained from the Parvovirus challenge along with the 2010 (4901) and 2011 (7353) Chilko samples (later referred to as lineage 3), and the other two differentiating the two 2012 samples.

Divergence between sequences within lineage 3 (the 2011 samples) over the entire parvovirus genome was $\leq$ 0.005 (0.5%) whereas divergence varied between 1.5% (between B1540 and B1342) and 2.4% between lineages (Table II).

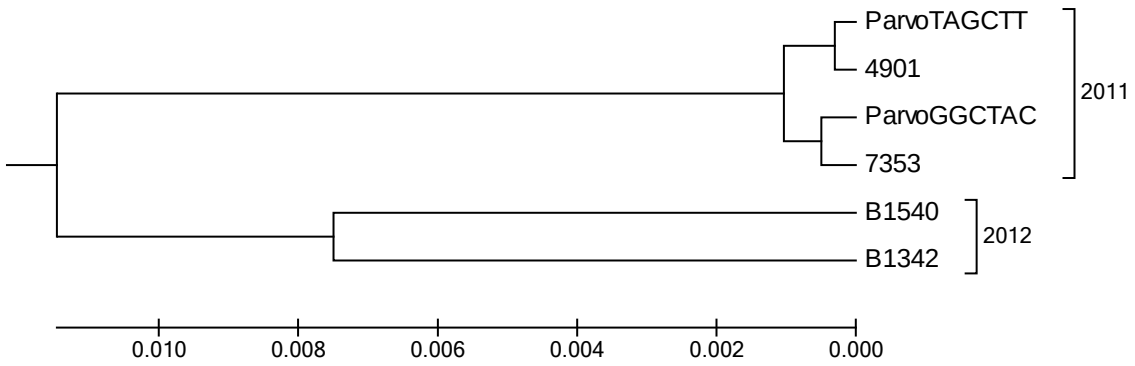


Figure 2. Phylogenetic Tree of Parvovirus in Sockeye salmon. The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 0.03108175 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site.

	ParvoTAGCTT	ParvoGGCTAC	4901	B1540	7353	B1342
ParvoTAGCTT						
ParvoGGCTAC	0.005					
4901	0.001	0.001				
B1540	0.023	0.022	0.021			
7353	0.001	0.001	0.001	0.023		
B1342	0.024	0.024	0.023	0.015	0.024	

Table II. Estimates of Evolutionary Divergence between Sequences. The number of base differences per site from between sequences are shown. Divergence between the 2011 and 2012 sequence groups was 0.023

Using the backdrop of the sequence variation characterized in sockeye salmon, eight QPCR assays were designed to known conserved regions of the virus (Figure 3). Primers were designed within the Replication and Capsid regions, which reflect the two open reading (protein coding) frames within the Parvovirus genome. Existing sequence along with published Parvovirus sequences from other species was used to identify gene regions. Primer Express3.1 was used to generate TaqMan Primers and Probes for use in QPCR.

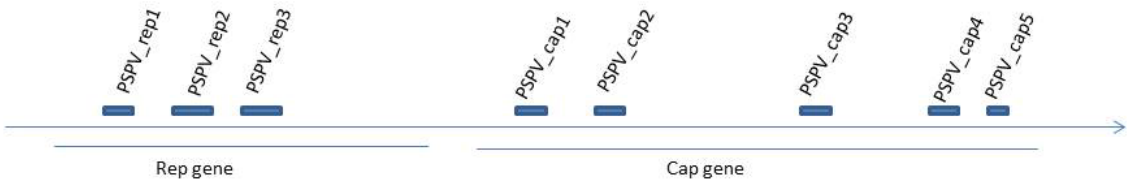


Figure 3. Placement of QPCR assays throughout the PSPV genome. Three assays located in the replication gene (rep1-3) and five assays situated in the cap gene (cap1-5).

All TaqMan assays were assessed for efficiency on positive PSPV tissue from sockeye salmon. In addition, we assessed assay sensitivity by amplifying serial dilutions of a positive pool sample (Table III).

Primer Set	Assay Length (pb)	R^2	Slope	Efficiency	Sensitivity limit
PSPV_rep1	63	0.999	-3.37	98.03	1:800
PSPV_rep2	135	0.997	-3.54	91.64	1:100
PSPV_rep3	81	0.999	-3.43	95.68	1:400
PSPV_cap1	94	0.999	-3.35	98.84	1:400
PSPV_cap2	114	0.999	-3.37	98.03	1:400
PSPV_cap3	68	0.999	-3.41	96.45	1:800
PSPV_cap4	109	0.997	-3.42	96.1	1:1600
PSPV_cap5	74	0.999	-3.36	98.48	1:800

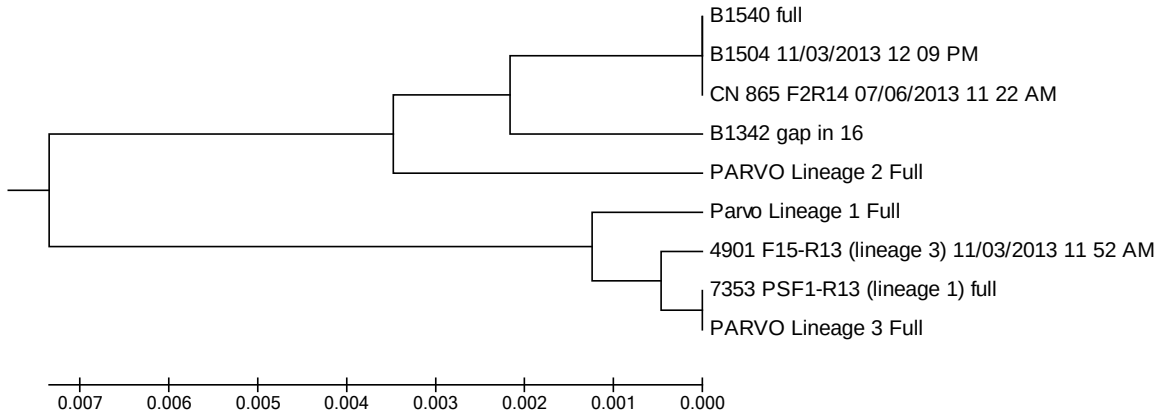
Table III. Primer combinations located throughout the PSPV genome assessed for slope, efficiency and sensitivity. Four primer combinations had a greater than 1:800 sensitivity (rep1, cap3-5), while one primer set was slightly less efficient than all others (rep2).

PSPV primers from each gene region assay were tested, re-combined and all positive products were further assessed by PCR within gene sets to obtain longer sequence reads. All 8 primer sets were tested on both sockeye and Chinook salmon. For the most part, we saw little variation in detection between primer sets based on sockeye salmon DNA, and relatively high prevalence of the virus was detected (>40% overall positive for sockeye smolts, with a range between stocks of 0 to >90%. Notably, low CTs for PSPV were found in >30% of the positive sockeye. In adults collected in 2008-2012, the expressed PSPV form was found at a prevalence of 30% over four tissues surveyed (gill, liver, brain and kidney), with the highest loads present in liver. Screening of over 600 Chinook samples on the ABI 7900 RealTime PCR platform uncovered extremely low loads of Parvovirus in a small percentage of samples (<1%). The addition of more primer sets did not significantly enhance the number of positives detected.

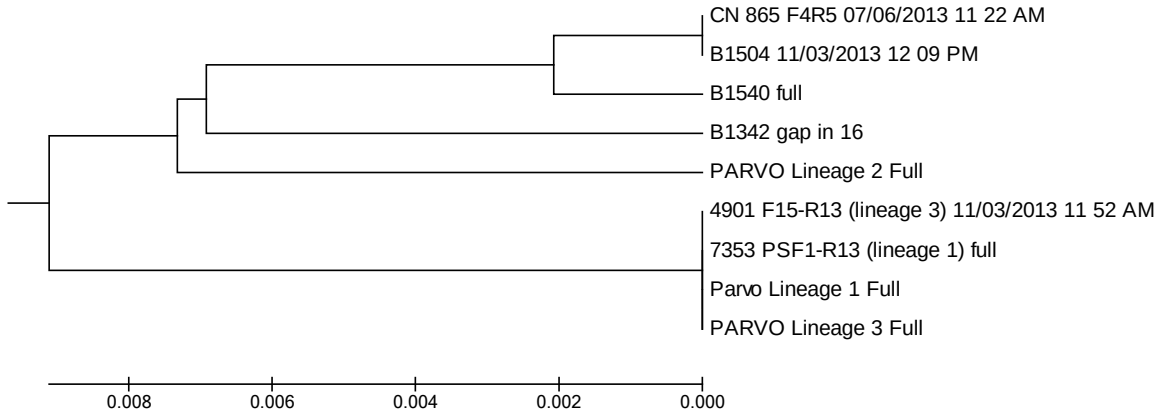
Five 96-well plates of Chinook samples (440 samples and associated controls) were also run on a higher sensitivity platform (the Fluidigm BioMark™; see section below for more details), which identified 22 potentially positive Chinook samples, 8 at CT's within the sensitivity of the ABI 7900. Following Fluidigm identification of potentially positive samples, sequencing was performed to obtain Chinook specific PSPV sequence. We were only able to obtain a longer sequence read from one positive Chinook sample, a 2008 Lower Adams (Shuswap system) stock salmon captured in the SOG.

The longer Chinook sequence was obtained with PCR using primers spanning the rep1-rep3, an area between rep3 and cap1, and cap4-cap5. PCR reactions were also carried out with the QPCR primers previously designed to the 454 sequence (Rep68F4-Rep68R5). These three PCR fragments (540bp, 241bp and 349bp) were sequenced using Life Technologies BDT 3.1 Sanger sequencing kit and analyzed on the Life Technologies ABI3730XL capillary sequencer. This produced a sequence representing a total of 881bp in the rep gene and 352bp in the cap gene.

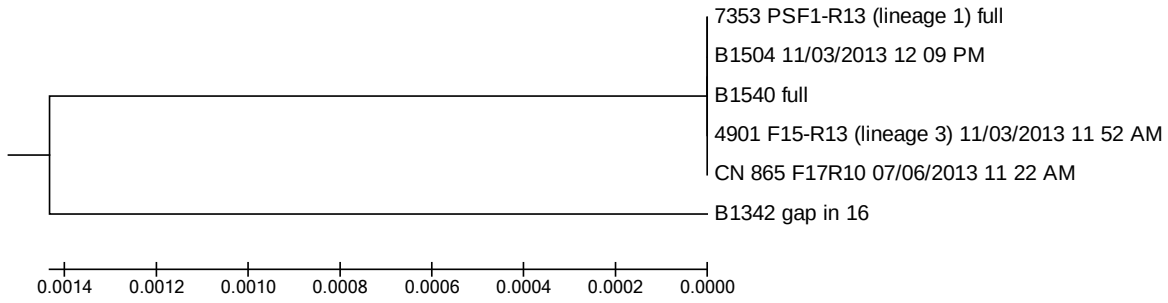
The Chinook sequence showed 100% homology to the 1504 Lower Shuswap sockeye salmon (Figure 4), which suggests a Shuswap system parvovirus sequence.



Rep1-3 region 540 bp
UPGMA tree, pairwise distances



F4R5 region 241 bp
UPGMA tree, pairwise distances



Cap4-5 region 349 bp
UPGMA tree, pairwise distance

Figure 4. Relationship of Chinook parvovirus (CN 865) sequence to PSPV in sockeye. Note the Chinook CN 865 and both sockeye B1540 and B1504 originate from the Shuswap system.

Objective 3: Molecular assessments of viral load in Chinook salmon

Organization of samples and extraction of nucleic acids

We have amassed collections of approximately 5000 Chinook salmon smolts taken from 2008-2012 (summer) that span stocks from Washington to SE Alaska. An additional 3800 samples have been collected since the fall of 2012. These include samples both from the freshwater natal rearing areas and hatcheries (for a subset of stocks) through to the first 9 months in the ocean. For stocks with a prolonged residence on the southern BC coast, e.g. Fraser River and East Coast Vancouver Island (ECVI) stocks that that rear in the Strait of Georgia, we have samples from multiple time-points during early marine residence that can be used to assess shifts in microbe profile within years. For highly migratory stocks, our collections generally cover a single time-point in their migrations.

Samples from stocks experiencing a large range in current productivity trends were included in our baseline extractions, as per the Table IV (also outlined in the original proposal).

High Conservation--Low productivity	
Fraser River Spring 4_2	Stream
Fraser River Early Spring 5_2	Stream
Fraser River Spring 5_2	Stream
West Coast Vancouver Island	Ocean
Puget Sound	Ocean
Snake River	Stream
Upper Columbia Spring	
Moderate Conservation/productivity	
Fraser River Summer 5_2	Stream
Fraser River Summer Lower Fraser 4_1	Ocean
East Coast Vancouver Island-Lower Georgia Strait	Ocean-Stream
Low Conservation--High productivity	
Fraser River Fall 4_1	Ocean
Fraser River Summer Thompson 4_1	Ocean
Nass	Ocean
Northern Mainland	Ocean
Southern Mainland	Ocean
Upper Willamette	Ocean
Stikine	Ocean
SE Alaska	Ocean

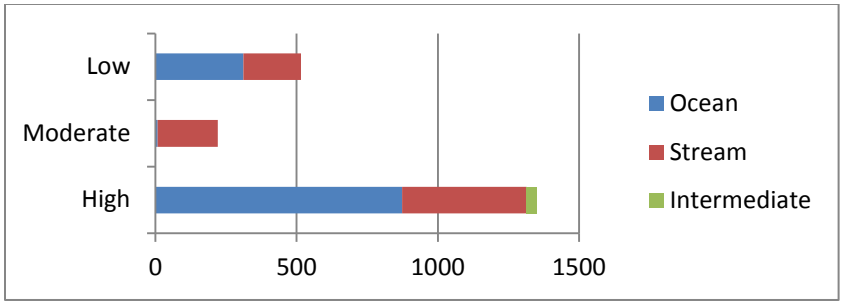
Table IV. Stocks of high, moderate, and low conservation concern included in our baseline.

Among our collections, we identified individuals that contained at least four of five key tissues (gill, heart, brain, liver, kidney) for extraction. Previous research on sockeye salmon showed that all of these tissues can carry the parvovirus, but the liver generally shows the highest loads and the earliest positives after exposure (parvovirus challenge study). While our primary goal was to conduct extractions that would maximize our ability to detect true positive fish, we had also envisaged these extractions to be useful for surveillance of other microbes in future, which

necessitated the bias towards samples with 4-5 tissues available. The two tissues that were not always present between samples were brain and heart. In 2012 onward we began alternating brain sampling between histology and molecular preservative; hence up to 50% of collections in 2012 were missing brain. In addition, in 2011 we did not take heart samples on two major cruises.

Final extraction plates for 2000 samples with five tissues were completed, and homogenates were prepared for the remaining projected 600 samples. Extracted samples include ocean and stream type Chinook salmon collected from 2008-2012 from high, moderate and low conservation stock groups (Figure 5A). The large sample sizes in the high conservation group are primarily attributed to those stocks and sample points for the ECVI and WCVI. Additionally, the middle upper Fraser River (moderate conservation concern) and South Thompson and Stikine (both of low conservation concern) have large sample sizes over a trajectory of sampling sites and seasons (Figure 5B). The sampling in the Strait of Georgia and in the high seas contained Strait of Georgia (SOG) Chinook samples primarily from the Fraser system. Of note, approximately 500 Chinook samples from stocks identified in this project collected from 2007-2010 have nucleic acid extracted from liver, gill, brain and muscle along with corresponding microarray data (data not shown). Together, the existing extracted collection of nucleic acids, both RNA and DNA, serve as a powerful baseline of samples for which to survey the microbiome Pacific coast Chinook.

A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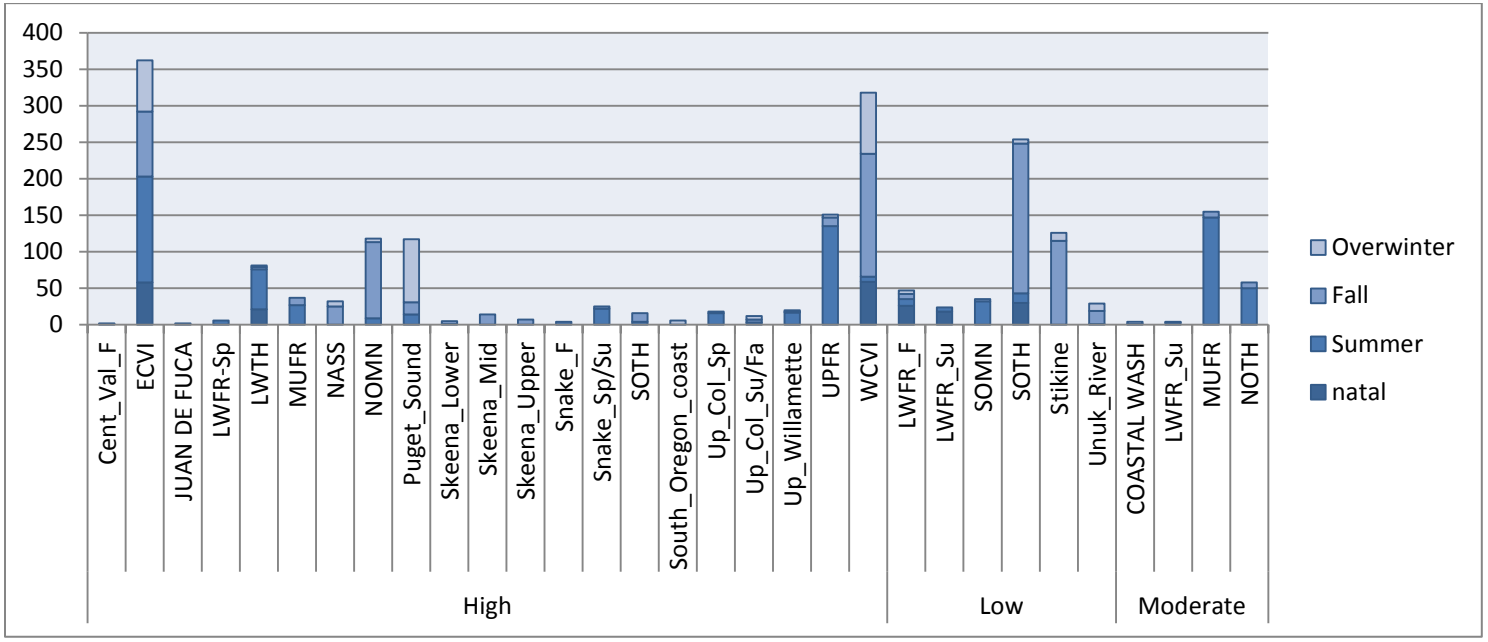
B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Skeena_Mid
Skeena_Upper
Snake_F
Snake_Sp/Su
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South_Oregon_coast
Up_Col_Sp
Up_Col_Su/Fa
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UPFR
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SOMN
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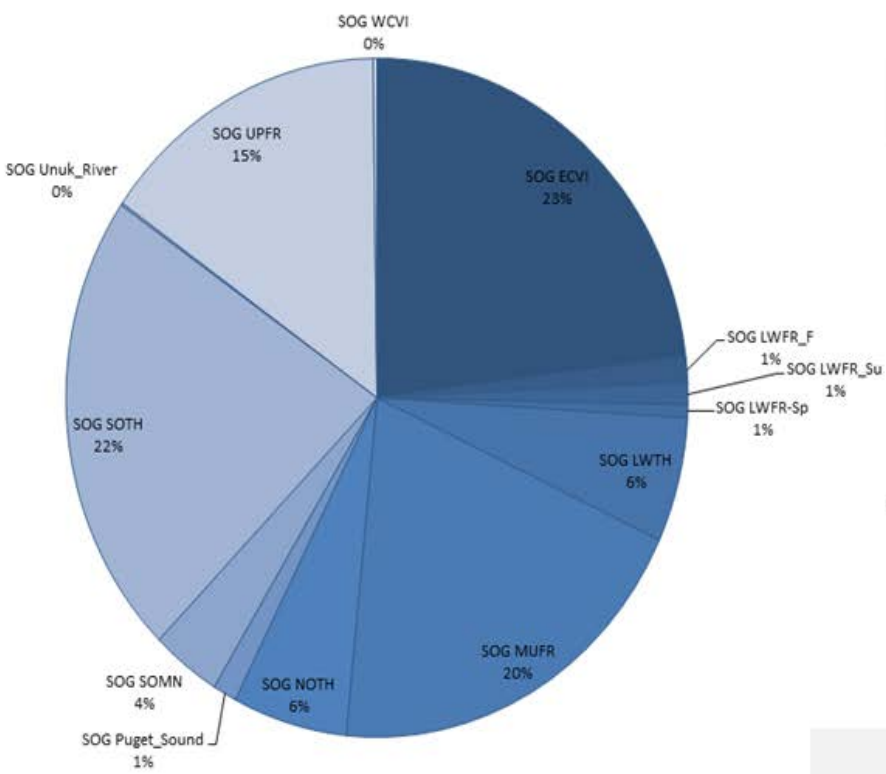
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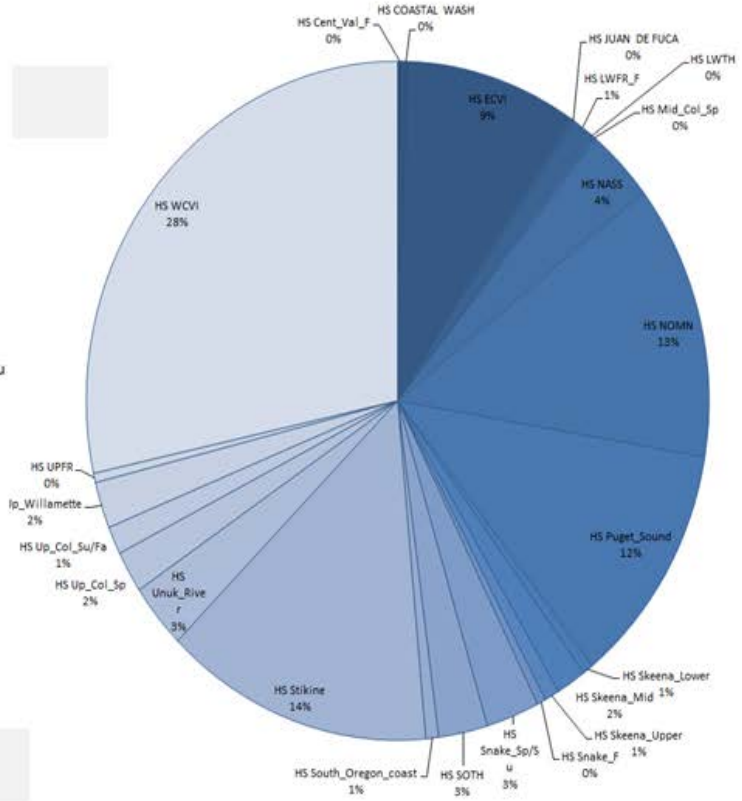
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HS Stikine
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HS Snake_Sp/Su
3%
HS Snake_F
0%
HS South_Oregon_coast
1%
HS SOTH
3%

Figure 5. Final extraction plates of four and five tissues available for surveillance of microbes include ocean and stream type Chinook salmon from High, Moderate and Low conservation groups (see A). The conservation groups are further delineated into stock management region codes with colour bars denoting the sampling season (B). Samples collected and extracted during the years 2008-2012, with stocks sampled in the Strait of Georgia and in the high seas/west coast Vancouver Island (C).

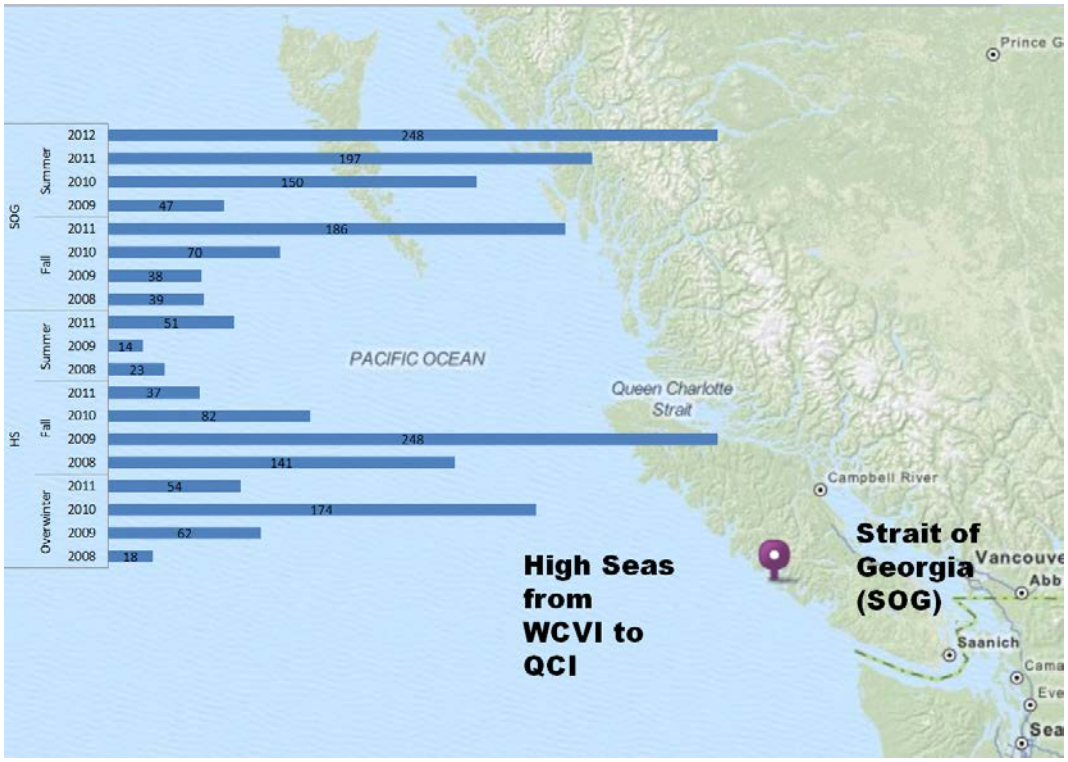

Figure 6. Annual collections during the summer and fall cruises in the Strait of Georgia and the high seas (including areas from the Southern tip of Vancouver Island to the Queen Charlotte Islands), as well as the high seas overwinter cruises. Sample sizes vary among years with large sample collections in the high seas for both 2009 and 2010 and significant sample sets for the SOG in 2011 and 2012. Additional samples for all years were collected at natal sites for both hatchery and wild Chinook (data not shown).

Initial Parvovirus surveillance of the Chinook samples on the ABI 7900 did not yield very many positives (<1% as noted in the previous sequencing section). Moreover, the loads that we were observing (CT > 30) were potentially indicative only of a carrier state, not likely that associated with an active infection (but note that research directly linking Parvovirus with disease has not yet been completed, so associations between Parvovirus CT (load) and viral activity are still speculative). The low load and prevalence of this virus in Chinook salmon smolts indicated to us that it was unlikely to be a significant factor in the variation in survivorship of Chinook salmon in the early marine environment; hence we decided not to expend further effort and funds towards obtaining data on all 2,600 fish. Instead, we decided to apply a portion of the samples in a preliminary analysis of a larger array of microbes using a platform under development for a new project, the Strategic Salmon Health Initiative, co-funded by Genome BC, Fisheries and Oceans, and the Pacific Salmon Foundation. Virtually all of the 38 microbes under assessment at the time we applied them to Chinook salmon (we are ultimately targeting 45 microbes) are associated with diseases in salmon worldwide (although a few have only speculative links with disease, including parvovirus). By applying this platform, we determined that we could obtain preliminary data to assess which microbes are carried at high prevalence and/or load in Chinook smolts migrating in the early marine environment, and which may emanate from the freshwater environment. While it was beyond our funding envelop to apply this technology to all 2,600 fish, given that many of the microbes being surveyed had not been previously characterized in Chinook salmon, we felt this information would provide a more holistic view of the microbes Chinook salmon are exposed to in the critical smolt out-migration stage that could guide future research activities exploring the role of microbes in early marine survival.

Background on the Fluidigm BioMark™

Since the Cohen inquiry, the Molecular Genetics lab at PBS has been developing and evaluating a novel high throughput platform for microbe surveillance capable of assessing the prevalence and load of 45 microbes (in duplicate) across 96 samples at once. This platform, the Fluidigm BioMark™, was developed originally for use in quantitative RT-PCR (qRT-PCR) and single nucleotide polymorphism (SNP) detection, but had not previously been utilized to assess presence and load of microbes. We have been using the platform for qRT-PCR of host biomarkers associated with our recently completed FishManOmics project, as well as for SNPs associated within our stock ID program.

In the development of this system for use on microbes, we began by assessing the efficiency, sensitivity, and reproducibility (on our second qPCR platform—an ABI 7900) of published microbe assays on the BioMark, and then moved on to developing new assays for microbes that have no existing assays available. We are targeting the development and evaluation of 45 microbes. While this process is ongoing, we have a number of assays that have been already assessed for performance across our two qPCR platforms, although full assay and platform

validations are not yet completed. We have recently begun conducting preliminary evaluations of wild collected salmon to determine the range of microbes that may be present, and so that we can identify positives for sequence characterization. Given remaining funds from the SEF project (but not the hold-back, which we did not ask for), we included five of the plates of Chinook salmon extracted for the Parvovirus assessments in these preliminary evaluations, and below we present an overview of our findings; Note that these findings should be considered preliminary until which time the platform evaluation is complete. That said, the results we provide are only for assays that we have sequence confirmed and (partial) assessments across both our platforms. Other assays, while run, are still being evaluated and will not be presented.

Preliminary Microbe Surveillance on the Fluidigm BioMark

We ran the BioMark microbe assay analyses on 5 plates that contained a combination of RNA and DNA extractions from tissue pools containing gill, liver, kidney, brain and heart; RNA and DNA are important to maximize the range of microbes resolved. These five plates were chosen as they represented a large range of stocks and years, and contained both freshwater and marine samples. We used microfluidic qPCR technology to simultaneously run duplicate reactions from a total of 48 TaqMan assays for 38 microbes and 1 housekeeping gene (some microbes have multiple assays). The assays were obtained from the published literature or designed in house with "primer express" (Life technology). A preamplification (specific target amplification: STA) reaction was performed prior to the microfluidic qPCR. The initial STA reaction is a multiplex PCR, which uses a combined set of all primers at 1/20 of concentration run over 14 PCR cycles to enrich for the targets of interest. This step is required for microfluidics PCR because the assay volumes are 1/1000th those used in normal qPCR (7 nl as opposed to up to 10 µl), hence without enrichment, the sensitivity to detect low copy number samples would be severely reduced. Final QPCR assays on STA samples are run singularly, with residual primers from the STA removed.

TaqMan assays were performed in duplicate using a Dynamic Array 96.96 chip (for Fluidigm BioMarkTM). Relevant negative and positive controls were included in each run. Aliquots of 5µl of sample premix (1x Taqman PCR master mix (Lifetech), 1x GE sample loading reagent (Fluidigm), and 2.25 µl 5-fold diluted STA product) and 5 µl of the assay premix (1x assay loading reagent (Fluidigm), 10 µM each primers, and 3µM probe) were loaded into 96.96 chip and mixed with an IFC controller HX (Fluidigm). The PCR was performed under the following conditions: 50oC for 2 min, 95oC for 10 min, followed by 40 cycles of 95oC for 15 sec, 60oC for 1 min. The quantification cycle (Cq, also known as the threshold cycle, CT) was determined using Fluidigm Real-Time PCR Analysis software 3.0.2 (Fluidigm). The result was exported as heatmap csv file, then imported to GenEx (www.multid.se) for data preparation and statistical analysis. The data from the five dynamic arrays were combined within GenEx, with averages taken for the duplicated samples. If only one of the duplicates was positive, the sample was treated as negative for that microbe. We also applied an artificial positive control (APC) to our clone standards to ensure assay performance and to identify incidences of positive clone

standards contamination. The microbe assay probes were labeled with 6-FAM, while the APC probes were labeled with NED. If any sample had APC (NED) positives with any microbe, the sample was treated as negative for that microbe. We used GenEx to run Pearson correlation test, T-tests and Anova's to compare microbes profiles among different years, ocean and stream-types. We consider all data emanating from these analyses preliminary.

Results of BioMark Study

In total, 440 Chinook samples were screened for 38 microbes. Twenty of the 38 microbes amplified products in at least one sample. The prevalence of most microbes increased from fresh water to the marine environment, except *Ichthyophthirius multifiliis* (IMF) which is a fresh water parasite. Microbes varied by prevalence and load. The most prevalent microbe was the microsporidian parasite *Desmozoon lepeophtherii* (NUC), with about 65% of fish testing NUC positive, but only one fish carried a low CT (<20) indicative of a high load (Fig. 7). Similarly, while 41% of fish were positive for another microsporidian parasite, *Nucleospora salmonis* (NSP), none of them had a low CT (<20). Alternately, 33% of fish were positive for a third microsporidian parasite, *Parvicapsula minibicornus* (PM-Dup), 34% of which had high loads (CT<20), with the highest prevalence of high load samples for PM-Dup observed in 2009 out-migrants. This parasite is known to be transmitted in estuarine environments and is associated with pre-mature mortality of adult sockeye salmon in the Fraser River (Jones et al., 2003), but its association with survival of smolts is unknown. Four additional parasites, including *Facilispora margolisi* (FACI), *Ichthyophonus hoferi* (ICH_H), *Myxobolus insidiosus* (MINSID), and *Parvicapsula pseudobranchicola* (Parvi2), were present in lower prevalence, but 10-20% of positives carried relatively high loads. The Piscine Reovirus (PRV) was the only virus of significant prevalence and load.

Statistical analysis showed that prevalence and load for some microbes varied significantly over years, locations, seasons and life-history-type. For example, fish sampled in summer had higher loads of harmful algal bloom species *Heterosigma akashiwo* (HAS), fish sampled in fall had higher loads of Parvi2, fish sampled in winter had lower loads of NUC.

If we make the assumption that microbes observed at higher loads (i.e. greater copy number) have a greater potential to be associated with active infections in the fish at the time of sampling, by using load, we can potentially differentiate carrier or early exposure states from active infections. Eleven of the microbes carried CT's in at least one fish that were <20, indicating a high load sample (note that CT <20 is a somewhat arbitrary cut-off). The highest prevalence among positives of low CT samples were in PM_DUP (33.6% of samples positive in the marine environment carried high loads—totalling 43 fish), *Renibacterium salmoninarum* (BKD) (20%, but this was only a single fish out of 6 positives), ICH_H (18.7% marine—23 fish), MINSID

(15.9% marine—33 fish), Parvi2 (15% marine—32 fish), and FACI (15% marine—3 fish). In freshwater, IMF was at low CT in 25% of positive fish (5 fish), but no marine fish.

Microbes showing divergent patterns between ocean- and stream-type life histories in the marine environment included HAS and Parvi2. *Sphaerothecum destructens* (Sphaer) and to a lessor degree Viral erythrocytic necrosis virus (VER), FACI and IMF were also influence by life-history type when intermediate life-history types were included (ANOVA). HAS was significantly influenced by a large number of variables, with year, environment (FW/SW), and season predominating. ICH_H and NSP were influenced primarily by year. MINSID, PM_DUP, and NSP showed an interaction between life-history type and season that was stronger than either alone.

The results presented are backed by at least some sequence data on each of the microbes discussed; however, as we have not completed assay or platform validations, the data should be considered preliminary and exploratory. Even so, we have confidence that this technology will provide highly relevant and useful information to managers once validated and applied across a broad spectrum of samples, and including contrasts between stocks currently under different productivity states.

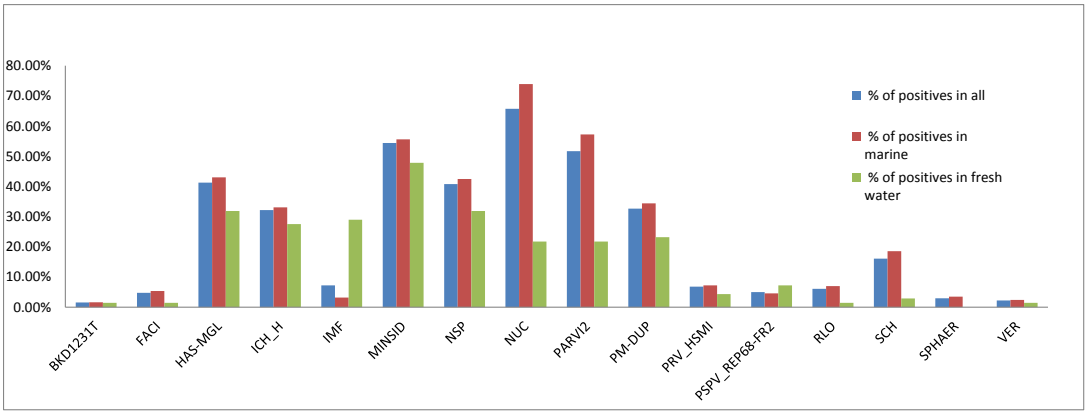

Figure 7. Prevalence of various microbes in Chinook salmon smolts sampled in freshwater (green), marine (red) and overall (blue). Microbes not described in the text include RLO (bacteria associated with Strawberry disease) and SCH (chlamydia-like bacterial microbe).

Discussion

A large proportion of Chinook salmon stocks in Southern BC and Washington have been declining in productivity, and there is as yet very little understanding of the factors controlling these declines. Most in the scientific community expect that variability in productivity among stocks is likely controlled by a combination of stressors rather than a single factor. Diseases in general, and viral diseases in particular, came into particular focus at the Cohen Commission of Inquiry and the various think-tanks surrounding this inquiry as having the potential to impact population dynamics to the degree that we have observed in salmon. Sandell and colleagues studying the potential role of parasites and BKD on early marine survival (2010) found that in years whereby marine rearing conditions were poor for salmon, disease was not tolerated, and fish that entered the ocean already affected by disease-causing microbes did not survive.

In the new Strategic Salmon Health Initiative, we have extensively leveraged funds, including the 100K from this SEF grant, to attain the information required both to understand the role of infectious agents of disease in salmon declines and to develop tools and models that can be used by managers. Given that in this SEF project we have already identified and extracted key Chinook salmon from stocks of interest within our archives, to be further supplemented by collections taking place in 2012 through 2014, Chinook will be the first species that we analyse on the 45 microbe surveillance platform under development; hence there will be an immediate benefit to the Chinook Technical Committee of the PSC in broad-based information on microbe exposure for Chinook salmon smolts, and the types of analyses we envisaged for Parvovirus alone will be expanded to include the majority of known salmon pathogens.

In this broader SSHI project, if we show that there is a relationship between microbiome loads, or shifts in microbe loads and productivity of stocks, there may exists some capacity for mitigation, especially within hatcheries. Rapid molecular-based screening of broodstock could be used to minimize vertical transmission and improved (informed) husbandry practices could minimize horizontal transmission. Importantly, even if we cannot mitigate some microbes, the knowledge and tools developed using the baseline of surveillance samples generated from this project could lead to enhanced forecasting of year-class strength in future, if associations between specific microbes and survival are found. Specifically, we envision that pre-season forecasts could benefit from knowing if there is any anticipated mortality associated with microbes from screening of smolts, which could be used for planning purposes. As well, information such as microbe status of smolts that can help explain or describe stock-specific run failures can also be used to help direct future cost-effective recovery methods and programs.

It is important that we are clear that the presence of a microbe alone is not indicative of a disease state. While SSHI project will initially conduct surveillance research to determine what microbes are present in BC salmon, when and where they are first detected, and what microbes are present in loads and in association with histopathological changes that may indicate a disease state, follow up microbe challenge research will be undertaken on the potentially most important

emerging microbes identified to begin to assess whether, and under what conditions specific microbes may associate with or cause disease in hatchery and wild fish. It is this information that will ultimately be critical to determining the potential for population-level effects of microbes.

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HUMAN RESOURCES

Dr. Kristi Miller: Project lead responsible for ensuring all aspects of the project are carried out on time and budget, and responsible for the experimental designs.

Dr. Marc Trudel: Head of the High Seas Salmon Section for DFO and was instrumental in providing an ocean platform for the collection of the samples used in this project.

David Patterson: Head of the Environmental Watch Program for DFO and was instrumental in organizing and carrying out our smolt collection program associated with the project.

Amy Tabata and Tobi Ming: Technicians responsible for carrying out laboratory analysis associated with the salmon Parvovirus.

Norma: Collection, GSI compilation

Karia: Design, sub-sampling, homogenization and RNA extractions

Stephen Yu and Jackson Price: Co-op students who assisted with sample archiving, sub-sampling, database updates, and DNA extractions.

Project Schedule

The Milestones and timelines (of both Parties) for this Project are as follows:

Date	Milestones/Accomplished	Responsible Party
2012		
May	Genetic Stock ID 2011	DFO (complete)
May	PSC Funding Release $50K to DFO	PSC (complete)
June	Initial Primer development and testing	DFO (complete)
July	Sequencing; Development of quantitative assay; DNA extractions	DFO (complete)
July-October	Sample prep, DNA extractions	DFO (complete)
Nov-Dec	Add 2012 to Sample prep, DNA extractions, GSI	DFO (**added**)
Aug-Dec	Parvoviral quantitative assays run	DFO (terminated after 600 samples run)
September	PSC Funding Release $36.57K to DFO	PSC (complete)
2013		
January	RNA Extractions, Normalization, cDNA (n=1248)	DFO (**added**)
Feb-March	BioMark Microbe assessments (n=475)	DFO (**added**)
April-May	Data Analysis	DFO (complete for Fluidigm)
April	Osmoregulatory assessments with Parvovirus	DFO (**not done**)
April	PSC Funding Release $9.68K to DFO	PSC(**not requested**)
June	Archive and subsample DNA extractions	DFO (complete)
July-August	Preparation of report to the PSC; submit report	DFO (complete)

Project Expenditures

Expenditures by Fiscal Year: May 1, 2012 to June 30, 2013

Fiscal Year – 2012-2013 Description	Financial Contribution to DFO	Actual Costs
Salary – Researcher (Ind)		
Salary –Technician (Ind)		
Salary – Coop Student	12,600	8,412
Salary - EBP		
Travel	880	
General administrative -	7,870	
Laboratory Materials and Supplies	65,220	78,158
Grand Total	86,570	86,570

*Additional funds were not requested for the fiscal year 2013-2014. The Financial Contribution to DFO represents the details for DFO's Recoverable Project Expenditures.