

Evidence of a hydraulically challenging reach serving as a barrier for the upstream migration of infection-burdened adult steelhead

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Anadromous fishes such as steelhead trout, *Oncorhynchus mykiss*, are exposed to a suite of infectious agents and migratory challenges during their freshwater migrations. We assessed infectious agent load and richness and immune system gene expression in gill tissue of Bulkley River (British Columbia, CA) steelhead captured at and upstream of a migratory barrier to evaluate whether infectious burdens impacted migration success. We further considered the potential influences of water temperature, sex and fish size on host infectious agents and transcription profiles. There were eight infectious agents detected in steelhead gill tissue, with high prevalence of the bacteria *Candidatus Branchiomonas cysticola* (80%) and *Flavobacterium psychrophilum* (95%) and the microparasite *Sphaerothecum destruens* (53%). Fish sampled at the falls had significantly greater relative loads of *Ca. B. cysticola* and *F. psychrophilum*, higher infectious agent richness and differential gene expression compared to fish captured upstream. *Flavobacterium psychrophilum* was only associated with immune gene expression (particularly humoral immunity) of fish sampled at the falls, while water temperature was positively correlated with genes involved in the complement system, metabolic stress and oxidative stress for fish captured upstream. This work highlights interesting differences in agent–host interactions across fisheries and suggests that hydraulic barriers may reduce the passage of fish with the heaviest infectious agent burdens, emphasizing the selective role of areas of difficult passage. Further, this work expands our knowledge of infectious agent prevalence in wild salmonids and provides insight into the relationships between infectious agents and host physiology.

Key words: Adult, disease, microbes, rainbow trout, salmon, spawning

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Introduction

Infectious agents (pathogenic microparasites including viruses, bacteria, fungi and protozoa) are widespread in aquatic environments (Price, 1980) and play an integral role in shaping aquatic ecosystems through disease processes (e.g. Cotner and Biddanda, 2002; Suttle, 2005; Smith, 2007). Infectious diseases have resulted in declines in some wild fish populations (Marty et al., 2003; Steinbach Elwell et al., 2009) and have been hypothesized as a factor contributing to collapse in others (Pearson et al., 1999; Gibson-Reinemer et al., 2017). Infectious agents in an active state induce changes to host physiology that can reduce performance of fish in the wild (i.e. disease; Jeffries et al., 2014a; Miller et al., 2014; Teffer et al., 2017; Bass et al., 2019). Fish have evolved immune mechanisms to protect against and reduce the cost of disease (Uribe et al., 2011), though ultimately there are fitness consequences associated with host-immune responses regardless of disease development (Zuk and Stoehr, 2002). Consequently, understanding infection dynamics and host physiological response in wild fish populations is important to provide context for monitoring any population scale changes. The study of infectious agent dynamics in wild fish has been limited due to challenges associated with data collection and the large spatial scale of these processes (Peeler and Taylor, 2011). Recent advances in molecular genetics (Lawson Handley, 2015) have allowed for rapid and cost-effective quantification of gene expression, including the use of high-throughput quantitative reverse transcriptase polymerase chain reactions (PCR) on the BioMarkTM microfluidics platform, which has recently been validated for detection and quantification of salmon infectious agents (Miller et al., 2016) and detection of a molecular signature indicative of viral disease (Miller et al., 2017). This platform can help deduce when an infectious agent has moved from a carrier state to actively replicate and cause damage to the host, based on the host-immune response (Casadevall and Pirofski, 1999; Miller et al., 2014).

Infectious agent communities are dynamic and their prevalence among and within wild fish populations are highly dependent on both biotic and abiotic factors. Intrinsic biological differences of potential host species such as life-history stage (Castro et al., 2015), size (Boerlage et al., 2011; Molina-Fernandez et al., 2015), age (Marty et al., 2003), condition (Rohlenová et al., 2011) and sex (Richards et al., 2010; Teffer et al., 2017) may alter individual susceptibility to infection. For example, infectious hematopoietic necrosis virus causes significant mortalities in juvenile salmonids but is relatively nonpathogenic in adult fish (Traxler et al., 1997). Further, sexually mature female rainbow trout, *Oncorhynchus mykiss* (Linnaeus), have demonstrated greater parasitaemia and mortality from the blood parasite *Cryptobia salmositica* than sexually mature males (Currie and Woo, 2007). Environmental conditions may affect infectious agent virulence and host vulnerability to these organisms (Wedemeyer, 1970; Rottmann et al., 1992; Marcos-Lopez et al., 2010; Sepahi et al., 2013).

Temperature, in particular, has a critical influence on infectious agent-fish host infection dynamics given the governing role the environment has on fish physiology (Brett, 1971; Fry, 1971; Yada and Tort, 2016) and infectious agent productivity (e.g. Thoen et al., 2016). Understanding the factors that contribute to infectious agent prevalence and host physiology is exceptionally important for anadromous fish that undertake challenging, long-distance migrations and experience a suite of physiological changes and novel agents upon returning to freshwater to complete their reproductive cycle (Cooke et al., 2011; Miller et al., 2014).

Migratory Pacific salmonid species typically enter rivers to spawn after completing the marine portion of their life-cycle (Schaffer, 2004). Pacific salmonids have been in decline across much of their distribution (Beamish et al., 1999) and there have been notable shifts in survival of return migrating adult salmon (Cooke et al., 2006; Hinch et al., 2012). Research has highlighted several potential sources of prespawn mortality including increasing water temperatures (Hinch et al., 2012; Bowerman et al., 2018), fisheries captures (Baker and Schindler, 2009) and infectious agents (Traxler et al., 1998). The impacts of which may be exacerbated in areas of difficult passage (Hinch and Bratty, 2000).

Steelhead *O. mykiss* (Walbaum) are unique to other Pacific salmonids in their potential iteroparity and overwintering behaviour prior to spawning (Scott and Crossman, 1973). During their spawning migrations, steelhead may remain in rivers for up to 10 months, increasing their potential exposure to accumulated thermal units and to infectious agents relative to other anadromous salmonids. Steelhead provide an ideal model to study the impact of infectious agents on migratory fish because their condition does not decline as rapidly and severely during migrations relative to semelparous salmon that undergo natural senescence. Despite growing evidence that infectious agents can have negative impacts on wild salmon populations (Jeffries et al., 2014a; Teffer et al., 2017; Bass et al., 2019), the influence of infectious agents on wild steelhead remains relatively unexamined. The Pacific Northwest maintains some of the last remaining entirely wild steelhead runs on Earth, with the rivers of the Skeena watershed, British Columbia, supporting many of these wild populations.

Adult steelhead returning to spawn in the Bulkley River of British Columbia, Canada, must undertake a physiologically challenging migration through Witset Falls 314 km into their migration. Fish can be observed holding at the base of the falls before making their ascent to spawning grounds. The Wet'suwet'en First Nation have a long-standing salmon fishery where they dip net fish holding in eddies as they attempt to ascend the falls. They also conduct a steelhead mark-recapture program in collaboration with the British Columbia Ministry of Forests, Lands, Natural Resource Operations and Rural Development—Skeena Region, with the recapture occurring

part way up the falls. Above the falls, there is a high-effort catch-and-release recreational angling fishery.

To determine if the falls act as a migration barrier for weaker fish we evaluated the condition of steelhead based on the transcription of biomarkers known to be associated with immunity, stress, osmoregulation and metabolism, as well as infectious agent presence and relative load at and above this natural barrier to migration. We hypothesized that infectious agent load would impact fish migration success (i.e. highly infected fish would have a low success rate at passing the migration barrier). We also explored relationships between the relative loads of infectious agents and host biomarkers and predicted that differential immune stimulation would be associated with a subset of infectious agents with the greatest pathogenic potential. We further considered the influence of water temperature, sex and fish size on host biomarkers and infectious agent load and richness. We predicted that host biomarkers of immunity and stress would be generally upregulated with warmer temperatures, as observed in previous temperature challenge studies (Jeffries et al., 2012a, 2014b) and would be greater for females, as they have shown higher stress and prespawn mortality during spawning migrations (Kubokawa et al., 1999; Teffer et al., 2017).

Methods

Study site and collection

Adult steelhead were captured from 23 September to 29 October 2016 by dip net and angling and nonlethally sampled for gill tissue. All fish captured by dip net were from Witsset Falls (314 rkm; height, 15 m) by Wet'suwet'en fishers. Steelhead captured by dip net were transferred in <5 s to a transport sling, which a runner then transported to a water-filled sampling trough (total air exposure of <25 s). Angling was completed upstream of Witsset Falls (325–407 rkm) by local or guided anglers that agreed to assist with the study. Anglers used a combination of fly fishing, spin fishing and centre pin fishing and various sizes of flies, inline spinners and artificial worms. Fish were transferred directly into a sampling trough positioned in the river upon landing (no air exposure period exceeded 30 s). The date of sampling differed between capture methods, which represented inherent differences in the fisheries we worked with to sample migrating steelhead. The Wet'suwet'en salmon fishery operates from early August until the last week in September at Witsset Falls while salmon and steelhead are actively migrating. The recreational fishery operates primarily between mid-September to early November and is typically completed upstream of the falls where there is greater and safer access to the river. Steelhead anglers almost exclusively target slow moving water or refuge sites where steelhead are known to hold prior to overwintering in the river (Keefer et al., 2009). These sampling groups therefore reflect fish in two different stages of migration and freshwater acclimation, which has been previously confirmed by telemetry (Twardek et al., 2019).

Once landed and secured in the water-filled sampling trough (or recovery bag), steelhead were sampled nonlethally for ~0.5 mg of gill tissue (2–3 filament tips) using sterilized diagonal cutting pliers to prevent tearing (see Cooke et al., 2005 for details on the technique). The presence of infectious agents and corresponding physiological changes can be effectively detected in gill tissue even when it is not the primary infectious tissue (Mendonca and Arkush, 2004; Tavares et al., 2016; Miller et al., 2017; Teffer et al., 2017) and many studies investigating infection status have used this method (e.g. Cornwell et al., 2013; Teffer et al., 2017; Bass et al., 2019). Gill tissue was stored in 1.2 ml of RNAlater® solution (Qiagen, MD, USA) in 1.5 ml microtubes and stored at –20°C for future genomic analyses. Nonlethal gill biopsy sampling was undertaken due to conservation concerns for Bulkley steelhead. All steelhead were assessed for fish length (FL; mm) and sex and were sampled and released within 30 min of capture, during which they remained submerged in water. Surface water temperatures (°C) at the point of capture were taken using a handheld digital thermometer (Taylor Precision Digital Thermometer, #9847, Taylor USA, Oak Brook, IL, USA) as an indication of the broader scale patterns in temperatures experienced by fish throughout the river and season. All sampling was conducted in accordance with the guidelines of the Canadian Council on Animal Care as administered by Carleton University (protocol 106247).

Sample analyses

All sample analyses were completed at the Pacific Biological Station (Fisheries and Oceans Canada). To determine the abundance of infectious agents and host gene expression simultaneously, high-throughput nanofluidic quantitative PCR (qPCR; Fluidigm BioMark™ Dynamic Array, CA, USA; outlined in Jeffries et al., 2014a and Miller et al., 2014, 2016) were undertaken on gill RNA using assays for 47 infectious agents known or suspected to cause disease in salmon (Supplementary Table S1) and 59 genes with a broad range of immune pathways of defence and stress responses. The magnitude of host response (measured herein via host gene expression) can be used to evaluate the relative likelihood an infectious agent is causing damage and therefore disease to the host (Casadevall and Pirofski, 1999; Miller et al., 2014, 2017). To provide an overview of the processing method, each sample was independently homogenized with a MM301 mixer mill (Restch Inc., PA, USA) and aliquots of the aqueous phase (containing genetic material) were taken. RNA quantity (A260) and purity (A260/280) were evaluated by spectrophotometry, and samples were normalized accordingly. RNA was used to construct cDNA (Invitrogen™ SuperScript™ VILO™ CA, USA, cDNA Synthesis Kit) that underwent a specific targeted amplification (STA) to provide sufficient template molecules for qPCR on the Fluidigm BioMark (impacts of STA on the analytical performance of infectious agent assays presented in Miller et al., 2016). Samples were then treated with ExoSAP-IT® PCR Product Cleanup (MJS BioLynx Inc., ON, Canada)

to remove unincorporated nucleotides and primers and were diluted 5-fold with DNA Suspension Buffer (Teknova, Hollister, California). Sample and assay mixes were loaded onto the Fluidigm Dynamic Array using an IFC Controller HX, and the GE 96 × 96 Standard v1.pcl. (TaqMan®) protocol was used for qPCR. TaqMan assays to infectious agents were designed to target RNA, which can assess microparasites in an active state through detection of mRNA, and allows for simultaneous screening of RNA viruses (Miller et al., 2016) and host genes (Miller et al., 2017). A subset of samples with strong activation of a biomarker panel associated with a viral disease development (VDD) state (Miller et al., 2017), but that did not test positively for any viruses in our initial panel, was screened for 10 additional salmon viruses recently discovered in BC salmon (Gideon Mordecai, unpublished data). On each dynamic array, we included a negative extraction control, a negative and positive cDNA control, a negative and positive control during the STA stage and a control that did not undergo pre-amplification (as described in Miller et al., 2016).

Samples and agent assays were run in duplicate during the final qPCR resulting in quadruplicate measures of infectious agent expression while biomarker assays and reference assays (78d16.1, MrpL40 and Coil-P84) were run singly, resulting in duplicate cycle threshold (CT) values for each sample. The BioMark real-time PCR analysis software was used to assign CT values for each reaction curve based on visual assessment of curve shape and replicate similarity (a negative scale from 0–40 corresponding to the maximum number of cycles completed on the BioMark). CTs were averaged for host biomarker and infectious agent assays. Samples that had two or more quadruplicates fail to amplify infectious agent products were treated as negative detections. Positive infectious agent detections were only assigned for samples with a CT below the 95% limit of detection, which varied for each infectious agent assay (Miller et al., 2016). Averaged CT scores underwent an efficiency correction using the formula $CT((\log(1 + \text{efficiency})/(\log(2))))$ as specified by the GenEx Software (www.multid.se). Biomarker assays with efficiencies outside 1.0 ± 0.2 were considered failed and were removed from analysis (Supplementary Table S2). Biomarker data were normalized against reference genes (developed in-house by the Molecular Genetics Laboratory at the Pacific Biological Station, Nanaimo, BC) and pooled sample using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001; Teffer et al., 2017).

Statistical analyses

Prior to analysis, infectious agent abundance data were transformed to a positive scale by subtracting each CT value from 40 (the maximum number of cycles completed on the BioMark as per Bass et al., 2017). This measurement reflects the relative abundance or ‘loading’ for each microbe between individuals and is hereby termed ‘relative load’. The variable ‘agent richness’ was also calculated from infectious agent data

and was defined as the number of individual infectious agents found within each fish.

To test the relationship between the relative loads of infectious agents and the predictor variables of sampling location, water temperature at the time of sampling, sex and fish size, we used multiple regression models. Two separate multiple regression models used the relative loads of either *Flavobacterium psychrophilum* or *Candidatus Branchiomonas cysticola* as response variables, limiting the data to positive infectious agent detections only. Only these two agents were present in enough fish to merit analysis of effect. Diagnostic plots suggested that the relative load models for both infectious agents had minimal deviations from normality. To test the relationship between infectious agent richness and the predictor variables of sampling location, water temperature at the time of sampling, sex and fish size, we used a Poisson regression model to account for count data and a right skewed distribution. To evaluate the relationship between relative loads of *Ca. B. cysticola*, *F. psychrophilum* and *Sphaerothecum destruens* we used Spearman’s rank correlations for all individuals with positive detections.

Kruskal’s nonmetric multidimensional scaling (NMDS) was used to evaluate relationships between sampling location and host gene expression values. NMDS is an ordination technique that uses nonlinear dimensionality reduction to visualize relationships between observations for multiple response variables and is an effective statistical approach for gene expression data (Taguchi and Oono 2005). A Bray–Curtis distance matrix was created based on the gene expression values for each fish (R function ‘metaMDS’; package ‘vegan’). Dimensionality of the ordination was determined as the fewest number of axes needed to reduce the disagreement between rank orders of observed and predicted distances to appropriate levels (stress < 0.2; Kruskal, 1964). Biomarker scores were determined using weighted averages of all predicted distances. The external variable sampling location was fit into the ordination using the ‘envfit’ function (package ‘vegan’) to test for differences in gene expression above and at the falls. The envfit function fits external variables to predicted points by maximizing their correlation. Separate NMDS analyses for fish sampled at the falls and above the falls were completed to determine whether infectious agents and other external variables were having unique impacts on fish physiology at different stages in the migration. These two NMDS analyses were completed for fish at and above the falls fitting the relative loads of *F. psychrophilum* and *Ca. B. cysticola*, agent richness, water temperature, sex and FL as external variables. Only infectious agents with high prevalence were fit to the ordination to reduce bias associated with agents that had only a few positive detections (as per Teffer et al., 2017). Continuous variables (agent loads, agent richness, water temperature and FLs) were plotted as vectors that represent maximal correlation of each variable with the ordination. Vector lengths were shortened and biomarker labels were adjusted to increase

Table 1: The prevalence and relative loads (40-CT) of infectious agents present in adult Bulkley River steelhead across fisheries/sampling locations.

Assay name	Agent name	Type	Prevalence (%)		Relative load (40-CT)	
			At falls	Above falls	At falls	Above falls
c_b_cys	<i>Candidatus Branchiomonas cysticola</i>	Bacterium	93	80	27.6 (22.4–32.1)	23.9 (15.9–31.7)
fl_psy	<i>Flavobacterium psychrophilum</i>	Bacterium	100	92	22.6 (18.7–24.2)	18.0 (13.2–23.6)
pch_sal	<i>Piscichlamydia salmonis</i>	Bacterium	13	4	22.7 (21.7–23.8)	13.1
lo_sal	<i>Loma salmonae</i>	Parasite	20	0	15.8 (14.7–16.7)	0
pa_ther	<i>Paranucleospora theridion</i>	Parasite	13	0	17.1 (15.0–19.3)	0
te_bry	<i>Tetracapsuloides bryosalmonae</i>	Parasite	0	4	0	24.7
sp_des	<i>Sphaerothecum destruens</i>	Parasite	66	40	21.4 (15.8–24.7)	19.1 (14.4–25.2)

Each assay lists the abbreviation, infectious agent name, type of agent, prevalence and mean relative load (range) by location. Mean relative loads were calculated using positive infectious agent detections only

plot comprehension. Significance was assessed at $\alpha < 0.05$. Where applicable, means and standard errors are presented. All statistical analyses were conducted in R Version 3.4.3 (R Core Team, 2015), and figures were constructed in Sigma Plot Version 11.0.0.75.

Results

Fifteen steelhead (598 ± 62 mm; 53% female) were captured by dip net at Witset Falls (314 rkm) between 23 and 29 September 2016. An additional 25 steelhead (688 ± 16 mm; 68% female) were captured by angling (325–407 rkm) between 23 September and 29 October 2016. Sampling temperatures remained cool for fish both at ($8.6 \pm 0.4^\circ\text{C}$; $7.0\text{--}11.5^\circ\text{C}$) and above ($6.2 \pm 0.3^\circ\text{C}$; $3.0\text{--}10.3^\circ\text{C}$) the falls. Sampling temperature was closely correlated with date ($r = -0.76$; $P < 0.01$) highlighting its usefulness as a proxy for broad scale changes in temperatures experienced as the sampling period progressed.

Infectious agents

Seven infectious agents were initially detected in Bulkley River steelhead gill tissue, though only two were sufficiently prevalent to evaluate drivers of their abundance and their influence on host physiology. *Candidatus Branchiomonas cysticola* (80%), *F. psychrophilum* (95%) and *S. destruens* (53%) were the most prevalent infectious agents, with low prevalence of *Loma salmonae* (20%), *Piscichlamydia salmonis* (13%), *Paranucleospora theridion* (13%) and *Tetracapsuloides bryosalmonae* (4%; Supplementary Table S1). The prevalence of all agents was higher in fish captured at the falls than above (Table 1), except for *T. bryosalmonae* that was only detected in one fish above the falls. The relative load of *Ca. B. cysticola* was not significantly correlated with water temperature at the time and location of sampling ($R^2 = 0.03$; $P = 0.25$), sex ($P = 0.97$) or FL ($R^2 = 0.03$; $P = 0.33$) but was significantly greater for fish sampled at the falls than

above ($t = 2.86$; $P < 0.01$; Fig. 1A). The relative load of *F. psychrophilum* was not significantly correlated with water temperature ($R^2 = 0.13$; $P = 0.94$), sex ($P = 0.07$) or FL ($R^2 = 0.01$; $P = 0.31$) but was significantly greater for fish sampled at the falls than above ($t = 4.37$; $P < 0.01$; Fig. 1B; Table 2). The relative loads of *Ca. B. cysticola* and *F. psychrophilum* were not closely correlated ($r_s = 0.28$), neither were *Ca. B. cysticola* and *S. destruens* ($r_s = 0.28$) nor *F. psychrophilum* and *S. destruens* ($r_s = 0.39$). Agent richness was also significantly greater ($\chi^2 = 11.79$; $DF = 1$; $P < 0.01$) for fish sampled at the falls (3.1 ± 0.3 agents) compared to fish above (2.1 ± 0.2 agents; Fig. 1C; Table 3). Water temperature ($P = 0.19$), sex ($P = 0.12$) and FL ($P = 0.94$) were not significant predictors of agent richness.

Host gene expression

Gene expression data were successfully reduced into a 2D ordination (stress, 0.18; Fig. 2). The genes most positive on NMDS1 included RSAD, IFIT5, Mx, NFX, DEXH, VAR1, X52Ro and IFI44A, which are indicative of a VDD response when co-expressed (Miller et al., 2017). Five individuals had a value >0.18 on NMDS1, suggesting they were in a state of VDD. Four of these individuals were captured above the falls and one was captured at the falls. Further screening of these samples with a more comprehensive panel of 10 viruses newly discovered through application of the VDD panel in BC salmon (Gideon Mordecai, unpublished data) revealed a novel strain of cutthroat trout virus (CTV-2) detected in two of the five samples (data not shown). The other three samples may have a yet to be identified virus inducing this viral disease signature.

Sampling location was significantly associated with the ordination gradient ($P < 0.01$). Fish sampled at the falls (earlier in migration during the active upstream movement phase) were closer in ordination space to osmoregulatory genes typically elevated during saltwater preparation (HBA and CA4). Fish sampled upstream of the falls (more advanced in

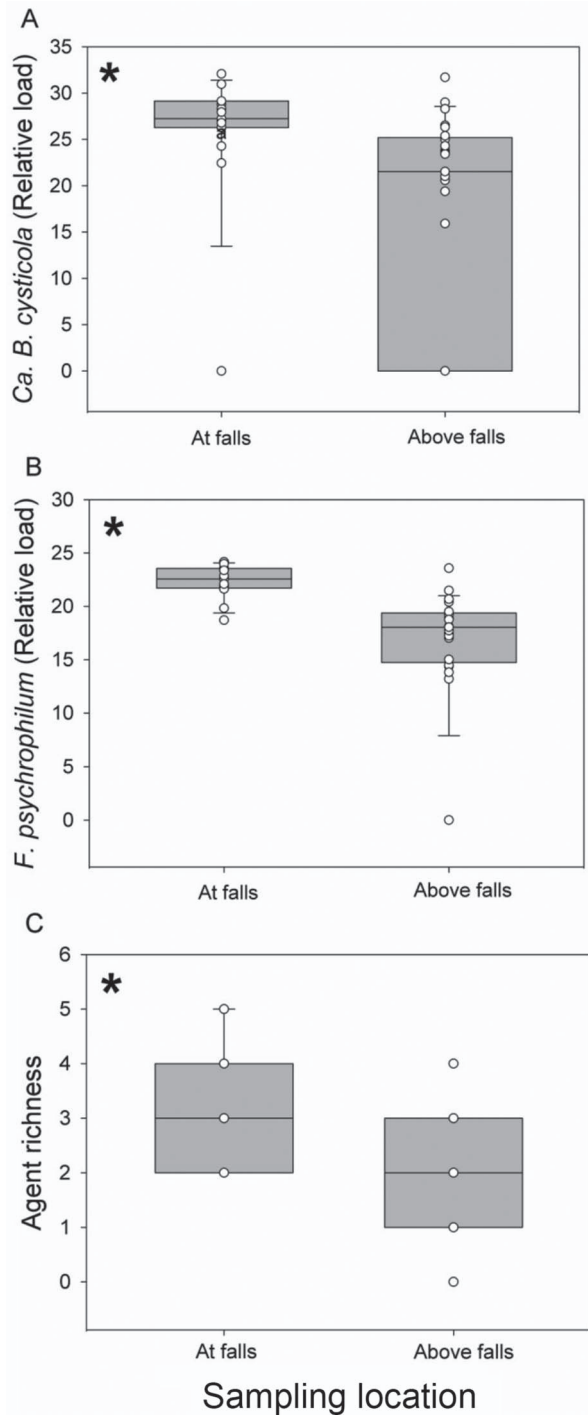


Figure 1: Boxplots depicting the relative load of (A) *Ca. B. cysticola* (B) *F. psychrophilum* and (C) agent richness in the gill tissue of steelhead sampled above the falls and at the falls. Asterisks denote a statistically significant difference ($P < 0.05$).

migration) were closer in ordination space to genes related to stress and immunity such as IL-17D, IL8 and MMP13, particularly humoral immune genes (IgMs and IgT).

Gene expression of fish captured by dip net at Witset Falls (earlier in migration)

Gene expression data were reduced to a 2D ordination (stress, 0.15) for the fish captured at Witset Falls (Fig. 3A). Relative load of *F. psychrophilum* was correlated with the ordination gradient ($R^2 = 0.52$; $P = 0.01$) being slightly positive on NMDS1 and negative on NMDS2. Relative load of *F. psychrophilum* was close in ordination space to SAA and opposite to IgT. Neither the relative load of *Ca. B. cysticola* ($R^2 = 0.30$; $P = 0.13$), infectious agent richness ($R^2 = 0.10$; $P = 0.51$), water temperature ($R^2 = 0.03$; $P = 0.82$), sex ($P = 0.37$) nor FL ($R^2 = 0.15$; $P = 0.51$) were correlated with the ordination gradient.

Gene expression of fish captured by angling upstream of Witset Falls (later in migration)

Gene expression data were reduced to a 2D ordination (stress, 0.16) for the fish captured above Witset Falls (Fig. 3B). Neither infectious agent load, *F. psychrophilum* ($R^2 = 0.09$; $P = 0.51$) or *Ca. B. cysticola* ($R^2 = 0.15$; $P = 0.32$), was correlated with the ordination gradient, nor was infectious agent richness ($R^2 = 0.04$; $P = 0.73$). Water temperature had a significant relationship with the ordination gradient ($R^2 = 0.27$; $P = 0.03$) and was close in ordination space to C3 and C7 and opposite to CA4. Neither sex ($P = 0.73$) nor FL ($R^2 = 0.05$; $P = 0.52$) was correlated with the ordination gradient.

Discussion

Wild steelhead captured by dip net below Witset Falls were found to have greater infectious agent richness, higher relative loads of *Ca. B. cysticola*, and *F. psychrophilum*, and differential gene expression compared to fish captured upstream of the falls by angling. We hypothesize that higher infectious burdens (in gill tissue) in fish sampled below the falls may have impacted the ability of fish to ascend the falls (i.e. migratory culling; Bradford et al., 2010; Altizer et al., 2011). Below, we discuss prominent differences between these groups of fish and how those differences may contribute to the observed findings. Fish sampled at Witset Falls were sampled earlier in the season and further downstream suggesting lower freshwater preparedness. This is consistent with the elevated expression of CA4 and HBA in this group of fish which are important in the earlier stages of freshwater acclimation (Houde et al., in review) and higher expression of humoral immune genes (IgMs and IgT) and B2M, MHCIIb and IRF1 that tend to increase over the course of migration in sockeye salmon (Teffer et al., 2017). Radio telemetry work has indicated that steelhead upstream of Witset Falls represent successful migrants that are holding at or near spawning sites, while fish at Witset Falls are in the active stage of upstream migration (Twardek et al., 2019; Økland et al., 2001).

Table 2: Multiple regression output predicting the relative load of *Ca. B. cysticola* and the relative load of *F. psychrophilum* in the gill tissue of steelhead captured in the Bulkley River, BC.

Variable	Relative load: <i>Ca. B. cysticola</i>			Relative load: <i>F. psychrophilum</i>		
	Estimate ± SE	t-value	P	Estimate ± SE	t-value	P
(Intercept)	32.427 ± 6.124	5.295	<0.001	14.531 ± 3.431	4.235	<0.001
Fishery/location: at falls**	4.795 ± 1.676	2.861	0.008	4.066 ± 0.929	4.372	<0.001
Water temperature	-0.472 ± 0.399	-1.182	0.248	0.016 ± 0.224	0.071	0.943
Sex: male	0.053 ± 1.394	0.038	0.970	1.456 ± 0.786	1.852	0.073
FL	-0.008 ± 0.008	-1.001	0.326	0.004 ± 0.004	1.031	0.310

The model includes fisheries/sampling location and sex as categorical variables and water temperature (°C) and FL (mm) as continuous variables. Asterisks denote a statistically significant difference ($P < 0.05$)

Table 3: Poisson regression output predicting infectious agent richness in the gill tissue of steelhead captured in the Bulkley River, BC.

Variable	Agent richness		
	χ^2	DF	P
Fishery/location**	11.791	1	<0.001
Water temperature	1.739	1	0.187
Sex	2.511	1	0.113
FL	0.005	1	0.944

The model includes fishery/sampling location and sex as categorical variables and water temperature (°C) and FL (mm) as continuous variables. Asterisks denote a statistically significant difference ($P < 0.05$)

Sampling location—fisheries selectivity

Witset Falls may act as a natural barrier influencing infection dynamics during steelhead migration, and successful migrants (angled fish) may be biased towards fish with lower infectious agent loads relative to actively migrating fish at Witset Falls (i.e. migratory culling; Altizer et al., 2011; Risely et al., 2018). Witset Falls constitutes the most difficult hydraulic reach of the Bulkley River. Steelhead are known to delay their migration at this point, resulting in large densities of steelhead below the falls, and ~8–12% of steelhead that enter the canyon/falls area do not successfully migrate upstream of the falls (Welch et al., 2009; Twardek et al., 2019). Confinement of steelhead below this migratory barrier may increase exposure to infected individuals and correspondingly increase infectious agent prevalence and loads at this location (Bebak-Williams et al., 2002). Direct fish to fish transfer of infections has been observed for *Ca. B. cysticola* in presmolt Atlantic salmon, *Salmo salar* (Linnaeus), suggesting transfer between steelhead may also occur during confinement (Wiik-Nielsen et al., 2017). Increased agent loads (including *Ca. B. cysticola* and *F. psychrophilum*) can entail reduced swimming capabilities, lethargy, negative effects on movement

and decreased survival (Schachte, 1983; Kent et al., 1989; Barber et al., 2000; Wagner et al., 2003; Risely et al., 2018), potentially explaining why fewer heavily burdened fish were observed upstream of the falls. Evidence to this effect comes both from the finding that *F. psychrophilum* was correlated with gene expression of fish at the falls but not those that successfully migrated above to holding sites, and the finding of three agents capable of causing gill disease primarily detected in fish at the falls. If these agents were negatively impacting the osmoregulatory or oxygen-carrying capacity of the host, or causing inflammation, we cannot discount the possibility that they may have impacted the ability of the fish to ascend the falls. A concurrent telemetry study evaluating steelhead migratory survival following angling suggested that infectious agent-attributed mortality during fall migration is low for steelhead captured upstream of Witset Falls (Twardek et al., 2018). This study found one mortality (1.5% of fish) that could not be readily explained by the capture event itself, which indicates that infectious agent-induced mortality is minimal for fish upstream of the falls (Twardek et al., 2018). The notion of migratory culling is partly supported by the described telemetry work (Twardek et al., 2018, 2019), but future telemetry studies should additionally include gill agent-transcriptome analyses such as performed herein.

As presented above, our data indicate that fish caught in the dip net fishery had higher infectious burdens and potentially greater pathological effects of *F. psychrophilum* than those caught upstream by angling. Previous research has highlighted that various fishing gears have sampling bias that may select for fish with phenotypes that make them more vulnerable to capture (Diaz Pauli et al., 2015; Philipp et al., 2015). The Wet’suwet’en dip net fishery at the falls takes advantage of congregations of fish resting within small eddies as they attempt to ascend the falls. It is possible that fish with greater infectious agent burdens spend more time resting in these eddies and may be more vulnerable to capture by this fishery. Conversely, angling has been suggested to capture fish with higher metabolic rates (Hessenauer et al., 2015), lower metabolic stress (Louison et al., 2017) and higher activity levels (Alós et al., 2012), which would likely be correlated

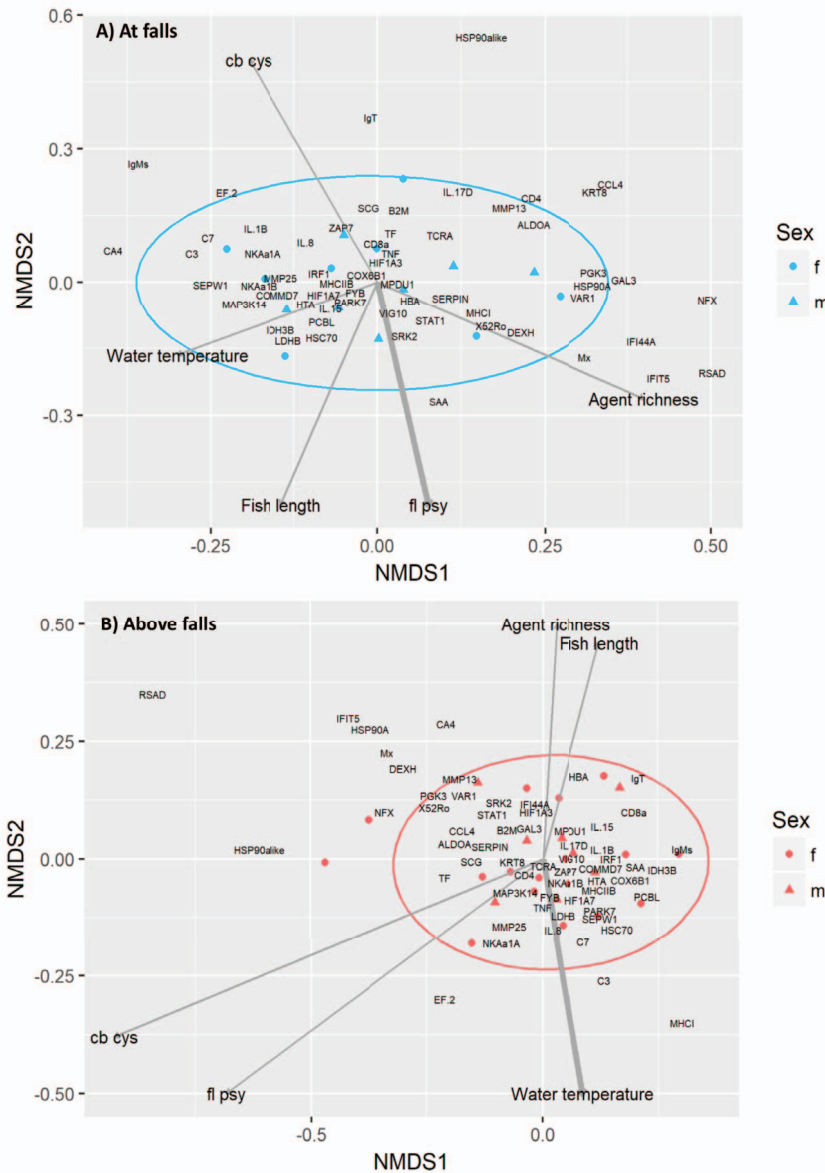


Figure 3: NMDS plots of host gene expression in the gill tissues of Bulkley River steelhead ($n = 40$) caught **(A)** at Witset Falls and **(B)** above Witset Falls. The shape of each point reflects sex (circle reflects female; triangle, male). Vectors represent external variables fit into the ordination including the relative loads of *Ca. B. cysticola* and *F. psychrophilum*, agent richness, water temperature and FL. Bolded lines indicate significant vectors. Confidence ellipses (95%) are shown in blue and red. In plot (A) the most influential variables on NMDS1 are RSAD (5.0%), NFX (4.9%) and IFIT5 (4.2%) and on NMDS2 are HSP90alike (8.6%), IgT (5.8%) and SAA (4.2%). In plot (B) the most influential variables on NMDS1 are RSAD (8.6%), HSP90alike (6.2%) and IFIT5 (4.3%) and on NMDS2 are RSAD (8.6%), MHCI (3.1%) and IFIT5 (4.3%).

downregulation of immune genes has also been observed in adult sockeye salmon, *Oncorhynchus nerka* (Teffer et al., 2017) and immune challenged wild rainbow trout responding to *Flavobacterium* spp. (TNE, IFN and CXCR8; Wellband and Heath, 2013) suggesting there may be a relationship between immunosuppression and *F. psychrophilum* (Barnes

and Brown, 2011). This association between host immunity and *F. psychrophilum* was only present for fish captured at Witset Falls, which suggests the agent was causing damage to these fish (i.e. disease; Casadevall and Pirofski, 1999). It could be that fish with heavy burdens of *F. psychrophilum* were less capable of surpassing the hydraulically challenging

swim through Witsset Falls related to this damage (Kent et al., 1989; Risely et al., 2018).

Sphaerothecum destruens and low prevalence infectious agents

Approximately half of the Bulkley River steelhead tested positively for *S. destruens*, a eukaryotic generalist parasite that can infect fish in both freshwater and marine ecosystems (Kent, 2011; Andreou and Gozlan, 2016). *Sphaerothecum destruens* has resulted in sublethal consequences for infected cyprinids including inflammation, lesions and cell death (Andreou et al., 2011). *Sphaerothecum destruens* was also associated with mortality in winter-run juvenile chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), from the Sacramento River, though juvenile rainbow trout seemed to be more resilient to infection (Arkush et al., 1998). Three other infectious agents known to cause gill disease and associated mortality were observed primarily in fish caught in the dip net fishery at the falls: *P. theridion*, *P. salmonis* and *L. salmonae* (Nowak and LaPatra, 2006; Nylund et al., 2010, 2011; Schmidt-Posthaus et al., 2012; Speare and Lovy, 2012). However, as these agents were not highly prevalent, we did not attempt to quantify their influence on host gene expression. Given previously reported consequences of these agents, future studies with larger sample sizes should consider the influence that they may have on salmonid host gene expression. Further research is also needed to understand the transmission requirements for these agents though it is known that *L. salmonae* may be transmitted in both marine and freshwater systems (Tucker et al., 2018).

Patterns in the expression of a suite of genes associated with viral disease response in salmon were identified in five individuals of this population and drove differentiation along NMDS1, depicting the strongest physiological driver in our data. This response is characterized by strong upregulation of RSAD, IFIT5, Mx, NFX, DEXH, VAR1, X52Ro and IFI44A (Miller et al., 2017). However, the initial infectious agent panel used in this study did not indicate a viral infectious agent was present. Additional screening of these individuals with a suite of novel viruses recently identified in BC salmon (Mordecai, unpublished data) indicated that a novel strain of CTV-2 was present in some but not all of these fish. We speculate that other as yet unidentified viruses may be present in the remaining fish. This is the first data showing that the novel CTV-2 strain is present in steelhead, though other strains of this virus are known to be widely distributed throughout trout populations in the United States (Batts et al., 2011). While we do not yet know if the CTV-2 strain is pathogenic, the previously characterized strain is not considered to be highly pathogenic, with no observed mortality in rainbow trout, cutthroat trout (*Oncorhynchus clarkia*, Richardson) and kokanee salmon (*O. nerka*) following waterborne exposure to the virus (Hedrick et al., 1991). Given the individuals with CTV-2 detection show molecular evidence indicative of a viral disease state, further examination of

this viral strain for pathogenic potential is warranted in steelhead.

Temperature and host physiology

Despite cool water temperatures well below the upper temperature tolerance for *O. mykiss*, temperature had an influence on gene expression of fish upstream of Witsset Falls (Yada and Tort, 2016). The most positive associations were with genes related to the complement system (C3 and C7), metabolic stress (LDHB), and oxidative stress (SEPW1). Although water temperature was not significantly correlated with gene expression in fish sampled earlier in the migration at Witsset Falls, these genes were also in similar ordination space as water temperature in this group of fish. Expression of genes in the complement system indicates that a component of the innate immune system is increasing with temperature (Bowden, 2008), though many other innate immunity biomarkers had neutral or negative relationships with temperature. LDHB was also greater at warmer temperatures which is consistent with the observation that lactic acidosis in white muscle and blood generally increases with warmer temperatures in rainbow trout and steelhead (Kieffer et al., 1994; Twardek et al., 2018). Despite evidence of higher levels of metabolic products with increasing water temperature, glycolytic genes were generally not upregulated with temperature. This relationship has been observed in wild rainbow trout in similar temperature conditions as the Bulkley River, with increased resting state expression of glycolytic genes (PK and PEP3K) with temperature (Wellband and Heath, 2013). Water temperature also showed a positive association with SEPW1, which functions as an antioxidant (Whanger, 2009), and is a known temperature responsive gene (Akbarzadeh et al., 2018). SEPW1 was also elevated in adult sockeye salmon held at water temperatures >19°C compared to 13–14°C (Jeffries et al., 2014b). Water temperature was not correlated with other well-characterized thermal-responsive genes (Akbarzadeh et al., 2018) such as those encoding heat shock proteins (e.g. HSP90a and SERPINH1). Previous work evaluating the influence of thermal stress on the transcriptome of sockeye and pink salmon, *Oncorhynchus gorbuscha* (Walbaum), indicated upregulation of heat shock proteins (HSP90AA1, HSPAB1 and SERPINH1) with warm temperatures (Miller et al., 2009; Evans et al., 2011; Jeffries et al., 2014b). Our data suggest that the relatively low water temperatures observed on the Bulkley River were likely not altering protein conformation or protein aggregation in steelhead to a degree that would necessitate changes in the expression of this family of chaperone proteins.

Conclusions

Positive detections of eight infectious agents of bacterial, viral, fungal, myxozoan and other eukaryotic origin were found in steelhead from the Bulkley River. The majority of fish sampled were infected with *Ca. B. cysticola*, *F.*

psychrophilum and *S. destruens*. Despite a small sample size, interesting differences were observed across sampling sites at and above Witsset Falls. Infectious loads of most identified infectious agents were greater in fish at vs. above the falls including three agents capable of causing gill disease (*L. salmonae*, *P. theridion* and *P. salmonis*). The relative load of *F. psychrophilum* was associated with the physiology of fish only at the falls, suggesting this agent may impact the migration of success of fish at this barrier. Fish upstream of the falls were further along in their migration, and observed differences in their physiology were consistent with later-stage migration. Our findings provide insight into the relationship between physiology and infectious agents and potential links to migratory outcomes in wild Steelhead. If salmonids with the highest infectious agent burdens are unable to reach spawning sites and successfully reproduce, then there may be broad scale consequences to recruitment across salmon populations. We encourage future research to evaluate the migratory culling hypothesis for long-distance salmonid migrations by combining tools in molecular genetics and biotelemetry (see Miller et al., 2011, as example). Future work should also seek to validate disease susceptibility and impacts of the agents identified in steelhead using histopathology.

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Supplementary material

Supplementary material is available at *Conservation Physiology* online.

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