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Serial sampling reveals temperature associated response in transcription profiles and shifts in condition and infectious agent communities in wild Atlantic salmon

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Abstract

1. Anadromous Atlantic salmon (*Salmo salar*) populations have declined across their Southern distributions in North America. While river temperature has been identified as a central factor influencing migration behavior and over-winter survival, little research has addressed the prevalence of infectious agents in wild Atlantic salmon populations. Further, current understanding of how temperature may interact with fish condition to influence infection outcomes in the wild is limited.
2. The objective of this research was to characterize the change in individual condition and pathogen dynamics as salmon acclimatized to the freshwater environment during the spawning migration. Serial individual sampling (non-lethal gill biopsy and microwave fatmeter readings) was accomplished by repeated capture and release of 27 marked individuals, which revealed how lipid content, infectious agent prevalence and relative loads, and stress and osmoregulatory transcripts changed during the initial month of their annual spawning migration.
3. Relative infection burden (a composite metric representing the overall pathogen profile) and transcription profiles were modeled with freshwater residency, river temperature, and lipid content. Infectious agents *Tetracapsuloides bryosalmonae*, *Candidatus* Branchiomonas *cysticola*, *Flavobacterium psychrophilum*, *Paranucleospora theridion*, and *Piscichylamidia salmonis* were detected in the population. Relative infection burden and microbial pathogen species richness increased over the course of the study.
4. Water temperature and time since fresh water entry were related to salmon transcriptional response, but not relative infection burden, highlighting the metabolic cost associated with warming temperatures and dynamic nature of pathogen infection profiles in migratory fish species.
5. This work is the first to provide a comprehensive screening of microbial pathogen species in wild Atlantic salmon in the region, and the first to employ a unique study design that facilitates serial sampling without imposing holding stress. The high prevalence and relative load of *T. bryosalmonae* observed may demonstrate increased exposure to transmission pathways as a result of migratory barriers or

elevated susceptibility to infection during spawning migrations in anadromous species.

KEYWORDS
climate change, disease ecology, fisheries, gene expression, migration

1 | INTRODUCTION

Infectious agents are ubiquitous across ecosystems and species, yet very little is known about their natural distributions and prevalence in wild populations (Walton et al., 2016). This is particularly true for aquatic organisms because outbreaks and disease related mortality events are not easily observable. Typically, it is not until major mortality events occur that infectious agents are studied (e.g. whirling disease *Myxobolus cerebralis;* Nehring and Walker 1996). Migratory species in particular present a major challenge for understanding disease dynamics because of their typically large distributions, complex life-histories, and use of multiple environments (Altizer et al., 2011). As a result, there is comparatively little known about infectious disease ecology in populations of migratory animals (McVicar, 1997; Miller et al., 2014).

Atlantic salmon (*Salmo salar*) populations have experienced a marked decline throughout the majority of its range over several decades, and conservation efforts limiting harvest have had little impact (Forseth et al., 2017; ICES, 2020; Parrish et al., 1998). Despite numerous hatchery programs, moratoriums on fishing, and habitat restoration, populations in Atlantic Canada, including those that were previously considered stable, have experienced unprecedented decline (ICES, 2020). While clear links between temperature and marine survival have been identified in Pacific salmon (Siegel & Crozier, 2019), factors that mediate Atlantic salmon survival remain poorly understood (Russell et al., 2012; Soto et al., 2018). Recent work suggests environmental conditions associated with the freshwater phases of Atlantic salmon life-history play a much larger role in marine survival than previously thought (Gregory et al., 2019). For anadromous species already facing demanding physiological changes related to changes in osmoregulation, the metabolic cost imposed by warming water temperatures may induce changes in energy allocation. At the same time, environmental stressors, including increased temperature, have been demonstrated to negatively impact fish immune function and increase infectious disease outbreaks in fishes (Adlard et al., 2015; Snieszko, 1974). Increased temperature can also influence the proliferation and development of infectious agents through both direct (e.g. shorter generation times) and indirect (e.g. changes in vector abundance) pathways (Adlard et al., 2015), further complicating potential outcomes of increased water temperatures. The effects of infectious agents are now being considered as a potential factor influencing population declines, however the majority of this research addresses transfer of specific infectious agents from aquaculture facilities to wild populations (reviewed in Lafferty et al., 2015; Olivier, 2002) rather than investigating infectious agent prevalence and loads in wild populations. As a result, researchers are not able to make comparisons over time or predict how changes to environmental conditions and species distributions may impact infectious agent infection dynamics and consequent wild fish survival.

While experimental studies address fine-scale responses of host/pathogen systems to increasing temperatures (e.g. Strepparava et al., 2018), in situ studies are required to understand how multiple factors interact to influence infectious agent communities and host condition in wild fish. A protocol for assessing the presence and relative load of infectious agents has recently been developed using high-throughput molecular nanofluidic qPCR (Miller et al., 2016). This platform can assess host gene expression in numerous biomarkers simultaneously to characterize transcription profiles, as well as the presence and productivity of infectious agents as determined by RT-qPCR amplification (Miller et al., 2014). This protocol has been used to better understand infectious agent community dynamics within and between populations (Bass et al., 2017), investigate shifts in genetic profiles associated with shifts in salinity and smoltification (Houde, Schulze, et al., 2019) and temperature (Jeffries et al., 2012; Teffer et al., 2019), and characterize genetic profiles indicating the early stages of disease development and viral infection in salmonids (Miller et al., 2017). This screening technique requires only a small amount of tissue, facilitating non-lethal and repeated sampling in wild salmon and allowing the investigation of natural changes to infectious agent communities and host response in the face of environmental and anthropogenic pressures (Bass et al., 2017; Jeffries et al., 2012; Miller et al., 2014).

This study sought to characterize the progression of adult Atlantic salmon condition and infectious agent prevalence and relative loads during up-river migration. Using a Fisheries and Oceans Canada counting fence as a barrier to migration, Atlantic salmon were intercepted and returned to the downstream side of the fence to create a naturalized in-river holding area. Study fish underwent repeated non-lethal sampling to quantify the development of infectious agent prevalence across time along with the relative load, host transcriptional profiles, and fat content for a period of 32 days before fish were passed upstream to continue migration to spawning grounds. Our objectives were to (a) to determine infectious agents present in the population; (b) assess changes in infectious agent burden and salmon condition over time spent in freshwater; (c) assess relationships between in-river temperature and infectious agent burden; and (d) characterize the

relationship between infectious agent burden and host transcriptional profiles.

2 | METHODS

Twenty-seven anadromous Atlantic salmon were intercepted at a Fisheries and Oceans Canada (DFO) counting fence at the Campbellton River enumeration facility in Campbellton, Newfoundland, Canada during the up-river migration after ocean feeding between 16 June and 20 July 2017. The Campbellton River Atlantic salmon population is a small population (total count of 2,751 grilse and 226 large salmon in 2016; Fisheries and Oceans 2017) that is not augmented by hatchery stock. All salmon included in the study were maiden one sea winter fish, also termed grilse, which make up a vast majority of individuals in this population and are easily identifiable by size class (Downton et al., 2001). The fence is located approximately 150 m up-river from the estuary and beyond tidal influence as a result of the system's relatively high grade in the lower reach. The river below the counting fence is high flow and made up of large boulder riffles and several smaller pools, approximately 1.5 m depth at the deepest areas. On the first day of the study, fish were individually dip netted out of the counting trap, transferred to an aerated cooler, and walked approximately 100 m down river to a release site (Figure 1). Here, they were quickly sampled for 2 × 2 mm of gill tissue from the second gill arch on the left side of the fish. Gill tissue has been demonstrated to be transcriptionally responsive to both environmental stressors and infectious agents (Miller et al., 2014), and for use in non-lethal pathogen screening in salmonids (Teffer & Miller, 2019). Samples were immediately transferred to RNAlater® in 1.5 ml microtubes using sterilized tools. An anterior and posterior muscle lipid measurement was taken with a Distell microwave Fatmeter Sensor (Fish Fatmeter Model-FM 692, Distell) as per manufacturer's instructions, and fish were tagged with unique identification codes using highly visible white Floy® spaghetti tags for clear identification of tagged fish. The counting fence trap is video monitored and includes a live-stream to a cabin adjacent to the fence. The gate to the trap is connected to a switch in the cabin that remotely closes the fence, or it can be shut manually at the fence. Consequently, we were able to monitor the trap for tagged fish and immediately process individuals observed to enter the trap. The sampling process was repeated each time a tagged fish entered the counting fence trap during the study period (32 days). Microtubes containing gill samples were transferred to 4°C for 24 hr and then frozen at −20°C prior to transfer to −80°C and shipped to the DFO Molecular Genetics Laboratory in Nanaimo, BC on dry ice for analysis.

The microwave fatmeter was used to take a measurement along the anterior and posterior of the fish's body (Crossin & Hinch, 2005) and the measurements were subsequently averaged to yield a single fat content estimate at each sampling event. Water temperature was obtained from a monitoring station located at the counting fence approximately 0.5 m below the water surface at the time the fish was sampled. All fish handling was conducted under Animal Care Protocol 101057 issued to Carleton University, and Fisheries and Oceans Canada Scientific Collection Permit NL-3235-16.

2.1 | RT-qPCR to quantify infectious agent loads and salmon biomarker expression

Gene expression and infectious agent prevalence and relative loads were examined in gill tissue collected from study salmon using high-throughput qPCR on the Fluidigm BioMark Dynamic Array™ Gene Expression platform. All assays were run on isolated RNA after extraction, purification, normalization, and amplification. TaqMan™ assays used in screening were designed to target microbe RNA to allow identification of RNA viruses and microparasites in active states. Consequently, qPCR quantification represents infectious agent productivity rather than absolute quantification, described hereafter as relative load. Details on sensitivity and specificity of each assay, sequence sources, and repeatability of the following RNA extraction, normalization, targeted amplification, and final HT-RT-qPCR using this platform are outlined in the Canadian Science Advisory Secretariat validation of disease screening in salmonids (Miller et al., 2016). To determine what infectious agents were present

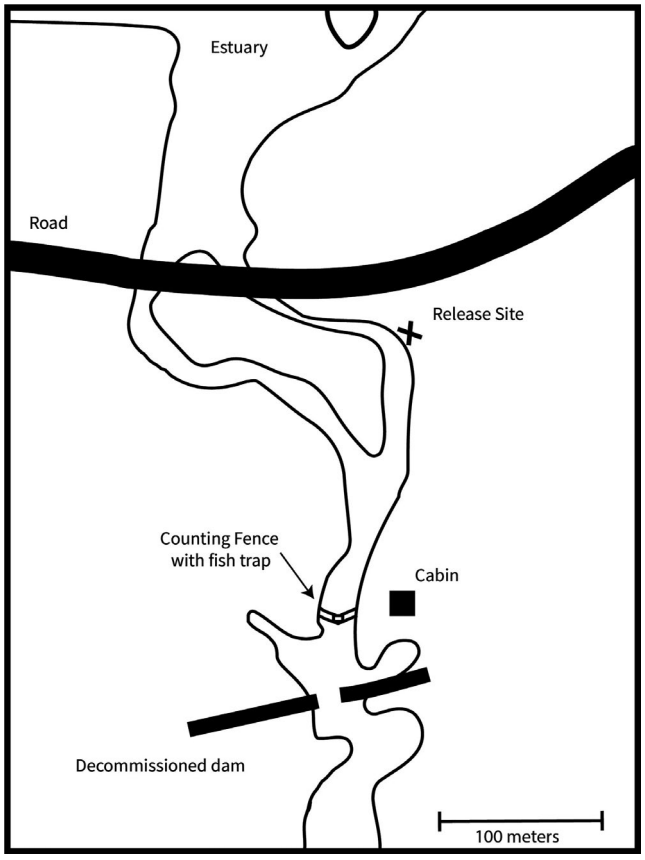

FIGURE 1 Schematic of study site at the Campbellton River, Newfoundland

within the sample population, a subset of amplified product from all samples was pooled and screened for the presence of 46 infectious agents found in both marine and freshwater environments (Table 1). Based on this initial screen, all positively detected agents were included in the final assay panel, and selected host biomarkers were added (Table 2). Infectious agent assays were run in duplicate against each sample, while biomarkers were run as singletons. Assays with efficacies <80% were considered failed and removed from analysis. All samples were processed as per methods outlined in Miller et al. (2016) and Teffer et al. (2017).

In brief, RNA extraction was completed using mechanical abrasion in a MM301 mixer mill (Restch Inc.) and Tri-reagent™ followed by addition of 1-bromo-3-chloropropane and purified using Magmax™-96 Microarrays Kits on a Biomek FXP automated liquid handler. Purified RNA quantity and quality was assessed using a Beckman Coulter DTX 880 Multimode Detector and sample RNA concentrations were normalized to 62.5 ng/μL. RNA was then converted to cDNA using SuperScript® VILO™ DNA synthesis kit (Life Technologies) following manufacturer's instructions. Specific amplification of target transcripts was performed using primer pairs corresponding to all assays using 1x TaqMan Pre-amp MasterMix as per manufacturer's instructions (Applied Biosystems). Unincorporated primers were removed using Exo-SAP-IT™ (Affymetrix) and sample was diluted 1:5 with DNA suspension buffer. The effect of this pre-amplification step on final quantification has been investigated thoroughly and was not found to influence the interpretation of results (for extensive detail see Miller et al., 2016). The resulting sample material and assays were loaded directly on to Fluidigm 96.96 Dynamic Array™ integrated fluidic circuit chip for qPCR. 5:1 serial dilution of pooled sample was used to track biomarker efficiency, while combined serial dilutions of artificial construct controls for infectious agent sequences with known copy number were added to the Dynamic Array last and used to track efficiency of each assay on each run. Artificial construct controls contained an extra probe to track potential contamination (see Miller et al., 2016). A series of negative processing controls for RNA extraction, cDNA synthesis, and pre-amplification were also included, as well as a pooled positive control sample of all samples used in the study.

Cycle threshold (C_t) is reported for each biomarker assay using relative expression in the form of $2^{-\Delta\Delta C_t}$ using the averaged expression of three housekeeping genes and the C_t value of a pooled control sample (Livak & Schmittgen, 2001). Infective agents are presented as relative load by subtracting the observed C_t value from the total PCR cycles for each qPCR run (i.e. 40-Ct). Infective agents were only considered detected if their C_t was above the assay-specific limit of detection (LOD; 95% level of confidence) as described in Miller et al. (2016), and detected in both duplicate samples. If an infective agent was known to be in the sample population based on detections above the LOD and detected within an individual in subsequent samples, visual assessment of amplification curves was used to confirm positive detections below the LOD.

An index of relative infection burden (RIB) was used to summarize the infective agent load and diversity of agents detected within each individual for each sample. Using this index, both high loads and common and rare pathogens contribute to the burden as follows:

$$\mathrm{RIB} = \sum_{i \in m}^{m} \frac{L_i}{Lmax_i},$$

where the relative load of i_{th} infectious agent L_i is divided by the maximum load for that agent observed within the population (i.e., $L\ \max_i$), and then summed across all agents found in the sample.

2.2 | Statistical analysis

Each fish was sampled at any point it was captured in the counting fence during the 32 day study period. Consequently, samples range from one to six per individual. To visualize changes in infectious agent relative loads and fat content over time, line plots for each individual by study day were constructed. For all subsequent analyses, the study was broken into three periods to facilitate data reduction techniques without pseudoreplication. Period 1: tagging; period 2: 8–20 days from release; and period 3: 21–32 days from release. In cases where more than one sample from an individual fell within the same period ($n = 11$), only the last sample within a period was included in analyses. Not all fish were sampled in each study period.

R statistical software version 3.6.1 was used for all data analysis and visualizations. Data were analyzed by constructing statistical models according to each specific research question. Changes in fat content and RIB across study periods was assessed independently by repeated measures ANOVA. Change in infectious agent species richness was tested by chi-squared test of independence. To investigate whether temporal variation and fish condition was related to RIB, a generalized mixed effect model was constructed with RIB as the response and fat content, temperature (log transformed), study period, and their interaction as fixed effects, and fish id as a random effect. Nonmetric multidimensional scaling with Bray-Curtis dissimilarity distances with 95% confidence ellipse centered on study period was used to visualize patterns in expression of 49 immune, stress, and osmoregulatory associated genes across the three time periods. Significance of separation of transcription profile was investigated using Analysis of Similarity (ANOSIM), and the relationship of RIB, temperature, and fat content was run to visually assess global trends in gene expression using the *Envfit* functions using the *vegan* package (version 2.5-6; Oksanen 2015). To model transcriptional responses to fish condition, infection dynamics, and temperature within each study period, permutational multivariate analysis of variance using based on Bray-Curtis dissimilarity matrices was used (*adonis* function in *vegan*). This function performs a permutated MANOVA to investigate the variation in matrices attributable to specified factors. Ordinations based on biomarker expression for all genes were

TABLE 1 Abbreviations, names, and groups of infectious agents detected in migratory adult Atlantic salmon collected in the Campbellton River system using high-throughput qPCR

Group	Infectious agent	Assay ID	Taqman primer sequences (5'–3') probe sequence (FAM-5'–3'-MGB)	P	E	Sequence source
Bacteria	*Aeromonas hydrophila*	ae_hyd	F—ACCGCTGCTCATTACTCTGATG / R—CCAACCCAGACGGGAAGAA P—TGATGGTGAGCTGGTTG	0.00	0.99	Lee et al. (2006)
	Aeromonas salmonicida	ae_sal	F—TAAAGCACTGTCTGTTACC / R—GCTACTTCACCCTGATTGG / P—ACATCAGCAGGCTTCAGAGTCACTG	0.00	0.98	Keeling et al. (2013)
	Candidatus Branchiomonas cysticola	c_b_cys	F—AATACATCGGAACGTGTCTAGTG / R—GCCATCAGCCGCTCATGTG / P—CTCGGTCCCAGGCTTTCCTCTCCCA	**0.96**	0.95	Mitchell et al. (2013)
	Candidatus Piscichlamydia salmonis	pch_sal	F—TCACCCCCAGGCTGCTT / R—GAATTCCATTTCCCCCTCTTG / P—CAAAACTGCTAGACTAGAGT	**0.07**	0.97	Nylund et al. (2008)
	Flavobacterium psychrophilum	fl_psy	F—GATCCTTATTCTCACAGTACCGTCA/R-TGTAAACTGCTTTTGCACAGGAA P—AAACACTCGGTCGTGACC	**0.74**	0.92	Duesund et al. (2010)
	Gill chlamydia	sch	F—GGGTAGCCCGATATCTTCAAAGT / R—CCCATGAGCCGCTCTCTCT / P—TCCTTCGGGACCTTAC	0.00	0.97	Duesund et al. (2010)
	Piscirickettsia salmonis	pisck_sal	F—TCTGGGAAGTGTGGCGATAGA / R—TCCCGACCTACTCTTGTTTCATC / P—TGATAGCCCCGTACACGAAACGGCATA	0.00	0.99	Corbeil et al. (2003)
	Renibacterium salmoninarum	re_sal	F—CAACAGGGTGGTTATTCTGCTTTC/ R-CTATAAGAGCCACCAGCTGCAA / P—CTCCAGCGCCGCAGGAGGAC	0.00	0.99	Powell et al. (2005)
	Rickettsia-like organism	rlo	F—GGCTCAACCCAAGAACTGCTT / R—GTGCAACAGCGTCAGTGACT / P—CCCAGATAACCGCCTTCGCCTCCG	0.00	1.00	Lloyd et al. (2011)
	Vibrio anguillarum	vi_ang	F-CCGTCATGCTATCTAGAGATGTATTTGA/R-CCATACGCAGCCAAAAATCA/ P—TCATTTCGACGAGCGTCTTGTTCAGC	0.00	0.98	In house
	Vibrio salmonicida	vi_sal	F—GTGTGATGACCGTTCCATATTT / R—GCTATTGTCATCACTCTGTTTCTT / P—TCGCTTCATGTTGTGTAATTAGGAGCGA	0.00	0.95	In house
Platyhelminth	*Gyrodactylus salaris*	gy_sal	F—CGATCGTCACTCGGAATCG / R—GGTGGCGCACCTATTCTACA / P—TCTTATTAACCAGTTCTGC	0.00	0.98	Collins et al. (2010)
	Nanophyetus salmincola	na_sal	F–CGATCTGCATTTGGTTCTGTAACA/RCCAACGCCACAATGATAGCTATAC / P—TGAGGCGTGTTTTATG	0.00	1.15	In house
Protozoa	*Ceratonova shasta*	ce_sha	F—CCAGCTTGAGATTAGCTCGGTAA / R—CCCCGGAACCCGAAAG / P—CGAGCCAAGTTGGTCTCTCCGTGAAAAC	0.00	1.02	Hallett and Bartholomew (2006)
	Cryptobia salmositica	cr_sal	F—TCAGTGCCTTTCAGGACATC / R—GAGGCATCCACTCCAATAGAC / P—AGGAGGACATGGCAGCCTTTGTAT	0.00	1.00	In house
	Dermocystidium salmonis	de_sal	F—CAGCCAATCCTTTCGCTTCT / R—GACGGACGCACACCACAGT / P—AAGCGGCGTGTGCC	0.00	0.97	In house
	Facilispora margolisi	fa_mar	F—AGGAAGGAGCACGCAAGAAC / R—CGCGTGCAGCCCAGTAC / P—TCAGTGATGCCCTCAGA	0.00	1.12	In house
	Ichthyophonus hoferi	ic_hof	F—GTCTGTACTGGTACGGCAGTTTC/ R-TCCCGAACTCAGTAGACACTCAA / P—TAAGAGCACCCACTGCCTTCGAGAAGA	0.00	0.98	White et al. (2013)
	Ichthyophthirius multifiliis	ic_mul	F—AAATGGGCATACGTTTGCAAA/R-AACCTGCCTGAAACACTCTAATTTTT / P—ACTCGGCCTTCACTGGTTCGACTTGG	0.00	0.97	In house
	Kudoa thyrsites	ku_thy	F—TGGCGGCCAAATCTAGGTT / R—GACCGCACACAAGAAGTTAATCC / P—TATCGCGAGAGCCGC	0.00	0.88	Funk et al. (2007)
	Loma salmonae	lo_sal	F—GGAGTCGCAGCGAAGATAGC/ R-CTTTTCCTCCCTTTACTCATATGCTT / P—TGCCTGAAATCACGAGAGTGAGACTACCC	0.00	1.05	In house

(Continues)

TABLE 1 (Continued)

Group	Infectious agent	Assay ID	Taqman primer sequences (5'–3') probe sequence (FAM-5'–3'-MGB)	P	E	Sequence source
	Myxobolus arcticus	my_arc	F—TGGTAGATACTGAATATCCGGGTTT / R—AACTGCGCGGTCAAAGTTG / P—CGTTGATTGTGAGGTTGG	0.00	0.92	In house
	Myxobolus cerebralis	my_cer	F—GCCATTGAATTTGACTTTGGATTA/ R-ACCATTCATGTAAGCCCGAACT / P—TCGAAGCCTTGACCATCTTTTGGCC	0.00	0.97	Kelley et al. (2004)
	Myxobolus insidiosus	my_ins	F—CCAATTTGGGAGCGTCAAA / R—CGATCGGCAAAGTTATCTAGATTCA / P—CTCTCAAGGCATTTAT	0.00	1.00	In house
	Neoparamoeba perurans	ne_per	F—GTTCTTTCGGGAGCTGGGAG / R—GAACTATCGCCGGCACAAAAG / P—CAATGCCATTCTTTTCGGA	0.00	1.07	Fringuelli et al. (2012)
	Nucleospora salmonis	nu_sal	F—GCCGCAGATCATTACTAAAAACCT / R—CGATCGCCGCATCTAAACA / P—CCCCGCGCATCCAGAAATACGC	0.00	1.06	Foltz et al. (2009)
	Paranucleospora theridion	pa_ther	F—CGGACAGGGAGCATGGTATAG / R—GGTCCAGGTTGGGTCTTGAG / P—TTGGCGAAGAATGAAA	**0.13**	1.16	Nylund et al. (2009)
	Parvicapsula kabatai	pa_kab	F—GTCGGATGATAAGTGCATCTGATT / R—ACACCACAACTCTGCCTTCCA / P—TGCGACCATCTGCACGGTACTGC	0.00	0.98	In house
	Parvicapsula minibicornis	pa_min	F-AATAGTTGTTTGTCGTGCACTCTGT/R-CCGATAGGCTATCCAGTACCTAGT/ P—TGTCCACCTAGTAAGGC	0.00	0.92	Hallett and Bartholomew (2009)
	Parvicapsula pseudobranchicola	pa_pse	F—CAGCTCCAGTAGTGTATTTCA / R—TTGAGCACTCTGCTTTATTCAA / P—CGTATTGCTGTCTTTGACATGCAGT	0.00	0.84	Jørgensen et al. (2011)
	Sphaerothecum destruens	sp_des	F—GCCGCGAGGTGTTTGC / R—CTCGACGCACACTCAATTAAGC / P—CGAGGGTATCCTTCCTCTCGAAATTGGC	0.00	1.00	In house
	Spironucleus salmonicida	sp_sal	F-AACCGGTTATTCGTGGGAAAG/R-TTAACTGCAGCAACACAATAGAATACT P—TGCCAGCAGCCGCGGTAATTC	0.00	0.95	In house
	Tetracapsuloides bryosalmonae	te_bry	F—GCGAGATTTGTTGCATTTAAAAAG / R-GCACATGCAGTGTCCAATCG / P—CAAAATTGTGGAACCGTCCGACTACGA	**0.55**	0.96	Bettge et al. (2009)
Virus	Atlantic salmon paramyxovirus	aspv	F-CCCATATTAGCAAATGAGCTCTATR-CGTTAAGGAACTCATCATTGAGCTT/ P—AGCCCTTTTGTTCTGC	0.00	0.97	Nylund et al. s taken from spawning (2008)
	Infectious haematopoietic necrosis virus	ihnv	F—AGAGCCAAGGCACTGTGCG / R—TTCTTTGCGGCTTGGTTGA / P—TGAGACTGAGCGGGACA	0.00	0.98	Purcell et al. (2013)
	Pacific salmon parvovirus	pspv	F—CCCTCAGGCTCCGATTTTTAT / R—CGAAGACAACATGGAGGTGACA / P—CAATTGGAGGCAACTGTA	0.00	0.96	In house
	Piscine myocardial virus	pmcv	F—TTCCAAACAATTCGAGAAGCG / R—ACCTGCCATTTTCCCCTCTT / P—CCGGGTAAAGTATTTGCGTC	0.00	0.96	Løvoll et al. (2010)
	Piscine Orthoreovirus	prv	F—TGCTAACACTCCAGGAGTCATTG / R—TGAATCCGCTGCAGATGAGTA / P—CGCCGGTAGCTCT	0.00	0.92	Wiik-Nielsen et al. (2012)
	Salmonid herpesvirus	omv	F—GCCTGGACCACAATCTCAATG / R—CGAGACAGTGTGGCAAGACAAC / P—CCAACAGGATGGTCATTA	0.00	1.11	In house
	Viral encephalopathy and retinopathy virus	ver	F—TTCCAGCGATACGCTGTTGA / R—CACCGCCCGTGTTTGC / P—AAATTCAGCCAATGTGCCCC	0.00	1.12	Korsnes et al. (2005)
	Viral erythrocytic necrosis virus	ven	F—CGTAGGGCCCCAATAGTTTCT /R—GGAGGAAATGCAGACAAGATTTG / P—TCTTGCCGTTATTTCCAGCACCCG	0.00	1.10	James Winton, pers. comm.
	Viral haemorrhagic septicaemia virus	vhsv	F—ATGAGGCAGGTGTCGGAGG / R—TGTAGTAGGACTCTCCCAGCATCC / P—TACGCCATCATGATGAGT	0.00	1.05	Garver et al. (2011)

Abbreviations: P, prevalence; E, assay efficiency

TABLE 2 Assay name and sequences for biomarkers of immune function and stress

Functional group	Assay name	Gene name	Primer and probe sequences	Source	E
Adaptive immunity	B2M	Beta 2-microglobulin	F—TTTACAGCGCGGTGGAGTC /R—TGCCAGGGTTACGGCTGTAC P—AAAGAATCTCCCCCCAAGGTGCAGG	Miller et al. (2016)	1.12
	CD8a	T-cell surface glycoprotein CD8 alpha chain	F—ACACCAATGACCACAACCATAGAG/R-GGGTCCACCTTTCCCACTTT P—ACCAGCTCTACAACTGCCAAGTCGTGC	Raida et al. (2008)	1.03
	IgMs	Immunoglobulin	F—CTTGGCTTGTTGACGATGAG /R—GGCTAGTGGTGTTGAATTGG P—TGGAGAGAACGAGCAGTTCAGCA	Raida et al. (2008)	1.05
	IgT	Immunoglobulin tau	F-CAACACTGACTGGAACAACAAGGT/R-CGTCAGCGGTTCTGTTTTGGA P—AGTACAGCTGTGTGGTGCA	Tadiso et al. (2011)	1.07
	MHCI	Major histocompatibility complex I	F—GCGACAGGTTTCTACCCCAGT /R—TGTCAGGTGGGAGCTTTTCTG P—TGGTGTCCTGGCAGAAAGACGG	Ingerslev et al. (2009)	1.06
	MHCII-B	Major histocompatibility complex IIβ	F—TGCCATGCTGATGTGCAG /R—GTCCCTCAGCCAGGTCACT P—CGCCTATGACTTCTACCCCAAACAAAT	Raida and Buchmann (2008)	1.00
	RSAD	Radical S-adenosyl methionine Domain-containing protein 2	F—GGGAAATTAGTCCAATACTGCAAAC/R–GCCATTGCTGACAATACT GACACT / P—CGACCTCCAGCTCC	Miller et al. (2016)	0.95
	TCRa	T cell receptor alpha chain	F—ACAGCTTGCCTGGCTACAGA/R—TGTCCCCTTTCACTCTGGTG P—CAGCGCACACAAGGCTAATTCG	Miller et al. (2016)	1.02
	ZAP70	Tyrosine-protein kinase (ZAP-70)	F—TCACCTCCGGACCTTTCATT/R—CCATGTGGGAAGCCTTTTCTT P—TCTTGTATGGTTTTCCTCC	Miller et al. (2016)	0.91
Innate immunity	C1Qc	complement C1q subcomponent subunit C	F—CGCCGGTGAGTGGAATCTA/R—CTTCTCCATCATGTGGTGTGCTA P—ACCTCCAAACATAGAAGAG	Miller et al. (2016)	0.94
	C3	Complement component 3	F—ATTGGCCTGTCCAAAACACA/R-AGCTTCAGATCAAGGAAGAAGTTC P—TGGAATCTGTGTGTCTGAACCCC	Raida and Buchmann (2009)	1.05
	C7	Complement factor	F—ACCTCTGTCCAGCTCTGTGTC /R—GATGCTGACCACATCAAACTGC P—AACTACCAGACAGTGCTG	Miller et al. (2016)	0.90
	CCL4	CC chemokine 4	F— TCTCTTCATTGCAACAATCTGCTT/R-ACAGCAGTCCACGGGTACCT P—CTACGCAGCAGCATT	Miller et al. (2016)	0.93
	GAL3	Galectin-3-binding protein precursor	F—TTGTAGCGCCTGTTGTAATCATAT/R-TACACTGCTGAGGCCATGGA P—CTTGGCGTGGTGGC	Miller et al. (2016)	1.12
	HERC6	Probable E3 ubiquitin-protein ligase	F-AGGGACAACTTGGTAGACAGAAGAA/R–TGACGCACACACAGCTA CAG / P—CAGTGGTCTCTGTGGCT	Miller et al. (2016)	0.96
	IL-15	Interleukin 15	F—TTGGATTTTGCCCTAACTGC /R-CTGCGCTCCAATAAACGAAT P—CGAACAACGCTGATGACAGGTTTTT	Raida et al. (2008)	1.14
	IL-17D	interleukin 17-delta	F— CAACAGAAGTGCGAACGATG /R—GATGCCACATCGCATAACAG P—TGGTCGAGTATCTTTCGTGTGTTTGC		1.04
	IL-1B	Interleukin 1-beta	F—AGGACAAGGACCTGCTCAACT/R-CCGACTCCAACTCCAACACTA P—TTGCTGGAGAGTGCTGTGGAAGAA	Raida et al. (2008)	0.93
	IL-8	Interleukin 8	F—GAGCGGTCAGGAGATTTGTC/R-TTGGCCAGCATCTTCTCAAT P—ATGTCAGCGCTCCGTGGGT	Ingerslev et al. (2009)	1.04

(Continues)

TABLE 2 (Continued)

Functional group	Assay name	Gene name	Primer and probe sequences	Source	E
	MMP13	Matrix Metallopeptidase 13	F—GCCAGCGGAGCAGGAA /R—AGTCACCTGGAGGCCAAAGA P—TCAGCGAGATGCAAAG	Tadiso et al. (2011)	1.02
	MMP25	Matrix Metallopeptidase 25	F—TGCAGTCTTTTCCCCTTGGAT/R-TCCACATGTACCCACACCTACAC P—AGGATTGGCTGGAAGGT	Miller et al. (2016)	0.93
	TNF	Tumour necrosis factor	F—CCCACCATACATTGAAGCAGATT/R—GGATTGTATTCACCCTCTAAA TGGA / P—CCGGCAATGCAAAA	Ching et al. (2010)	0.82
	UBE2Q2	ubiquitin-conjugating enzyme E2 Q2-like	F—GGCAGGACCACTTGAACGTAA/R—AGGCCTGCACTGAACCAGAT P—TGCTCATTCGGGTGCG	Miller et al. (2016)	0.87
Stress/ osmoregulation	DEXH	Altizer et al. (2011)	F—CCATAAGGAGGGTGTCTACAATAAGAT/R—CTCTCCCCCTTCAGCT TCTGT / P—TGGCGCGCTACGTG	Miller et al. (2016)	0.99
	EF-2	Elongation factor 2	F—AGGTCACAGCCGCCCTTAG/R—ACACAGTCTCTGTCTGCACACACA P—CGACTGCGTCTCAGGT	Miller et al. (2016)	0.87
	HBA	Hemoglobin subunit alpha	F—GCCCTGGCTGACAAATACAGA/R—GAGCAGGAACTGGAGTCCAAT P—ACCATCATGAAAGTCC	Miller et al. (2016)	0.98
	HIF1A	Hypoxia-inducible factor 1-alpha	F—TGGCAAATCTGCCTACGAATT/R—GCAGGCTCTTGGTCACATGA P—ATCATGCCCTGGACTC	Miller et al. (2016)	1.00
	HSP90a	Heat shock protein 90-alpha	F—AGTACCCTGTTGCACTGAGTTTTAAA/R—GAATGTTTCATTTCCCA TTGTTCA / P—ATTGGACTGGTAGATGTGT	Miller et al. (2016)	1.05
	HSP90ab1	heat shock protein 90-beta	F—GACACGGTGTTGGGTTGGTT /R—TTGCAGTCAACTCTCCATGCA P—TCATGTGCAACATAACAT	Miller et al. (2016)	1.14
	JUN	AP-1 Transcription Factor Subunit	F—TTGTTGCTGGTGAGAAAACTCAGT/R—CCTGTTGCCCTATGAATTG TCTAGT/P—AGACTTGGGCTATTTAC	Miller et al. (2016)	0.88
	LDHB	lactate dehydrogenase B	F—GTCACTGCTCCCATTTTACACTCTAG/R—CCCAAACTCCCTCCCAG ATAAC/P—CTGTTCTTAGCTTCCC	Miller et al. (2016)	0.93
	NKA_a3	Na/K ATPase α-3a (freshwater)	F—GGAGACCAGCAGAGGAACAG /R—CCCTACCAGCCCTCTGAGT P—AAGACCCAGCCTGAAATG	Stefansson et al. (2007)	1.15
	NKAa1-a	Na/K ATPase α-1a (freshwater)	F—TGGAATCAAGGTTATCATGGTCACT/R—CCCACACCCTTGGCAATG P—ATCATCCCATCACTGCGA	Miller et al. (2016)	1.09
	NKAa1-b	Na/K ATPase α-1b (saltwater)	F—GCCTGGTGAAGAATCTTGAAGCT/R—GAGTCAGGGTTCCGGTCTT P—CCTCCACCATTTGCTCA	Miller et al. (2016)	1.01
	PDIA4	protein disulfide isomerase associated 4	F—TGAGGTGCAGGACTTTTTTAAGAA /R—TCGTTGCTCTGTTTCCTGT P—ACATCCTGCCACTGGT	Miller et al. (2016)	0.92
	SHOP21	Salmon hyperosmotic protein 21	F—GCGGTAGTGGAGTCAGTTGGA /R—GCTGCTGACGTCTCACATCAC P—CCTGTTGATGCTCAAGG	Miller et al. (2016)	1.00
	Tuba1a	Tubulin alpha-1A chain	F—CTCTGCTGAGAAGGCCTACCAT /R—AGCAGGCGTTGGTGATGTC P—AGCAGCTGTCTGTTGC	Miller et al. (2016)	0.85

(Continues)

TABLE 2 (Continued)

Functional group	Assay name	Gene name	Primer and probe sequences	Source	E
Viral disease associated	CA054694	Mitochondrial ribosomal protein (VAR1)	F—CCACCTGAGGTACTGAAGATAAGACA/R—TTAAGTCCTCCTTCCTC ATCTGGTA / P—TCTACCAGGCCTTAAAG	Miller et al. (2016)	0.87
	CD9	CD9 antigen	F—CGCCACCACAACCAAGGT /R—TCCTCAGCCTCTTCTTCTTGAAG P—AGATCCCCAAGACTCTGTCAGACGCCT	Miller et al. (2016)	0.96
	IFI44A	IFN-induced protein 44–1	F—GCTAGTGCTCTTGAGTATCTCCACAA/R—TCACCAGTAACTCTGTATCATCCTGTCT/P—AGCTGAAAGCACTTGAG	Miller et al. (2016)	0.86
	IFIT5	Interferon-induced protein w tetratricopeptide repeats 5	F—CCGTCAATGAGTCCCTACACATT/R—CACAGGCCAATTTGGTGATG P—CTGTCTCCAAACTCCCA	Miller et al. (2016)	0.91
	IRF1	Interferon regulatory factor 1	F—CAAACCGCAAGAGTTCCTCATT/R-AGTTTGGTTGTGTTTTTGCATGT AG / P—CTGGCGCAGCAGATA	Miller et al. (2016)	1.12
	Mx	Antiviral protein	F—AGATGATGCTGCACCTCAAGTC /R—CTGCAGCTGGGAAGCAAAC P—ATTCCCATGGTGATCCGCTACCTGG	Eder et al. (2009)	0.86
	NFX	Zinc finger NFX1-type	F—CCACTTGCCAGAGCATGGT/R—CGTAACTGCCCAGAGTGCAAT P—TGCTCCACCGATCG	Miller et al. (2016)	0.91
	RPL6	Neoplasm-related protein C140	F—CGCCACCACAACCAAGGT /R—TCCTCAGCCTCTTCTTCTTGAAG P—AGATCCCCAAGACTCTGTCAGACGCCT	Miller et al. (2016)	1.07
	SRK2	Tyrosine-protein kinase FRK	F—CCAACGAGAAGTTCACCATCAA/R—TCATGATCTCATACAGCAAGA TTCC / P—TGTGACGTGTGGTCCT	Miller et al. (2016)	0.92
	VHSV-P10	VHSV-inducible protein-10	F—GCAAACTGAGAAAACCATCAAGAA/R—CCGTCAGCTCCCTCTGCAT P—TGTGGAGAAGTTGCAGGC	Miller et al. (2016)	0.96
	VHSVI-P4	VHSV-inducible protein-4	F—TGGCTTCCCACATTGCAA /R—CCTCCTCCCCCCTGCAT P—AGATGGAGACAGGAATG	Miller et al. (2016)	0.95
Reference	COIL	Coiled-coil domain-containing protein 84	F—GCTCATTTGAGGAGAAGGAGGATG/R—CTGGCGATGCTGTTCCT GAG / P—TTATCAAGCAGCAAGCC	Miller et al. (2016)	0.94
	MRPL40	39S ribosomal protein L40, mitochondrial precursor	F—CCCAGTATGAGGCACCTGAAGG/R—GTTAATGCTGCCACCCTCTCA P—ACAACAACATCACCA	Miller et al. (2016)	0.92

Abbreviation: E, assay efficiency.

modeled with temperature, fat content, relative infection burden, and infectious agent species richness, with study period included as a constraining strata for permutations. Heat maps of biomarker expression within each study period were constructed to further investigate gene-specific associations with each factor.

3 | RESULTS

On average, displaced salmon re-entered the fish counting trap after 11.7 days, with a range of 0.17–28 days between entries. Fish remained in good external condition with no visible abrasions, however demonstrated morphological changes typical of migratory salmon, primarily darkening from bright silver to darker brown towards the end of the study. Based on data collected by DFO fence staff, all fish survived for the duration of the study and continued migration up-stream after being passed through the counting fence. Study fish were observed holding in freshwater areas downstream of the fence, moving between higher flow areas and deeper pools. It is unknown if any individuals fell back to the marine environment in between sampling events. River temperatures were variable and ranged from 13.1°C to 21.7°C with an average of 17°C (Figure 2).

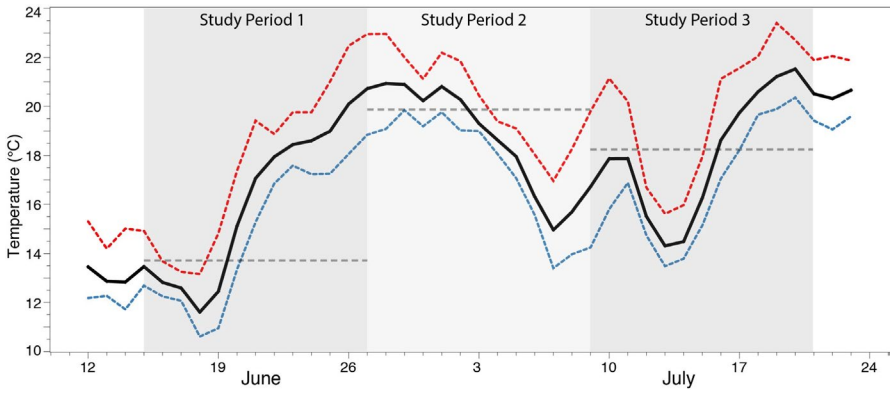

FIGURE 2 Water temperature of the Campbellton River over the course of the study represented as daily average (black), maximum (red), and minimum (blue). Average temperature for samples taken from Atlantic salmon for gene expression and infectious agent screening during each study period are indicated by vertical dashed lines

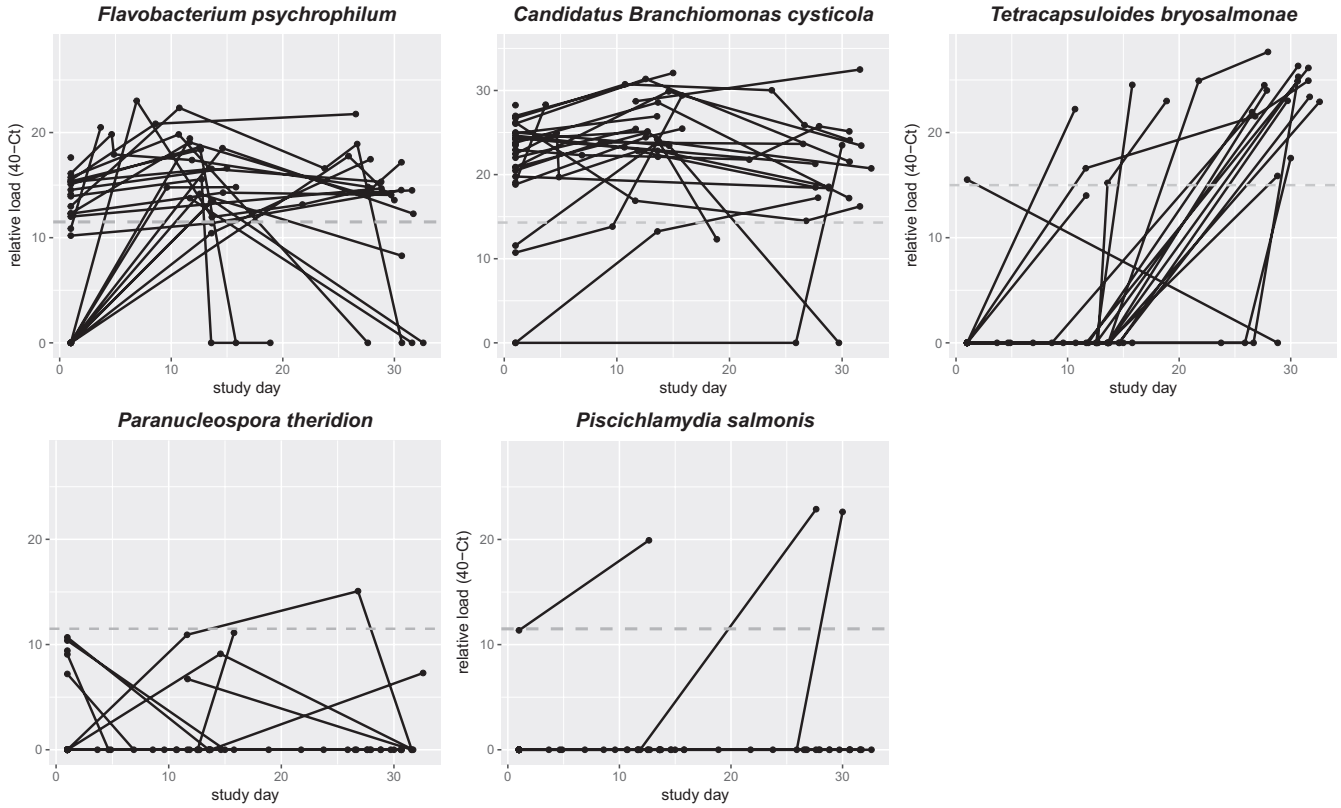

FIGURE 3 line plots of infectious agent relative loads from individual adult Atlantic salmon collected from the Campbellton River in Newfoundland, Canada. Each fish was externally tagged with an identification code and released downstream of a counting fence after each sample. Samples were opportunistically taken over the course of 32 days study period when fish re-entered a passive trap. Each line represents an individual fish ($n = 27$), with each point as a sample (1–6 per individual). Dashed line represends the 95% confidence limit of detection for each assay

A total of 70 samples from 27 grilse were included in analysis: 26 unique samples from individual fish were included in the first study period (samples taken at initial release, with one excluded due to low RNA yield), 21 in the second, and 17 in the third. All but two salmon screened positive for the presence of infectious agents upon initial sampling, and both of those individuals tested positive in subsequent samples. Infectious agents present in the sample population included the bacteria *Candidatus* Branchiomonas *cysticola* (associated with epitheliocystis) and *Flavobacterium psychrophilum* (causative agent of bacterial coldwater disease), and parasites *Paranucleospora theridion* (associated with proliferative gill disease), *Piscichlamydia salmonis* (associated with proliferative gill disease), and *Tetracapsuloides bryosalmonae* (causative agent of proliferative kidney disease; Table 1). Prevalence and relative loads of infectious agents increased throughout the duration of the study (Figure 3), reflected by increases in richness and RIB ($X^2 = 18.037$, $p = 0.0210$; repeated measures ANOVA $F_{2,40.2} = 12.223$, $p < 0.0001$; Figure 4). Species richness per host ranged from 0 to 4 species, and relative infection burden ranged from 0 in uninfected individuals to 3.04 in the most burdened individual (Figure 5). *Ca.* B. cysticola was the most prevalent infectious agent, present in 92% of fish at the beginning of the study, followed by *F. psychrophilum*. Co-infection was common among fish sampled; 69% of fish screened positive for two or more infectious agents upon initial sampling and co-infection increased to 100% of the population in study periods 2 and 3 (Figure 4). The most drastic change in prevalence and relative load was seen in the myxozoan *T. bryosalmonae*, where the prevalence increased dramatically over the course of the study. In comparison, *Ca.* B. cysticola prevalence and load remained relatively consistent, while *F. psychrophilum* demonstrated a clear increase during study period 2. Finally, fat content decreased on average for all individuals ($F_{2,37.9} = 32.563$, $p < 0.0001$; Figure 5). Mixed effect models found RIB to be significantly related to fat content but not in-river temperature or study period (Table 3).

Gene expression profiles were dissimilar among study periods, with a significant separation observed between initial sampling and subsequent samples in study periods 2 and 3 (ANOSIM $R = 0.27$; $p < 0.001$; Figure 6). Differences in gene expression within each study period were significantly associated with river temperature and not associated with lipid content or RIB (Table 4). Genes positively correlated with temperature were not specific to any single functional group, and the temperature effect was insignificant during the first study period. Transcriptional response over time suggests an up-regulation in genes associated with stress and immune function (hsp90a, MMP13, IL-15 and IgMs), and a down-regulation of genes associated with viral disease response (Mx, RSAD, DEXH, IFI44a), oxygen transport (HBA), and transcriptional regulation of protein biosynthesis (EEF2; Figure 7a). Transcriptomic response to river temperature was strongest in study period 2, when the average temperature upon sampling was 19.8 C (Figure 7b). Strong positive correlations were observed in the thermal stress associated molecular chaperones HSP90a and PDIA4. Innate immune associated inflammatory cytokine IL-15, inflammatory regulators

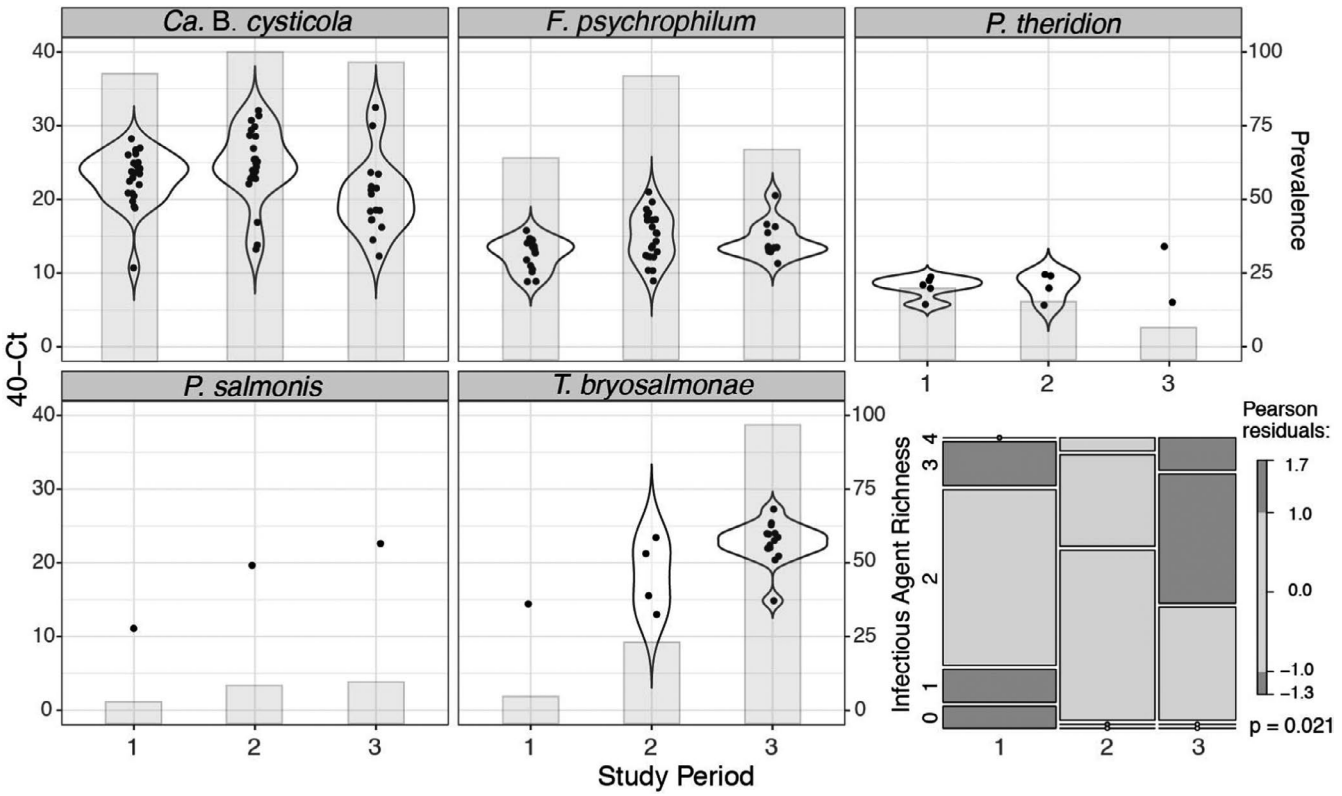

FIGURE 4 Infectious agent relative load, prevalence, and overall species richness from Atlantic salmon non-lethal gill tissue samples taken from spawning migrants in the Campbellton River, Newfoundland

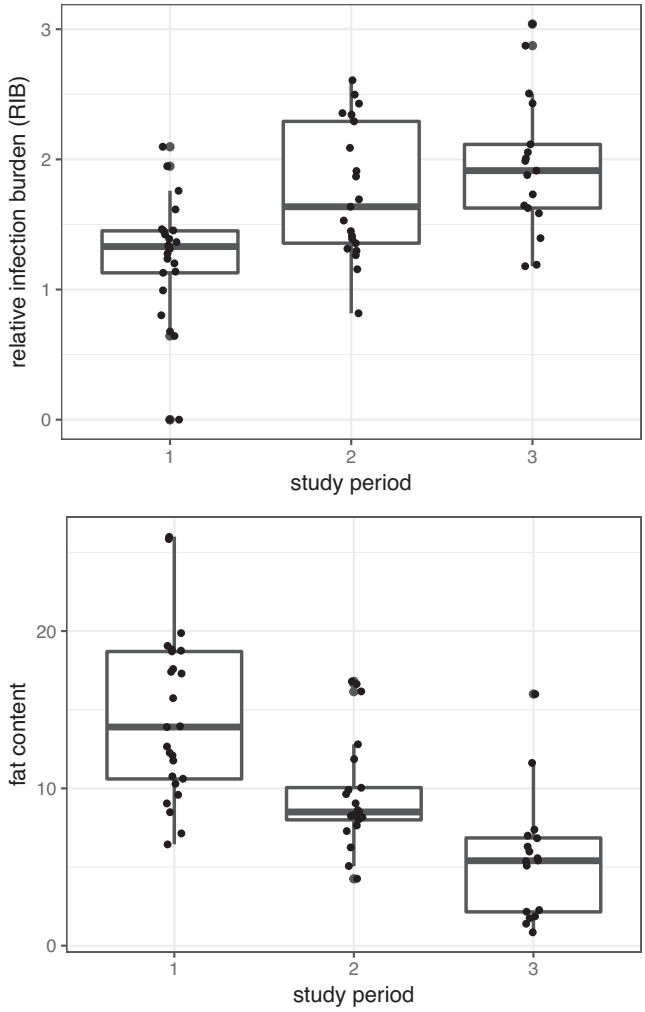

FIGURE 5 Change in relative infection burden (RIB) and fat content collected from individual Atlantic salmon sampled in three time periods over the course of a 32 day study

MMP13 and MMP25, and genes associated with adaptive immunity including the immunoglobulin IgT and T-cell stimulating kinase ZAP70 and associated TCRa receptor as well as antigen CD8a were positively correlated with temperature. Observed relationships shifted in study period 3, where temperature was more consistently associated with stress and osmoregulatory transcripts, however, observed correlations were not as strong as in study period 2. Expression of transcription factor EEF2, hemoglobulin component HBA, ribosomal protein RPL6, and sodium potassium ATPase component NKA-a3 were negatively associated with temperature, while molecular chaperones HSP90ab1, HSP90a and PDIA4, structural protein Tuba1a, and transcription factor JUN were positively correlated with temperature. Negative association with temperature were seen in inflammatory cytokines IL-17D and IL-15 and ribosomal protein RPL6. Individual genes were only associated with RIB in study period 3, predominantly represented by weak positive correlations of HBA, MHC1, and osmoregulatory sodium-potassium ATPase isoforms (Figure 8).

4 | DISCUSSION

This study was the first to assess temporal changes in infectious agent dynamics and broad scale transcriptional response in free-ranging migratory adult Atlantic salmon. Increase in relative infection burden, infectious agent richness, and decrease in fat content are consistent with previous work on migratory adult salmon upon freshwater re-entry (Jonsson, Jonsson, & Hansen, 1991, 1997; Miller et al., 2014). Infectious agents observed were known to be endemic to the region and have been detected in wild salmon in previous research (Kambestad, 2019; Starliper, 2011; Sterud et al., 2007; Teffer et al., 2020), however the prevalence of *T. bryosalmonae* observed was extremely high compared to recent work on Atlantic salmon in North America and Greenland (Teffer et al., 2020) and a potential association with rearing facilities should be further investigated.

4.1 | Fish condition and infectious agent communities

Decreased fish condition, reflected in fat content, is linked to metabolic shifts associated with energy allocation in adult salmon during spawning migrations (Bombardier et al., 2010). Migratory adult Atlantic salmon cease feeding upon re-entering freshwater. As a result, survival is dependent on available energy stores to be adequate to support up-river migration, gonadal maturation, spawning behavior, over-winter survival, and subsequent out-migration (Jonsson et al., 1991). Considering that investment in defense against infectious agents may reduce fecundity (Baalen, 1998), and the low probability of survival post spawning, down-regulation of immune-associated transcripts is a potential tradeoff that reallocates energetic resources towards reproduction and survival. Significant decreases in body condition and lipid reserves are related to the onset of anorexia and freshwater residency (Bombardier et al., 2010; Kadri et al., 1995), limiting the energy available for cellular maintenance and immune functions. This strategy is similar to that used by semelparous species (e.g. Pacific salmon; Bass et al., 2019) however may leave individuals more vulnerable to opportunistic infection. Increase in infectious agent species richness during exposure to freshwater is typical of migratory salmonids, but the decrease in *F. psychrophilum* observed in some individuals may indicate more immune activity than what is observed in semelparous species, where *F. psychrophilum* typically increases with time in freshwater for Pacific salmon (Bass et al., 2017; Teffer et al., 2017).

The increase in within-host infectious agent species richness observed over the course of the study corresponds to an increase in co-infection over the course of freshwater residency. The order in which infectious agents infect a host can determine the outcome of infectious agent-host interactions due to either synergistic or antagonistic interactions between or among pathogens. Opportunistic co-infection can occur as a consequence of decreased host immunocompetency in response to a present agent allowing for opportunistic infection by additional pathogens (Figueroa et al., 2017; Kotob

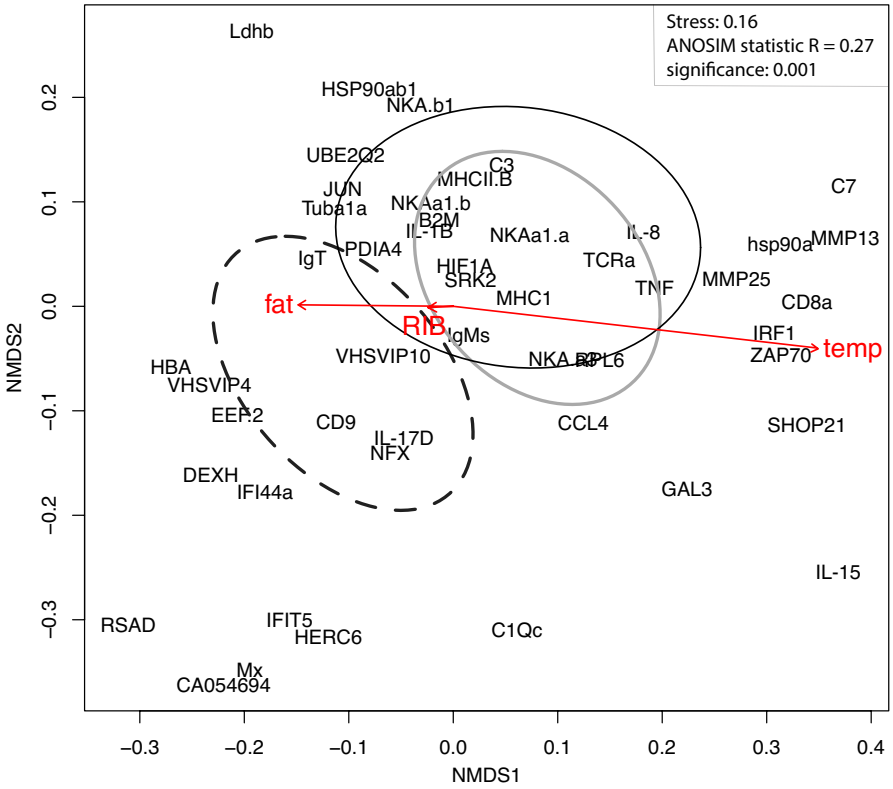

FIGURE 6 NMDS ordination using Bray-Curtis dissimilarity distances of expression of immune and stress associated transcripts non-lethally sampled from adult migratory Atlantic salmon. Ellipses represent each study period as follows: study period one, black dashed line; study period 2, solid black line; study period 3, solid gray line

Model term	*df*	*SS*	R^2	*F* value	*p* value
log(temperature)	1	0.558	0.130	9.19	0.001***
Fat content	1	0.0659	0.015	1.08	0.441
RIB	1	0.0398	0.009	0.656	0.726
Residuals	60	3.64	0.846		
Total	63	4.30	1.00		

TABLE 4 Results from Permutational Multivariate Analysis of Variance model with study period as strata for biomarker expression based on Bray-Curtis distances

et al., 2016). In other cases, an antagonistic relationship may exist, where the presence of an infectious agent inhibits the infection or virulence of another (Sofonea et al., 2017). Here, the relative loads of *F. psychrophilum* and *Ca.* B. cysticola decreased alongside a major increase in the prevalence and relative load for *T. bryosalmonae*. Whether this change represents a true antagonistic relationship between *T. bryosalmonae* and *F. psychrophilum* and *Ca.* B. cysticola or is a result of increased exposure to *T. bryosalmonae*, or decreased host immunocompetency requires further investigation.

4.2 | Transcription profiles

In the present study, salmon transcription profiles were more closely associated with river temperature than time spent in freshwater or RIB, likely driven by the strong response to elevated river temperatures observed during study period 2. The concurrent up-regulation of genes associated with stress and both adaptive and innate immunity in response to temperature may be linked with temperature stimulated metabolic activity, priming of immune defenses in response to stress, or in response to elevated infection (Sigh et al., 2004). Up-regulation of molecular chaperones such as HSP90 and PDIA4 has previously been observed in adult (Teffer et al., 2019) and juvenile (Houde, Akbarzadeh, et al., 2019) Pacific salmon experimentally exposed to thermal stress (also see Akbarzadeh et al., 2018), and Atlantic salmon post-smolts reared in high temperature conditions (Akbarzadeh et al., 2018; Jensen et al., 2015). However, heat shock proteins have also been associated with infection in Atlantic salmon exposed to sea-lice infestation (Provan et al., 2013). Higher-temperature conditions were also related with significant up-regulation of the majority of mRNA transcripts compared to low-temperature conditions (Jensen et al., 2015). The reduction of temperature-dependent expression observed in study period 3 may represent chronic temperature stress and metabolic shift, indicated by the observed down-regulation of transcription factors EEF2 and HBA. Reduced expression of transcription factors may indicate a reduction in protein synthesis as fish begin to shift metabolic activity under fasting conditions (Bombardier et al., 2010). The concurrent positive association between HBA

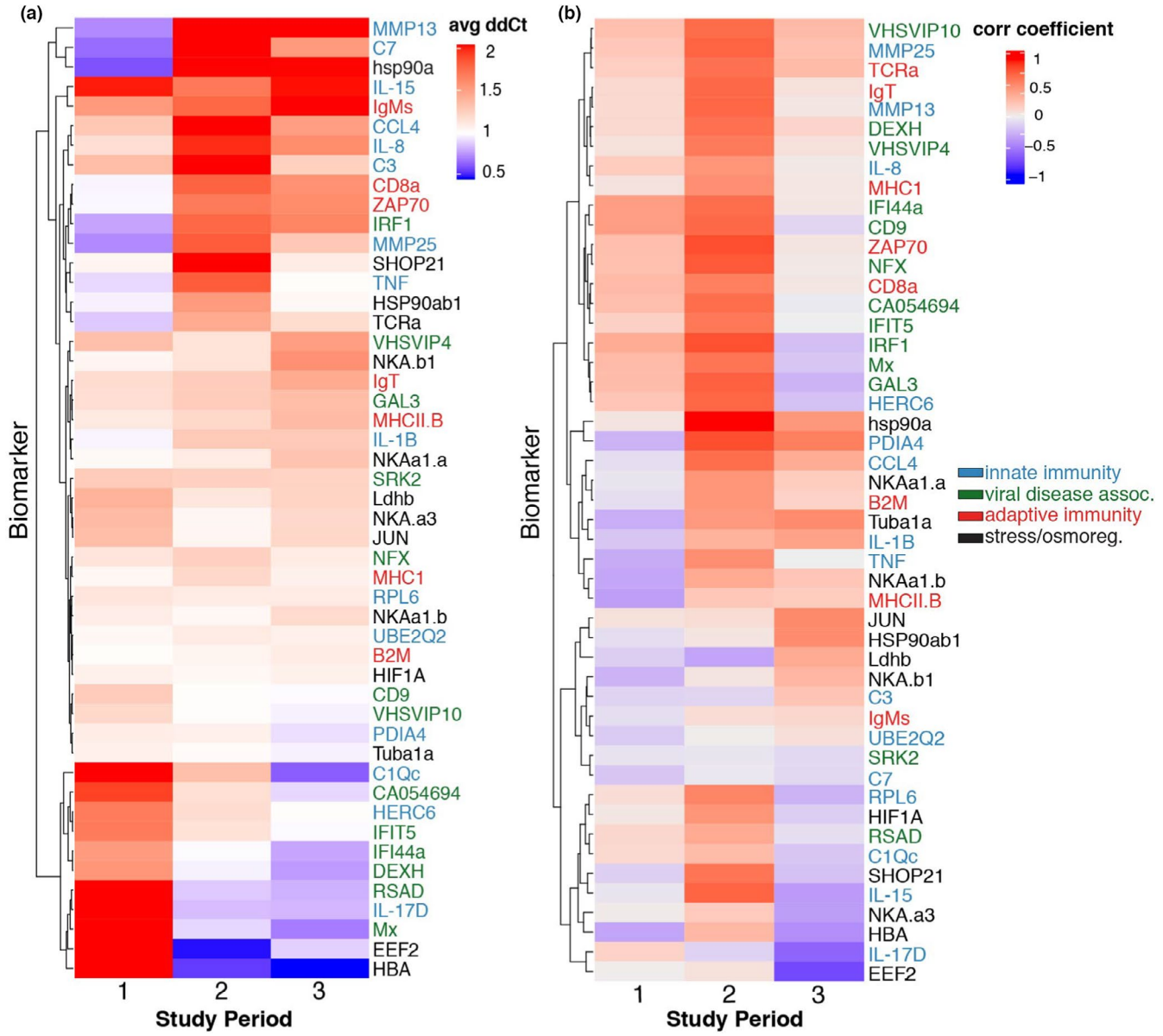

FIGURE 7 Heat maps of gene expression for transcripts associated with immune function and stress in Atlantic salmon. (a) Average fold change expression observed in each study period; (b) correlation of river temperature with gene expression for each transcript

with RIB in study period three may suggest metabolic shifts in response to infection. Taken with the coinciding positive association of adaptive immune complex MHC1 and biomarkers of genes associated with osmoregulation and ion balance (NKAa1-a, NKA-b1, and NKAa1-b; Shrimpton et al., 2005; Houde, Schulze, et al., 2019), high infection burden may be causing disruption in osmoregulation that could be exacerbated by chronic thermal stress (Akbarzadeh et al., 2018).

Elevated water temperature is known to result in metabolic stress in adult Atlantic salmon (Wilkie et al., 1997), and river temperatures are anticipated to rise across Atlantic salmon distributions in the coming years (Poesch et al., 2016). Here, temperature was more closely associated with transcription profiles than freshwater residency and relative infection burden, demonstrating the metabolic responsiveness and corresponding energy use imposed on fish under thermal stress. Adult anadromous Atlantic salmon have been shown to behaviourally thermoregulate at temperatures between 17–19°C during holding phases of their spawning migrations (Frechette et al., 2018; Moore et al., 2012). Barriers to migration such as dams and waterfalls can cause migration delay in Atlantic salmon (Thorstad et al., 2003; Twardek et al., 2019), however the high survival rate of fish in this study indicates that the barrier created by the fish fence does not represent a substantial immediate physiological challenge to the population. The fish counting fence restricted salmon to relatively high-flow habitat with extensive hydrological mixing and thus limited access to deep pools where cooler water would be available. Consequently, the observed transcriptional response may not represent the physiological status of fish able to access a larger diversity of

habitats, providing insight for future conditions where thermal refugia may not be available. Broad scale behavioural responses to temperature have already been observed in the study region, as timing of Atlantic salmon migrations in Newfoundland has shifted in response to climactic changes; River entry is now 12 days earlier in warm conditions, and later in years where ocean conditions are cooler and there is a higher amount of in-shore sea-ice (Dempson et al., 2017). The cumulative effects of reduced time at sea for feeding, potentially leading to reduced condition, and elevated metabolic stress associated with temperature, warrant further study to understand how wild fish are able to adjust both physiologically and behaviourally to a narrower energetic scope.

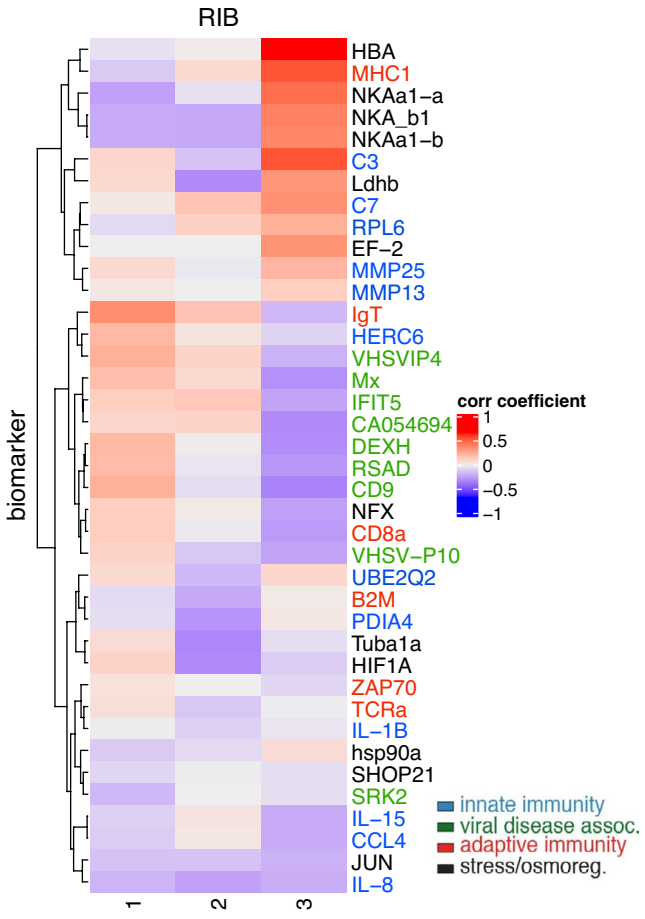

FIGURE 8 Heat map of correlation between gene expression and relative infection burden (RIB) from transcripts associated with immune function and stress in adult Atlantic salmon

4.3 | Tetracapsuloides bryosalmonae

Recent surveys of infectious agents from Atlantic salmon collected in three systems in New Brunswick revealed no positive detections in the Restigouche and St. John's rivers, however aquaculture escapees collected at the mouth of the Magaguadavic River carried *T. bryosalmonae* (Teffer et al., 2020). Similarly, hatchery screening of brook trout *Salvelinus fontinalis* detected *T. bryosalmonae* both in a facility dedicated to stock enhancement and in wild brook trout from Star Lake (DFO unpublished data), suggesting the myxozoan is present in the region however the majority of sampling to date has found the agent to be associated with fish rearing facilities. Whether or not *T. bryosalmonae* is endemic to the region or a result of intensifying aquaculture and stock enhancement facilities requires further investigation.

T. bryosalmonae is a myxozoan endoparasite of mainly salmonids that is the causative agent of the potentially fatal proliferative kidney disease (PKD). First attributed to mass mortalities in aquaculture, PKD has been associated with large-scale mortality events in wild juvenile Atlantic salmon (Sterud et al., 2007) and population declines of wild brown trout *Salmo trutta* in Europe (Wahli et al., 2002). A freshwater bryozoan is the primary host and thus required to complete the life cycle (Okamura et al., 2011). Bryozoans are benthic, clonal organisms that grow on the substrate. Infection with *T. brysalmonae* occurs when spores released from bryozoans come in to contact with fish gills and/or skin, using amoeboid cells to infiltrate fish tissue (Longshaw et al., 2002). The main site of infection is the kidney, however extrasporogenic stages undergo proliferation in the blood stream and can infect other organs, and spores passed in urine are infective to bryozoan hosts (Hedrick et al., 2004). The sudden increase in *T. bryosamonae* may be the result of study fish being constrained to habitat that held infective bryozoans, artificially increasing exposure risk compared to fish that are able to quickly transit the system.

Clinical pathology of PKD such as kidney hyperplasia and anemia and physiological impairment is positively associated with increased temperatures (Bruneaux et al., 2017; Morris et al., 2005). Consequently, anthropogenic factors associated with the spread and proliferation of *T. bryosalmonae* such as climate warming and barriers to migration have the potential to increase infection rates and pathogen distribution in wild fish (Harris et al., 2011). All fish sampled remained in high condition for the duration of the study, however clinical signs of disease are often not observed in adult salmon (El-Matbouli & Hoffman, 1994). Inter and intraspecific variation in

TABLE 3 Generalized Linear mixed effect model estimates of relative infection burden (RIB) with fat content, temperature and study period and their interaction as fixed effects and fish id as a random effect. Significance is given based on Satterhwaite's method

Model term	Mean Sq	*df*	ddf	*F* value	*p* value
Fat content	2.12	1	53	9.22	0.00371**
Log(temperature)	0.0655	1	53	0.278	0.600
Study period	0.216	2	53	2.86	0.0660
Log(temp)*study period	0.642	2	53	2.79	0.0706

adaptive immunity to *T. bryosalmonae* has been demonstrated, however survivors appear to develop resistance to subsequent infection and/or disease (Cauwelier et al., 2010). Further, reinfection has the potential to cause decreased immunocompetency and leave individuals susceptible to secondary infections. Adult salmon may also act as vectors, transferring from the bryozoan host to more susceptible juveniles (Sterud et al., 2007). Given the recent declines in population observed in the Campbellton River, the high prevalence of *T. bryosalmonae* observed here indicates this pathogen is present in the population, and whether or not the disease is contributing to population decline warrants further research.

5 | CONCLUSION

This is the first study to use a repeated measures sampling design to ascertain the progression of disease in wild salmonids. Many recent studies have illuminated the prevalence of various pathogens in wild populations (e.g. Bass et al. 2017; Teffer et al., 2020; Twardek et al., 2019) from a single timepoint to describe pathogen profiles. Study of changes in pathogen dynamics over time in wild fish is limited and over larger time scales (e.g. Chapman et al., 2020) or in captive settings (e.g. Teffer et al., 2017). We show that the pathogen profile can shift relatively rapidly as fish continue their migration and up- or down-regulate immune-related genes. Given the high pathogenic potential of *T. bryosalmonae*, additional monitoring and/or surveys should be executed to determine regional prevalence and potential location of bryozoan vectors. The association between RIB and fat content suggests that fish condition may play a central role in immunocompetency in the wild, however research reducing confounding environmental factors is required to corroborate this. Given the transcript responsiveness to elevated temperatures, future work investigating the potential immunological consequences of thermal shock and chronic thermal stress in wild Atlantic salmon under natural conditions where habitat such as thermal refugia is present is warranted. While the barrier associated with the fish fence may have created an artificial stressor, field research of this kind should be preferred over experimental holding studies, where confinement stress and artificial environments reduce the applicability of data to natural environment (Portz et al., 2006). Future field programs may also benefit from combining remote sensing methods, such as biotelemetry, and genetic screening molecular methods to understand the broad-scale impact of microbial pathogens on fish health, behaviour, and survival (Chapman et al., 2021).

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

DATA AVAILABILITY STATEMENT

Raw data were generated at the Molecular Genetics Laboratory at the Fisheries and Oceans Canada Pacific Biological Station. Data supporting the findings of this study are available from the corresponding author on request.

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