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A molecular assessment of infectious agents carried by Atlantic salmon at sea and in three eastern Canadian rivers, including aquaculture escapees and North American and European origin wild stocks

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Abstract

Infectious agents are key components of animal ecology and drivers of host population dynamics. Knowledge of their diversity and transmission in the wild is necessary for the management and conservation of host species like Atlantic salmon (*Salmo salar*). Although pathogen exchange can occur throughout the salmon life cycle, evidence is lacking to support transmission during population mixing at sea or between farmed and wild salmon due to aquaculture exposure. We tested these hypotheses using a molecular approach that identified infectious agents and transmission potential among sub-adult Atlantic salmon at marine feeding areas and adults in three eastern Canadian rivers with varying aquaculture influence. We used high-throughput qPCR to quantify infection profiles and next generation sequencing to measure genomic variation among viral isolates. We identified 14 agents, including five not yet described as occurring in Eastern Canada. Phylogenetic analysis of piscine orthoreovirus showed homology between isolates from European and North American origin fish at sea, supporting the hypothesis of intercontinental transmission. We found no evidence to support aquaculture influence on wild adult infections, which varied relative to environmental conditions, life stage, and host origin. Our findings identify research opportunities regarding pathogen transmission and biological significance for wild Atlantic salmon populations.

Key words: Atlantic salmon, bacteria, virus, infectious agent, disease ecology, qPCR

Introduction

Infectious agents such as viruses, bacteria, and other microparasites are ubiquitous in aquatic and marine environments (Marcogliese 2008; Lafferty 2017), yet their diversity among wild fish hosts remains largely undescribed. Logistical constraints of studying pathogens in the wild have limited

our understanding of naturally occurring infections in wild fishes (Miller et al. 2014). This knowledge is necessary to anticipate how changing environmental conditions may affect the virulence of endemic agents, the introduction of exotic agents, and associated disease development (Burge et al. 2014), which have the potential to drive host population dynamics and the economics of fisheries (Selakovic et al. 2014; Johnson et al. 2015; Lafferty et al. 2015). Most information on fish disease has been derived from aquaculture settings where fish are more easily observed in later stages of disease (Bakke and Harris 1998). However, extrapolation of this information to wild fishes may be misleading as conditions experienced by cultured fish differ greatly from wild fish. A prime example is the Atlantic salmon (*Salmo salar*), which is a key cultured species with well-established relationships between infection dynamics and survival in captivity (Bakke and Harris 1998). While farmed salmon are fed, handled, and held at high densities, wild salmon must hunt for prey, avoid predators, migrate across dynamic environments, and are generally found at relatively lower densities throughout most of their life history. These differences likely contribute to disparate disease outcomes between wild and cultured salmon, even for the same pathogen. To inform future research on how pathogens may influence host population dynamics, evaluations are needed that characterize infectious agents carried by wild and cultured Atlantic salmon.

Since the late 1980s, Atlantic salmon populations have experienced large declines in abundance over much of their range (Parrish et al. 1998; Klemetsen et al. 2003). Wild Atlantic salmon begin their lives in fresh water, rear in natal rivers for one to seven years, and then migrate to marine feeding areas in the North Atlantic Ocean; at maturity, adults return to natal rivers to spawn and then can migrate back out to sea (Jonsson and Jonsson 2011). Survival throughout marine migrations has been shown to be a key factor in observed population declines, yet these life stages remain relatively understudied and the causes of mortality unknown (Hansen and Quinn 1998; Sheehan et al. 2012). Pathogen transmission dynamics in marine habitats and how infections contribute to wild salmon mortality at sea is even less well known (Hansen and Quinn 1998). Substantial mixing of stocks occurs in the Labrador Sea near Greenland (Chaput et al. 2018), including both North American and European origin fish (Reddin and Friedland 1999; Sheehan et al. 2012). This mixing not only provides an opportunity for infectious agents to spread among individuals, but also geographical areas since salmon will eventually migrate back to natal freshwater systems to spawn (Madhun et al. 2018; Vendramin et al. 2019). Introduction of emerging pathogens or pathogen strains through intercontinental exchange may pose new risks to otherwise threatened populations in a way that is not easily controlled by anthropogenic activities. Although the transmission of agents may or may not result in disease or reduced host survival, understanding if and where pathogen exchange occurs is a crucial first step toward characterizing impacts on wild Atlantic salmon across their range, including the influence of human activities like aquaculture.

Many wild Atlantic salmon stocks come into contact with aquaculture facilities during seaward or spawning migrations, which may result in infectious agent exchange between farmed and wild fish (Heggberget et al. 1993; Garseth et al. 2018). There is also a persistent issue of escapees from salmon farms mixing with wild stocks, posing further opportunities for pathogen transmission in addition to potential interbreeding and fitness consequences for wild salmon (Castellani et al. 2018). Modeling studies of wild Pacific salmon (*Oncorhynchus* spp.) on the west coast of Canada have demonstrated decreased wild salmon productivity in association with exposure to Atlantic salmon farms with high macroparasite (sea lice) densities (Krkošek et al. 2011; Peacock et al. 2013). Microparasites such as viruses, bacteria, and various metazoan species certainly have the potential to impact wild salmon productivity, especially under poor environmental conditions (Burge et al. 2014).

Despite recent advances in our understanding of the diversity of infectious agents hosted by salmon on Canada's west coast (Bass et al. 2017; Di Cicco et al. 2017, 2018; Nekouei et al. 2018;

Tucker et al. 2018), our knowledge of infectious agents affecting wild Atlantic salmon on the east coast remains scant. Infectious agent surveys can be used to direct future research toward quantifying specific host interactions; for example, similarities in the composition of agent species and genotypes hosted by wild and cultured Atlantic salmon may indicate farm–wild pathogen exchange (Olivier 2002; Johansen et al. 2011). Importantly, coinfection (multiple agent species in one host) and superinfection (multiple agent genotypes in one host) are common in wild animals and the dynamics of these communities are linked to host fitness outcomes (Martin et al. 2012; Alizon et al. 2013; Sofonea et al. 2015). Host health and performance, especially in the wild, can be impacted by shifts in coinfection or superinfection prior to the occurrence of detectable or typical tissue changes (pathology) associated with disease (Brassard et al. 1982; Wiik-Nielsen et al. 2016; Downes et al. 2018). This complexity warrants an approach beyond traditional diagnostics to characterize transmission events that can influence disease processes in wild fish.

Molecular tools are rapidly increasing our ability to describe the pathogen dynamics of wild animal populations and can be cost-effectively applied to quantify an array of infectious agents (e.g., Miller et al. 2016). The minimal tissue requirements of molecular approaches allow for nonlethal tissue biopsy, which is especially useful for studying populations of conservation concern (Archie et al. 2009). Studies of wild Pacific salmon in British Columbia, Canada, have successfully applied high-throughput polymerase chain reaction (HT-qPCR) for infectious agent screening and host response characterization (Jeffries et al. 2014; Miller et al. 2014; Bass et al. 2017). As this HT-qPCR tool was developed to include assays to pathogens impacting salmon worldwide, the same approach is amenable to application in Atlantic salmon on the east coast of Canada. Additional molecular techniques, such as next generation sequencing (NGS), can provide further insight into how and where pathogens are exchanged in the wild; for example, hosts that carry phylogenetically similar viral strains likely share a transmission source and (or) location (Stimson et al. 2019). RNA viruses are especially useful for characterizing pathogen transmission dynamics due to their relatively high strain variability across spatial and temporal gradients (Stimson et al. 2019). By combining qPCR and sequencing approaches, we can not only quantify similarity in coinfection profiles based on location (marine sub-adults and freshwater adults) and source (wild and cultured escapees), but also conduct phylogenetic analysis of viruses to identify evidence of transmission at sea or between farmed and wild fish.

We tested two hypotheses using a molecular approach: (H_1) the Labrador Sea will comprise a melting pot of European and North American origin salmon as a potential area where pathogen exchange between fish of different continents occurs, and (H_2) adult salmon exchange infectious agents with cultured salmon during spawning migration, resulting in similarity in infection profiles based on the proximity of their natal rivers to aquaculture. Atlantic salmon were collected from marine feeding grounds near Greenland (sub-adults) and from three eastern Canadian rivers (mature adults) with variable aquaculture influences: distant from aquaculture (Restigouche River, nonthreatened wild population), proximal to aquaculture (St. John River, threatened wild population), and aquaculture escapees (Magaguadavic River, where resident wild population is extirpated). Tissue samples were evaluated for the presence and loads of 44 viruses, bacteria, and other microparasites known or expected to cause disease in salmon worldwide using a high-throughput Fluidigm BioMark platform (Fluidigm Corporation, San Francisco, CA, USA) and assay panel (Miller et al. 2014, 2016). To identify potential natural (marine stock mixing) and anthropogenic (aquaculture-wild) transmission routes, we compared viral isolates from this study with published sequences obtained from Atlantic salmon in previous studies worldwide. Our objective was to provide baseline data to inform future studies of the transmission and disease dynamics of wild Atlantic salmon.

Methods

Sample collection, preservation, and transfer

Biological sampling at all fish collection sites included a fork-length measurement, external morphology assessment, scale and tissue sampling, and external observation for macroparasites. Tissues were sampled from fish (~0.5 mg) using sterile tools and fixed in RNA*later* (Ambion, Austin, Texas, USA; 1.5 mL). Gill, heart, and kidney biopsy samples (multi-tissue) were taken from fish in Greenland and at the Magaguadavic and Restigouche rivers in 2017, whereas only kidney samples were taken from Greenland-sampled fish in 2016 and only nonlethal gill biopsies from fish of the threatened St. John River population in 2017. Gill biopsies have been shown to comprise most infectious agents detected in multi-tissue HT-qPCR analyses (Teffer and Miller, 2019).

To obtain samples from offshore marine waters where fish from North American and European stocks mix, wild salmon were collected over two years by commercial fishers using gill nets in the Labrador Sea along the coast of Greenland (Fig. 1). Direct acquisition of freshly landed fish and tissue sampling took place at local markets in Paamiut and Maniitsoq, Greenland, in September of 2016 ($N = 43$; kidney only, Paamiut) and 2017 ($N = 30$; multi-tissue, Maniitsoq), respectively. Tissues collected in Greenland in 2016 were stored at 4 °C for 30 d and then −20 °C for 13 months. Tissues collected in Greenland in 2017 were stored at 4 °C for 24 h and then −20 °C for 2 months. Greenland-collected samples were transported from Greenland to Quebec, Canada, on ice (stored at −20 °C at night during 3 d transport), and then shipped on dry ice to the Atlantic Salmon Federation (ASF) headquarters in Chamcook, New Brunswick, Canada, where they were stored at −80 °C until analysis.

Salmon bearing rivers were sampled in 2017, including the Restigouche (low aquaculture influence; $N = 30$), St. John (high aquaculture influence; $N = 30$), and Magaguadavic rivers (aquaculture escapees; $N = 17$; Table 1, Fig. 1). The Restigouche River sampling site is isolated from aquaculture

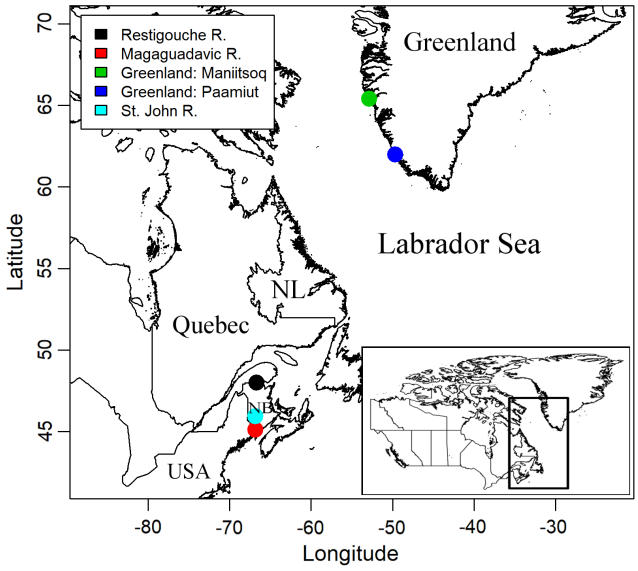

Fig. 1. Map of study area. Fish collection sites included three eastern Canadian rivers (Restigouche (black), St. John (red), and Magaguadavic (light blue) in New Brunswick (NB)) and offshore fishing areas located in the Labrador Sea, with fish obtained from commercial fishers at local markets in Maniitsoq (green) and Paamiut (dark blue), Greenland (marine fishing took place in offshore waters of Labrador Sea). Map assembled using the "sp" R package and RData from the GADM database of Global Administrative Areas, version 2.0.

Table 1. Adult salmon sampled for infectious agent screening using high-throughput qPCR.

Source location	Continent of origin	Year	Tissue type(s)	*N*	Fork length, mm (mean ± SD)
Labrador Sea near Greenland	North America	2016	Kidney	10	654 ± 24
		2017	Multi-tissue[a]	26	672 ± 55
	European	2016	Kidney	33	618 ± 25
		2017	Multi-tissue[a]	4	668 ± 20
Restigouche River	North America	2017	Multi-tissue[a]	30	836 ± 67
St. John River	North America	2017	Gill	30	572 ± 31
Magaguadavic River	North America	2017	Multi-tissue[a]	17	653 ± 47

[a]Gill, heart, and kidney.

influence and located near the head of tide. Restigouche fish were lethally sampled between 12 June and 1 July 2017 with the collaboration of Listuguj Mi'gmaq fishers following chain of custody procedures for sample preservation (see below). Restigouche tissue samples were stored at 4 °C for 24 h and then at −18 °C for ≤18 d, then transported on ice to the ASF headquarters in Chamcook, New Brunswick, (≤24 h transport) and stored at −80 °C.

In the St. John River, returning adult wild and hatchery (released at the juvenile stage) Atlantic salmon were sampled nonlethally for gill tissue at the Department of Fisheries and Oceans (DFO) Biodiversity Facility near the base of Mactaquac Dam situated 3 km above head of tide; this facility is approximately 100 km upstream from the river mouth and is proximal to the commercial salmon aquaculture industry. The St. John tissue samples were transported to the ASF headquarters on ice (≤2 h transport), stored at 4 °C for 24 h, and then stored at −80 °C.

The Magaguadavic River collection site is proximal to commercial Atlantic salmon aquaculture operations in the Bay of Fundy, and escaped salmon are a regular occurrence in the river (Carr 1995; Morris et al. 2008). Escapees were collected from a trap in a head of tide fish ladder, identified as escapees using external morphology and scale characteristics (Carr 1995), euthanized, and then transported on ice to ASF headquarters (20 min) for tissue sampling. Tissue samples were stored at ASF headquarters at 4 °C for 24 h and then stored at −80 °C.

Tissue samples from all locations were stored at the ASF headquarters in a −80 °C freezer for 50–255 d. All samples were shipped on dry ice to the DFO Pacific Biological Station, Nanaimo, British Columbia, on 1 February 2018 (1 d transport) and stored at −80 °C until analysis.

Laboratory protocols

Greenland fish were genotyped using genome-wide single-nucleotide polymorphisms (Jeffery et al. 2018) at the DFO Salmonids Section Population Genomic Lab to assign North American or European origin (Table 1). Infection profiles were evaluated at the DFO Molecular Genetics Laboratory, Pacific Biological Station, using the Fluidigm BioMark HT-qPCR platform and assay panel to quantify the presence and relative loads of 44 infectious agents in RNA extracted from preserved tissues (Table 2). Most assays included in the panel for this study have been analytically validated for specificity, sensitivity, repeatability, and reproducibility between platforms (Miller et al. 2016), with the exception of Atlantic salmon calicivirus (ASCV) and salmon gill poxvirus (SGPV), which were added after the initial panel was developed and validated (only specificity and sensitivity

Table 2. Assays included in the high-throughput qPCR panel tested on wild and cultures Atlantic salmon tissues.

Agent	Type	Abbreviation	Limits of detection	Primer and probe sequences	Accession no.	Assay reference
Atlantic salmon calicivirus[a]	Virus	ascv	27.14	F: ACCGACTGCCCGGTTGT R: CTCCGATTGCCTGTGATAATACC P: CTTAGGGTTAAAGCAGTCG	—	Gideon Mordecai
Infectious hematopoietic necrosis virus	Virus	ihnv	27.64	F: AGAGCCAAGGCACTGTGCG R: TTCTTTGCGGCTTGGTTGA P: TGAGACTGAGCGGGACA	NC_001652	Purcell et al. 2013
Infectious pancreatic necrosis virus	Virus	ipnv	27.63	F: GCAACTTACTTGAGATCCATTATGCT R: GAGACCTCTAAGTTGTATGACGAGGTCTCT P: CGAGAATGGGCCAGCAAGCA	—	Clouthier et al. 2014
Infectious salmon anemia virus	Virus	isav	26.12	F: TGGGCAATGGTGTATGGTATGA R: GAAGTCGATGAACTGCAGCGA P: CAGGATGCAGATGTATGC	EU118822	LeBlanc et al. 2010
Salmonid herpesvirus	Virus	omv	26.59	F: GCCTGGACCACAATCTCAATG R: CGAGACAGTGTGGCAAGACAAC P: CCAACAGGATGGTCATTA	—	Miller et al. 2016
Piscine myocarditis virus	Virus	pmcv	26.29	F: AGGGAACAGGAGGAAGCAGAA R: CGTAATCCGACATCATTTTGTGA P: TGGTGGAGCGTTCAA	HQ339954	Wiik-Nielsen et al. 2013
Piscine reovirus	Virus	prv	26.11	F: TGCTAACACTCCAGGAGTCATTG R: TGAATCCGCTGCAGATGAGTA P: CGCCGGTAGCTCT	—	Wiik-Nielsen et al. 2012
Salmon alphavirus	Virus	sav	26.28	F: CCGGCCCTGAACCAGTT R: GTAGCCAAGTGGGAGAAAGCT P: TCGAAGTGGTGGCCAG	AY604235	Andersen et al. 2007
Salmonid gill poxvirus	Virus	sgpv	25.15	F: ATCCAAAATACGGAACATAAGCAAT R: CAACGACAAGGAGATCAACGC P: CTCAGAAACTTCAAAGGA	—	Gjessing et al. 2015
Putative Totiviridae	Virus	toti	25.87	F: TCTGCGCGCTGCACCTA R: ATGCGGAGGAACTCACACACT P: CAAGTGCTACACTGCG	—	Gideon Mordecai
Viral erythrocytic necrosis virus	Virus	ven	24.85	F: CGTAGGGCCCCAATAGTTTCT R: GGAGGAAATGCAGACAAGATTTG P: TCTTGCCGTTATTTCCAGCACCCG	—	Purcell et al. 2016
Viral encephalopathy and retinopathy virus	Virus	venv	26.21	F: TTCCAGCGATACGCTGTTGA R: CACCGCCCGTGTTTGC P: AAATTCAGCCAATGTGCCCC	AJ245641	Korsnes et al. 2005
Viral hemorrhagic septicemia virus	Virus	vhsv	26.86	F: AAACTCGCAGGATGTGTGCGTCC R: TCTGCGATCTCAGTCAGGATGAA P: TAGAGGGCCTTGGTGATCTTCTG	Z93412	Jonstrup et al. 2013
Aeromonas hydrophila	Bacterium	ae_hy	28.67	F: ACCGCTGCTCATTACTCTGATG R: CCAACCCAGACGGGAAGAA P: TGATGGTGAGCTGGTTG	AY165026	Lee et al. 2006
Aeromonas salmonicida	Bacterium	ae_sal	25.61	F: TAAAGCACTGTCTGTTACC R: GCTACTTCACCCTGATTGG P: ACATCAGCAGGCTTCAGAGTCACTG	M64655	Keeling et al. 2013 (modified)

(*continued*)

Table 2. (*continued*)

Agent	Type	Abbreviation	Limits of detection	Primer and probe sequences	Accession no.	Assay reference
Flavobacterium psychrophilum	Bacterium	fl_psy	29.46	F: GATCCTTATTCTCACAGTACCGTCAA R: TGTAAACTGCTTTTGCACAGGAA P: AAACACTCGGTCGTGACC	—	Duesund et al. 2010
Ca. Piscichlamydia salmonis[a]	Bacterium	pch_sal	30.72	F: TCACCCCCAGGCTGCTT R: GAATTCCATTTCCCCCTCTTG P: CAAAACTGCTAGACTAGAGT	EU326495	Nylund et al. 2008
Piscirickettsia salmonis	Bacterium	pisck_sal	23.32	F: TCTGGGAAGTGTGGCGATAGA R: TCCCGACCTACTCTTGTTTCATC P: TGATAGCCCCGTACACGAAACGGCATA	U36943	Corbeil et al. 2003
Renibacterium salmoninarum	Bacterium	re_sal	25.91	F: CAACAGGGTGGTTATTCTGCTTTC R: CTATAAGAGCCACCAGCTGCAA P: CTCCAGCGCCGCAGGAGGAC	AF123890	Powell et al. 2005
Rickettsia-like organism	Bacterium	rlo	25.23	F: GGCTCAACCCAAGAACTGCTT R: GTGCAACAGCGTCAGTGACT P: CCCAGATAACCGCCTTCGCCTCCG	EU555284	Lloyd et al. 2011
Ca. Syngnamydia salmonis[a]	Bacterium	sch	27.9	F: GGGTAGCCCGATATCTTCAAAGT R: CCCATGAGCCGCTCTCTCT P: TCCTTCGGGACCTTAC	FJ897519	Duesund et al. 2010
Tenacibaculum maritimum	Bacterium	te_mar	26.71	F: TGCCTTCTACAGAGGGATAGCC R: CTATCGTTGCCATGGTAAGCCG P: CACTTTGGAATGGCATCG	—	Fringuelli et al. 2012b
Vibrio anguillarum	Bacterium	vi_ang	26.41	F: CCGTCATGCTATCTAGAGATGTATTTGA R: CCATACGCAGCCAAAAATCA P: TCATTTCGACGAGCGTCTTGTTCAGC	L08012	Miller et al. 2016
Aliivibrio salmonicida	Bacterium	vi_sal	25.84	F: GTGTGATGACCGTTCCATATTT R: GCTATTGTCATCACTCTGTTTCTT P: TCGCTTCATGTTGTGTAATTAGGAGCGA	AF452135	Miller et al. 2016
Yersinia ruckeri	Bacterium	ye_ruc	28.13	F: TCCAGCACCAAATACGAAGG R: ACATGGCAGAACGCAGAT P: AAGGCGGTTACTTCCCGGTTCCC	—	Keeling et al. 2012
Paramoeba perurans	Amoeba	ne_per	25.39	F: GTTCTTTCGGGAGCTGGGAG R: GAACTATCGCCGGCACAAAAG P: CAATGCCATTCTTTTCGGA	EF216905	Fringuelli et al. 2012a
Ichthyophthirius multifiliis	Ciliate	ic_mul	23.7	F: AAATGGGCATACGTTTGCAAA R: AACCTGCCTGAAACACTCTAATTTTT P: ACTCGGCCTTCACTGGTTCGACTTGG	IMU17354	Miller et al. 2016
Gyrodactylus salaris	Fluke	gy_sal	26.42	F: CGATCGTCACTCGGAATCG R: GGTGGCGCACCTATTCTACA P: TCTTATTAACCAGTTCTGC	—	Collins et al. 2010
Spironucleus salmonicida	Flagellate	sp_sal	26.05	F: GCAGCCGCGGTAATTCC R: CGAACTTTTTAACTGCAGCAACA P: ACACGGAGAGTATTCT	AY677182	Miller et al. 2016
Nanophyetus salmincola	Fluke	na_sal	24.3	F: CGATCTGCATTTGGTTCTGTAACA R: CCAACGCCACAATGATAGCTATAC P: TGAGGCGTGTTTTATG	AY269674	Miller et al. 2016

(*continued*)

Table 2. (*concluded*)

Agent	Type	Abbreviation	Limits of detection	Primer and probe sequences	Accession no.	Assay reference
Sphaerothecum destruens	Mesomycetozoea	sp_des	26.5	F: GGGTATCCTTCCTCTCGAAATTG R: CCCAAACTCGACGCACACT P: CGTGTGCGCTTAAT	AY267346	Miller et al. 2016
Facilispora margolisi	Microsporidian	fa_mar	30.55	F: AGGAAGGAGCACGCAAGAAC R: CGCGTGCAGCCCAGTAC P: TCAGTGATGCCCTCAGA	HM800849	Miller et al. 2016
Loma salmonae	Microsporidian	lo_sal	25.42	F: GGAGTCGCAGCGAAGATAGC R: CTTTTCCTCCCTTTACTCATATGCTT P: TGCCTGAAATCACGAGAGTGAGACTACCC	HM626243	Miller et al. 2016
Paranucleospora theridion[a]	Microsporidian	pa_ther	28.16	F: CGGACAGGGAGCATGGTATAG R: GGTCCAGGTTGGGTCTTGAG P: TTGGCGAAGAATGAAA	FJ59481	Nylund et al. 2010
Ceratonova shasta	Myxozoan	ce_sha	28.5	F: CCAGCTTGAGATTAGCTCGGTAA R: CCCCGGAACCCGAAAG P: CGAGCCAAGTTGGTCTCTCCGTGAAAAC	AF001579	Hallett and Bartholomew 2006
Myxobolus arcticus	Myxozoan	my_arc	26.8	F: TGGTAGATACTGAATATCCGGGTTT R: AACTGCGCGGTCAAAGTTG P: CGTTGATTGTGAGGTTGG	HQ113227	Miller et al. 2016
Myxobolus insidiosus	Myxozoan	my_ins	26.43	F: CCAATTTGGGAGCGTCAAA R: CGATCGGCAAAGTTATCTAGATTCA P: CTCTCAAGGCATTTAT	EU346375	Miller et al. 2016
Parvicapsula kabatai	Myxozoan	pa_kab	25.58	F: CGACCATCTGCACGGTACTG R: ACACCACAACTCTGCCTTCCA P: CTTCGGGTAGGTCCGG	DQ515821	Miller et al. 2016
Parvicapsula minibicornis	Myxozoan	pa_min	29.62	F: AATAGTTGTTTGTCGTGCACTCTGT R: CCGATAGGCTATCCAGTACCTAGTAAG P: TGTCCACCTAGTAAGGC	AF201375	Hallett and Bartholomew 2009
Parvicapsula pseudobranchicola[a]	Myxozoan	pa_pse	25.16	F: CAGCTCCAGTAGTGTATTTCA R: TTGAGCACTCTGCTTTATTCAA P: CGTATTGCTGTCTTTGACATGCAGT	AY308481	Jørgensen et al. 2011
Tetracapsuloides bryosalmonae	Myxozoan	te_bry	24.98	F: GCGAGATTTGTTGCATTTAAAAAG R: GCACATGCAGTGTCCAATCG P: CAAAATTGTGGAACCGTCCGACTACGA	AF190669	Bettge et al. 2009
Trypanoplasma salmositica	Protozoan	cr_sal	24.34	F: TCAGTGCCTTTCAGGACATC R: GAGGCATCCACTCCAATAGAC P: AGGAGGACATGGCAGCCTTTGTAT	—	Miller et al. 2016
Dermocystidium salmonis	Protozoan	de_sal	25.49	F: CAGCCAATCCTTTCGCTTCT R: GACGGACGCACACCACAGT P: AAGCGGCGTGTGCC	U21337	Miller et al. 2016
Ichthyophonus hoferi	Protozoan	ic_hof	24.17	F: GTCTGTACTGGTACGGCAGTTTC R: TCCCGAACTCAGTAGACACTCAA P: TAAGAGCACCCACTGCCTTCGAGAAGA	AF467793	White et al. 2013
Si:dkey-78d16.1 protein	Host reference	hkg	45	F: GTCAAGACTGGAGGCTCAGAG R: GATCAAGCCCCAGAAGTGTTTG P: AAGGTGATTCCCTCGCCGTCCGA	—	Miller et al. 2016

[a]Agents that have not been previously described as occurring in this region in peer-reviewed or publicly available grey literature.
Note: F, forward primer; R, reverse primer, P, probe

validated). Tissue preparation, nucleic acid extraction and normalization, cDNA synthesis, specific target amplification, incorporation of artificial control standards and processing controls, and dynamic array preparations were completed according to protocols described by Miller et al. (2016). The primers and probes used in this screening are listed in **Table 2**. Artificial positive controls (Chinook embryo cell control nucleic acids, infectious agent artificial control standards) and negative controls were included in the protocol and a second fluorescent NED-labeled dye (Applied Biosystems, Foster City, CA, USA) was included in all reaction chambers to detect laboratory contamination by artificial control standards. All singleplex HT-qPCR assays were run in duplicate on dynamic arrays. Limits of detection (LOD) specific to each assay (Miller et al. 2016; **Table 2**) were applied to the data at 95% detection confidence, which provides a measure of analytical sensitivity corresponding to the amount of analyte in a sample that is expected to produce a positive result 95% of the time. To be incorporated into the analysis, infectious agents needed to be detected in both duplicates at a quantification cycle (Cq) within the 95% LOD. HT-qPCR results are reported as copy number calculated using sample Cq (average of duplicates) and standard curves for each assay. We characterized infections as emerging versus endemic from a review of peer-reviewed and publicly available grey literature (e.g., government and organization reports); we classified the designation "emerging" for agents not previously known to occur in eastern Canada, recognizing that some may be endemic but simply not previously assessed. Throughout, we were careful not to assume the detection of an infectious agent was equivalent to the detection of disease.

Sequencing

A subset of Atlantic salmon samples in which piscine orthoreovirus (PRV-1) or infectious salmon anemia virus (ISAV) were detected were selected for sequence analysis using NGS to validate HT-qPCR detections and conduct phylogenetic analyses. All PRV detections described in this study refer to the PRV-1 genotype. Two North American origin Atlantic salmon collected in offshore waters near Greenland in 2017 were positive for ISAV, but only one had sufficient sequencing coverage for analysis. Three Atlantic salmon samples in which PRV-1 was detected were sequenced: one North American origin, marine-collected fish sampled at the Maniitsoq market, Greenland, in 2017 (J3575_NAM, multi-tissue, PRV-1 Ct of 12.4); one European origin fish sampled at the Paamiut market, Greenland, in 2016 (J3611_EUR, kidney only; PRV-1 Ct of 13.3); and one aquaculture escapee collected in the Magaguadavic River (J3542_MAG, multi-tissue; PRV-1 Ct of 19.1). PRV-1 was chosen as an ideal candidate to evaluate transmission potential at sea and between wild and farmed Atlantic salmon as it has widespread prevalence across the range of Atlantic salmon (and beyond) and was detected in both European and North American origin fish at sea and in aquaculture escapees in this study. To our knowledge, this is the first publication of the full genome sequence for PRV obtained from hosts collected in eastern Canada.

All NGS samples were processed on the same v2 300 Illumina MiSeq sequencing run PRV-1. Samples (J3575_NAM, J3611_EUR, and J3542_MAG) generated ~2.9, 3.0, and 2.4 mol/L post-trim reads, respectively, with average quality scores of 35.0 or greater. We applied target enrichment for all known viral genomes that infect salmon via the SureSelect^{XT} RNA Direct NGS target workflow (Agilent, Santa Clara, California, USA). A custom set of RNA target enrichment probes (120 base pairs (bp) in length and staggered along the exome or viral RNA) were designed to the genomes of salmonid, relevant fish, and other emerging viruses that were included in our infectious agent HT-qPCR screening platform. These sequences (435.384 kbp) and subsequent bait oligonucleotides included the PRV-1 and ISAV genomes. In the case of ISAV, multiple sequences were included for some segments to represent the various genogroups, hyper polymorphic region (HPR0), and sequences that were <85% homologous. Baits that failed the SureSelect quality assurance or quality control parameters and (or) significantly matched salmonid genes via blast searches were removed, leaving the final set of enrichment probes at 15 609.

We prepared the RNAseq library with the SureSelect Strand-Specific RNA library Prep kit (Agilent, Santa Clara, California, USA) according to manufacturer's instructions. The adaptor-ligated samples were purified with the Agencourt AMPure XP system (Beckman Coulter, Brea, California, USA). High sensitivity (HS) DNA chips were run on the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, California, USA) to determine the final library size and the Qubit dsDNA HS kit (Invitrogen, Carlsbad, California, USA) was used to determine the concentration. Hybridization of the adapted cDNA library with the viral SureSelect bait capture library (Agilent, Santa Clara, California, USA) was performed at 65°C for 24 h according to manufacturer's instructions. The cDNA library or capture library hybrids were captured on streptavidin magnetic beads and purified with the Agencourt AMPure XP system (Beckman Coulter, Brea, California, USA). Index tags were added to the postcaptured libraries through 14 rounds of amplification and purified using the Agencourt AMPure XP system (Beckman Coulter, Brea, California, USA). HS DNA chips were run on the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, California, USA) to determine the final library size, and the concentration was determined using the Qubit dsDNA HS kit (Invitrogen, Carlsbad, California, USA). Sample libraries were normalized to 4 nmol/L and denatured and diluted to obtain a final library of 20 pmol/L. The Atlantic salmon enriched RNAseq libraries were processed on one paired end v2 300 bp kit on the Illumina MiSeq System (Illumina, San Diego, California, USA), which included a 10% PhiX Control v3 Library spike-in to improve overall run quality.

Sequence analysis was performed using the Partek Flow software (Partek Inc., St. Louis, Missouri, USA). Adaptors and bases with Phred quality scores <30 were trimmed from both ends and reads less than 25 bp were removed. The remaining reads were aligned to the PRV genome segments of the Norwegian isolate Salmo/GP-2010/NOR (Palacios et al. 2010) using the BWA-MEM (Burrows Wheeler Aligner) software and algorithm with default parameters (Li 2013). SAMtools variant caller was utilized to determine SNPs using the default settings (Li et al. 2009; Li 2011). The consensus sequences were compared against all available sequences in GenBank (Benson et al. 2003) using the BLAST program (blast.ncbi.nlm.nih.gov/Blast.cgi) via the National Center for Biotechnology Information (Altschul et al. 1990) to identify their closest matches across each segment.

ISAV sequences were de novo assembled to enable unbiased assembly of a deletion which is known to occur on segment 6 of the genome that has been associated with virulence (Gagné and LeBlanc 2018). Adapters were removed using Trimmomatic and host-associated reads were removed by alignment to the Atlantic salmon genome using the Burrows–Wheeler aligner (Davidson et al. 2010; Li and Durbin 2010; Bolger et al. 2014). Unmapped sequences were de novo assembled using SPAdes (Bankevich et al. 2012). The viral genomic sequences were aligned using MUSCLE (within Geneious) (Edgar 2004), and an approximately maximum-likelihood phylogenetic tree were constructed using FastTree (Price et al. 2010). The trees were displayed and annotated using Figtree (available at tree.bio.ed.ac.uk/software/figtree/) and ggtree (Yu et al. 2018). PRV and ISAV segment consensus sequences for all sequenced samples were deposited into GenBank under the accession number series MN106286 to MN106316.

Statistical methodology

To quantify and visualize differences in infectious agent communities based on host group membership (Restigouche, St. John, Magaguadavic, Greenland-collected North American origin, and Greenland-collected European origin), we used nonmetric multidimensional scaling (NMDS) analysis and permutational multivariate analysis of variance (PERMANOVA; **Fig. 2**). Infectious agent loads were normalized to the maximum copy number for each agent (i.e., the quotient of each load value and the maximum load value of that agent in the study) prior to NMDS and PERMANOVA analysis. Any agent detected in fewer than two individuals was removed from the analysis as well as any host with no agents detected to reduce statistical bias ($N = 120$ fish included in NMDS). Community

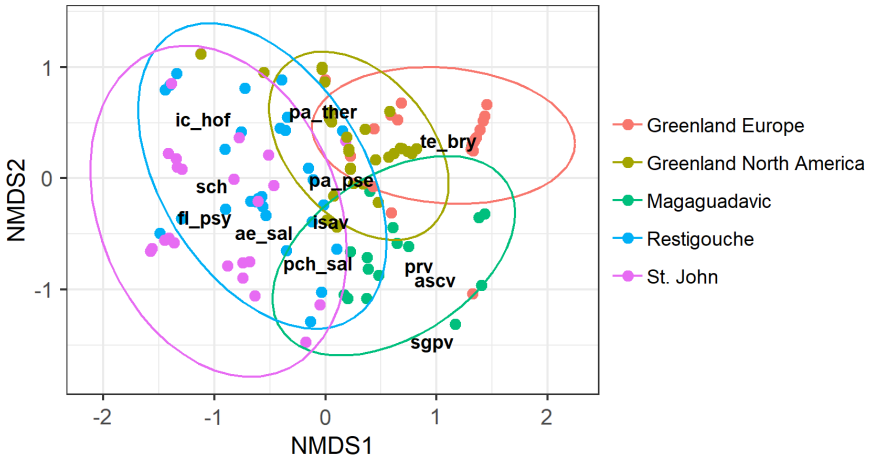

Fig. 2. A nonmetric multidimensional scaling (NMDS) analysis of infectious agent profiles determined using HT-qPCR of Atlantic salmon tissues. Adult Atlantic salmon were collected in offshore waters near Greenland (European or North American origin) and three New Brunswick rivers, comprising two wild populations (Restigouche, St. John) and aquaculture escapees (in the Magaguadavic River). Points correspond to individual fish, which are plotted according to load gradients of multiple infectious agents. Abbreviations are defined in Table 2.

composition was also visually represented by comparing how agent prevalence and community composition differed among groups; this was achieved by plotting the prevalence of each agent as a proportion of the cumulative prevalence (i.e., the sum of the proportional prevalence of all agents; Fig. 3).

As a cumulative infection metric, relative infection burden (RIB) was calculated for each fish as a composite score incorporating aspects of pathogen richness and loads:

$$\mathrm{RIB} = \sum_{i \in m}^{m} \frac{L_i}{L\max_i} \tag{1}$$

where for a given fish, the copy number of the ith infectious agent (L_i) is divided by the maximum copy number within the population for the ith infectious agent ($L_{\max i}$) and then summed across all agents (m) detected in the given fish (Bass et al. 2019). We used RIB as a community-level metric of cumulative infection burden, which comprises load, prevalence, and richness information when averaged across a host group to determine if fish sampled in the Labrador Sea in different years (2016 and 2017) could be pooled within continental stock assignment (North American or European origin). Linear regression was used to compare infection profiles between years within stock groups (i.e., identify any significant effect of sampling year within stock groups). Because of the right-skewed distribution of RIB, this variable was log-transformed to meet the assumptions of normality. Generalized linear models (GLM) were used to identify differences in infectious agent richness among groups (total unique agents per host). Where sample sizes allowed (≥10 detections in each group), analysis of variance was used to identify load differences between groups.

Results

Genotyping of marine-collected Atlantic salmon and annual infection differences

Among fish captured in the Labrador Sea near Greenland (marine-collected; Fig. 1), both European and North American stocks were represented in 2016 (kidney tissue only; European origin: $N = 33$,

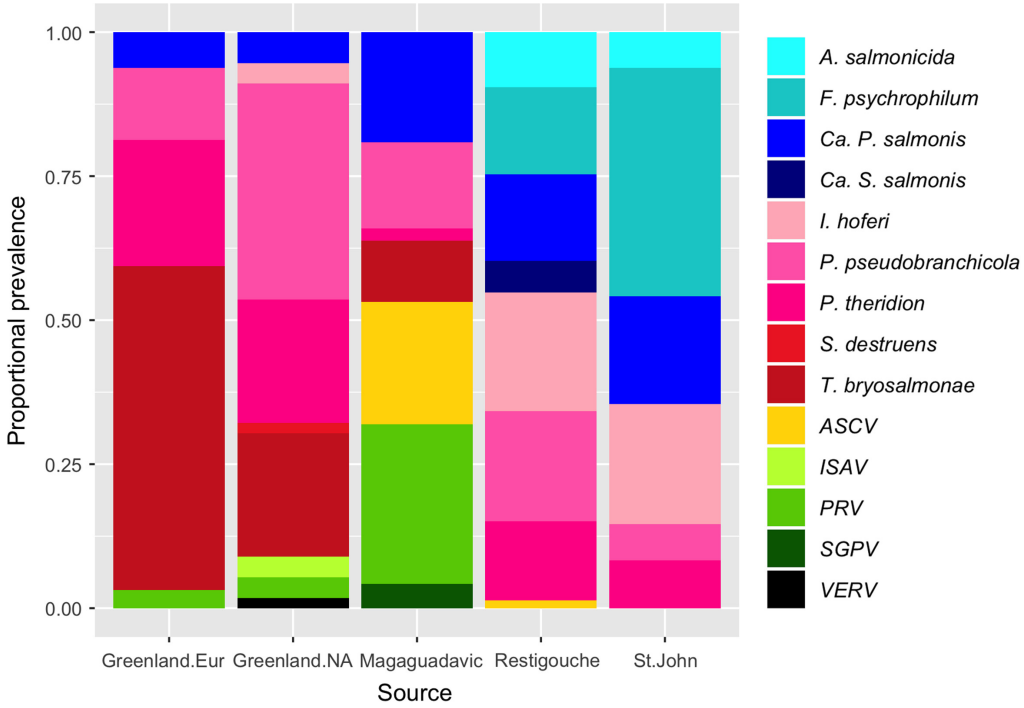

Fig. 3. Proportional prevalence of infectious agents determined using high-throughput qPCR of Atlantic salmon tissues from fish in marine (Greenland: European (EUR) origin and Greenland: North American (NA) origin) and riverine (Magaguadavic, Restigouche, and St. John rivers, New Brunswick, Canada) environments. Proportional prevalence is defined here as the prevalence of each infectious agent divided by the sum of prevalence values for all agents detected in each host group.

North American origin: $N = 10$) and 2017 (multi-tissue; European origin: $N = 4$, North American origin: $N = 26$). RIB did not differ significantly between years within continental stock groupings ($p > 0.05$ for both stock groups), so data from 2016 to 2017 were pooled within stock groups for subsequent analyses and reporting. Year-specific data for marine-collected fish can be found in Table S1.

Prevalence and load differences among collection locations and strains

Fourteen infectious agents were detected overall (both marine and freshwater samples), including four species of bacteria, five viruses, and five other microparasite species, commonly occurring as multiple infections within hosts (Table 3; Figs. 2, 3). PERMANOVA identified a significant effect of group (i.e., collection location and continental origin) on infection profiles ($r^2 = 0.35$, $p < 0.01$). Three NMDS axes sufficiently comprised variation in infection community profiles (stress = 0.07), with the majority of group separation comprised by the first two axes (Fig. 2). River-collected wild fish (Restigouche, St. John) showed the highest degree of overlap in NMDS positioning and the largest 95% confidence interval areas, suggesting high individual variability in infection profiles of freshwater-collected wild adults relative to other groups. Greenland-collected North American and European origin fish had similar NMDS positioning, though European origin fish loaded higher on axis 1 (furthest from river-collected wild fish). The escapee group was isolated from other groups on the NMDS plot, largely due to strong viral agent influences on infection profiles.

Table 3. Infectious agents detected using high-throughput qPCR of tissues (gill, kidney, or a pool of heart, gill, and kidney) from adult Atlantic salmon captured in the Labrador Sea near Greenland (marine) and in three rivers in eastern Canada.

	Marine: N. American origin (*N* = 36)			Marine: European origin (*N* = 37)			Magaguadavic River (*N* = 17)			Restigouche River (*N* = 30)			St. John River (*N* = 30)			Total
Agent	*N*	Mean	SD	*N*	Mean	SD	*N*	Mean	SD	*N*	Mean	SD	*N*	Mean	SD	*N*
Parasites																
P. pseudobranchicola	21	6463	15 914	4	538	384	7	1927	2716	14	1674	3924	3	1087	837	49
T. bryosalmonae	12	31 547	71 331	18	351 773	656 993	5	71 395	99 215	0	—	—	0	—	—	35
P. theridion	12	3395	5968	7	3697	5195	1	181	—	10	2807	6367	4	3527	6171	34
I. hoferi	2	385	482	0	—	—	0	—	—	15	1 608 948	3 587 305	10	338 474	433 839	27
S. destruens	1	1485	—	0	—	—	0	—	—	0	—	—	0	—	—	1
Bacteria																
Ca. P. salmonis	3	83 350	130 482	2	248 725	194 166	9	148 466	255 231	11	1 989 479	4 397 990	9	109 302	221 325	34
F. psychrophilum	0	—	—	0	—	—	0	—	—	11	3614	5175	19	2284	4426	30
A. salmonicida	0	—	—	0	—	—	0	—	—	7	2 805 611	7 186 680	3	139 877	241 677	10
Ca. S. salmonis	0	—	—	0	—	—	0	—	—	4	85	53	0	—	—	4
Viruses																
PRV	2	5980	8363	1	18 799	—	13	24	33	0	—	—	0	—	—	16
ASCV	0	—	—	0	—	—	10	188 248	383 841	1	10	—	0	—	—	11
ISAV	2	218	264	0	—	—	0	—	—	0	—	—	0	—	—	2
SGPV	0	—	—	0	—	—	2	227	81	0	—	—	0	—	—	2
VERV	1	22	—	0	—	—	0	—	—	0	—	—	0	—	—	1
Richness	—	1.6	1.1	—	0.9	0.9	—	2.8	1.3	—	2.4	1.3		1.6	1.3	—
RIB	—	0.23	0.5	—	0.14	0.3	—	0.21	0.34	—	0.36	0.4		0.12	0.23	—

Note: Total positive detections (*N*) and the mean and standard deviation (SD) of agent copy numbers, richness (total unique agents per host), and relative infection burden (RIB) are shown for each agent relative to the sampling location. RIB is a composite metric that incorporates richness and load information from all agents detected in each host; PRV, piscine orthoreovirus; ASCV, Atlantic salmon calicivirus; ISAV, infectious salmon anemia virus; SGPV, salmonid gill poxvirus; VERV, encephalopathy and retinopathy virus.

Among marine-collected adult Atlantic salmon, nine infectious agent species were detected, with greater richness among the North American origin group (nine agents; mean 1.6 agents per individual) than European origin fish (five agents; mean 0.9 agents per individual; GLM: $p = 0.004$; Fig. 3). Among marine-collected fish, all agents detected in the European origin group (*Parvicapsula pseudobranchicola*, *Tetracapsuloides bryosalmonae*, *Paranucleospora theridion*, *Candidatus* Piscichlamydia salmonis, and PRV-1) were also detected in the North American origin group; four additional agents were detected in the North American group (*Ichthyophonus hoferi*, *Sphaerothecum destruens*, ISAV, viral encephalopathy, and retinopathy virus (VERV)). Mean RIB was greater for North American origin marine fish (0.23) than European origin (0.14), but not significantly different ($F = 0.81$, $p = 0.37$). In the marine environment, prevalence among European origin fish was dominated by *T. bryosalmonae*, whereas *P. pseudobranchicola* was the most prevalent agent among North American fish (Fig. 3). Half of the European origin fish had positive detections of *T. bryosalmonae* (49%), whereas *P. theridion* (19%), *P. pseudobranchicola* (11%), *Ca.* P. salmonis (5%), and PRV-1 (3%) were detected

at lower prevalence. North American origin fish also carried *P. pseudobranchicola* (58%), *T. bryosalmonae* (33%), and *P. theridion* (33%) at moderate prevalence, whereas *Ca*. P. salmonis (8%), *I. hoferi* (6%), PRV-1 (6%), ISAV (6%), VERV (3%), and *S. destruens* (3%) were detected at lower prevalence. Except for *T. bryosalmonae* (European origin loads were greater; $F = 5.07$, $p = 0.032$), agent loads were similar between continental origin groups at sea (nonsignificant at $p > 0.05$ or insufficient detections for comparison).

Adult Atlantic salmon were collected from freshwater and brackish sites in the Magaguadavic ($N = 17$), Restigouche ($N = 30$), and St. John ($N = 30$) rivers in eastern Canada (**Fig. 1**). Aquaculture escapees sampled in the Magaguadavic River (multi-tissue) were unique in their infection profiles, which included three viruses, one bacterial species, and three other microparasites (**Fig. 3**). The Magaguadavic infection profile more closely resembled that of marine-collected fish than the wild river-sampled groups. Among escapees, PRV-1 was the most prevalent agent (76%), followed by ASCV (59%), *Ca*. P. salmonis (53%), and *P. pseudobranchicola* (41%), *T. bryosalmonae* (29%), salmonid gill poxvirus (SGPV) (12%), and *P. theridion* (6%).

The infection profile of returning adults from the St. John population (gill tissue only, threatened population with high aquaculture influence) was similar to those from the Restigouche River population, but with slightly lower richness (six agents). Agents detected in St. John fish included *Flavobacterium psychrophilum* (63%), *I. hoferi* (33%), *Ca*. P. salmonis (30%), *P. theridion* (13%), *P. pseudobranchicola* (10%), and *Aeromonas salmonicida* (10%).

The Restigouche population (multi-tissue, low aquaculture influence) had the greatest infectious agent richness of freshwater-sampled groups (eight agents), which included all agents detected in the St. John population plus additional bacterial, viral, and myxozoan agents. Among Restigouche fish, *I. hoferi* (50%), and *P. pseudobranchicola* (47%) were detected in approximately half of the sampled population, whereas *Ca*. P. salmonis (37%), *F. psychrophilum* (37%), *P. theridion* (33%), and *A. salmonicida* (23%) occurred at moderate prevalence, and salmon *Candidatus* Syngnamydia salmonis (13%) and ASCV (3%) at low prevalence.

The primary characteristics that differentiated infection profiles among river-collected groups were the enhanced viral richness and *T. bryosalmonae* prevalence in the Magaguadavic River sampled escapees relative to wild populations and the lower infectious agent richness in the St. John population. Infectious agent richness of the St. John population (mean = 1.6) was significantly lower than that of the Restigouche population (mean = 2.5; GLM: $p = 0.02$), whereas the escapee richness (Magaguadavic: mean = 2.8) was most similar to the Restigouche group ($p = 0.45$), but with very different agent composition. No significant differences in individual agent loads were identified, either due to low sample sizes (low power due to few positive detections) or nonsignificant ANOVA results. RIB was lowest overall in the St. John population but did not significantly differ among river-sampled groups (ANOVA: $p = 0.51$; **Table 3**).

PRV and ISAV sequence analysis

Analysis of segment six of the ISAV genome revealed that the strain identity in two North American origin fish at sea belonged to the "European" genotype (Gagné and LeBlanc 2018). One isolate (J3577) included the full-length HPR0 (identified by the absence of a deletion on segment six of the genome); the other isolate (J3574) did not have HPR coverage to reveal type.

From the PRV NGS analysis, a reference-guided assembly of J3575_NAM generated 228 846 total alignments (7.8% of total reads) to the Norwegian PRV Salmo/GP-2010/NOR 10 segment reference genome (Palacios et al. 2010), which resulted in 100× coverage for >99% of the genome and an average depth of 2568 reads. J3611_EUR generated 635 925 total alignments (25.7% of total reads) to the

reference genome, which resulted in 100× coverage for >99% of the genome with an average depth of 7009 reads. Finally, J3542_MAG generated 7762 total alignments (0.3% of total reads) to the reference genome, which resulted in 30× coverage for >79% of the genome with an average depth of 85 reads. Over all three samples, segments M2 (outer shell) and S1 (outer clamp/NS p13) displayed the greatest variation relative to the reference genome (97.2%/60–61 SNPs and 96.8%–97.2%/30–35 SNPs, respectively). For J3575_NAM and J3611_EUR, segments S3 (NS RNA) and L1 (core shell) displayed the least variation (99.5%/6 SNPs and 99.3%/28 SNPs, respectively), whereas for J3542_MAG, segments L3 (core RdRp) and L1 (core shell) displayed the least variation relative to the reference genome (99.6%/17 SNPs and 99.4%/23 SNPs, respectively).

BLAST searches and phylogenetic analysis of the S1 segment reveal that all three samples cluster with PRV-1a along with all other Canadian strains to date (Figs. 4, S1). The PRV genome sequences of

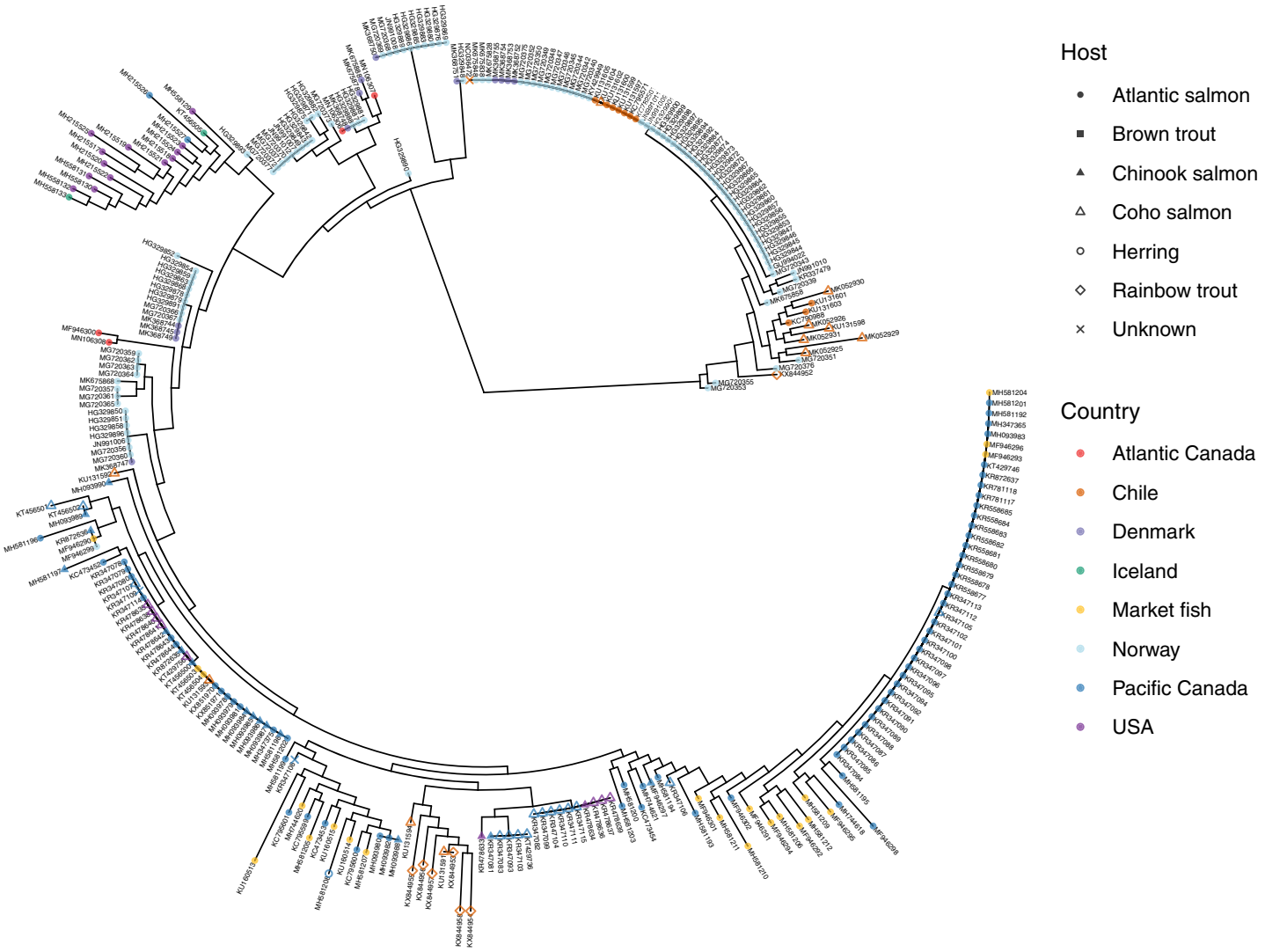

Fig. 4. PRV-1 phylogeny based on publicly available sequences trimmed to the shortest sequence included (732 nucleotides). Tip labels correspond to GenBank Accession numbers. Tip point shapes and colors correspond to the host and country of where each sample was collected. Samples collected in this study are labelled red (MN106306–MN106308).

J3575_NAM and J3611_EUR were virtually identical over all segments except for one SNP in both L3 (core RdRp) and S1 (outer clamp/NS p13; **Fig. 4**). These sequences were isolated from Atlantic salmon sampled at Maniitsoq market in September 2017 and Paamiut market in September 2016, respectively, after being caught in offshore marine waters near Greenland. The PRV genome sequence of J3542_MAG (aquaculture escapee caught in the Magaguadavic River in September 2017) was >99.0% homologous to the two Greenland-collected samples across all segments with the exception of segment M3 (NS factory), which was a 98.9% homologous. PRV isolates from the two marine-collected (Greenland) hosts clustered with wild and cultured Atlantic salmon from Norway (Garseth et al. 2013), wild fish from Denmark, and cultured fish in the Faroe Islands (Denmark). Alternatively, the escapee isolate J3542_MAG was most similar in the S1 segment (99.7%) to VT03022017-69 (accession No. MF946300) isolated from an Atlantic salmon recovered after escaping a farm near McNutts Island, Nova Scotia, Canada, in March 2017 (Kibenge et al. 2017; F. Kibenge, personal communication).

Discussion

We used HT-qPCR and viral sequencing to characterize variability in the infectious agent profiles and potential transmission dynamics of wild and cultured Atlantic salmon. We identified 14 agents in the tissues of salmon collected as sub-adults in the Labrador Sea near Greenland or as mature adults in three eastern Canadian rivers. Five of these agents, to our knowledge, have not been described as occurring in eastern Canada in peer-reviewed or publicly available literature, which included two bacteria (*Ca.* P. salmonis and *Ca.* S. salmonis), one virus (ASCV), one microsporidian (*P. theridion*), and one myxozoan parasite species (*P. pseudobranchicola*). SGPV and PRV-1 have only been reported to the International Council for the Exploration of the Sea (ICES) as occurring in this region (ICES 2018), and the S1 segment sequence of PRV-1 was isolated from a fish in Nova Scotia and is available in GenBank, but its geographic source is labeled only as "Canada" (Kibenge et al. 2017; F. Kibenge, personal communication 2019). To our knowledge, this is the first publication of the full genome sequence for PRV obtained from hosts collected in eastern Canada. Our results identify several opportunities for future research and a need to improve our knowledge of infectious agent transmission dynamics and disease potential among wild and cultured Atlantic salmon.

There were three key findings in this study. First, we identified both North American and European origin fish in marine waters off the coast of Greenland, providing further evidence to support this feeding area as a multi-continental melting pot (Hansen and Quinn 1998; Sheehan et al. 2012; Chaput et al. 2019). The similar infectious agent composition between North American and European origin hosts sampled in Greenland, and the finding of virtually identical PRV-1 genome sequences in fish of different continental origins supports the hypothesis of inter-continental transmission of pathogens in North Atlantic feeding areas where stocks mix (Madhun et al. 2018; Vendramin et al. 2019). Second, the high degree of similarity of the PRV-1 genome sequenced from two independent aquaculture escapees in eastern Canada suggests a common source or transmission of PRV-1 within aquaculture facilities that was distinct from the two wild fish sequenced in this study. Third, we found no significant effect of aquaculture proximity on infection profiles of wild returning adult salmon sampled in the St. John and Restigouche rivers of New Brunswick, Canada. Below we present an expanded discussion around these three findings.

Marine transmission potential between continental stocks

Phylogenetic analysis of the three PRV-1 detections sequenced in our study has uncovered potential transmission pathways for PRV-1 (and possibly other agents) between Europe and the Atlantic coast of North America. Nearly identical sequences of PRV-1 were isolated from European and North American origin fish sampled from marine feeding grounds near Greenland. This finding supports

the hypothesis that ocean feeding grounds, where fish from different continents converge, provide a natural pathway of agent transmission between Europe and North America (Gagné and LeBlanc 2018; Madhun et al. 2018; Vendramin et al. 2019). We found high homology between sequences of PRV-1 isolated from two escaped farm salmon in eastern Canada in 2017, one collected from the Magaguadavic River in New Brunswick (this study) and the other recovered in Shelburne Harbour, Nova Scotia (Kibenge et al. 2017; F. Kibenge, personal communication). PRV-1 S1 sequences from both aquaculture escapees differed from those isolated from wild fish at sea (Greenland-collected), and PRV-1 was not detected in either wild river-sampled population in this study. It is worth noting that the PRV-1 variants observed both in Greenland and in eastern Canada all clustered with the Norwegian "wild-type" variant, classified in some studies as PRV-1a (Kibenge et al. 2019) that, based on the S1 segment, is divergent from PRV-1b, has been proposed to be of higher virulence (Dhamotharan et al. 2019) and has been shown to be the causative agent of heart and skeletal muscle inflammation (HSMI) (Wessel et al. 2017). However, HSMI has been diagnosed in farmed Atlantic salmon in western Canada in association with PRV-1a (Di Cicco et al. 2017). We propose that natural routes of transcontinental transmission favor movements of less virulent pathogen strains, allowing more time, for example, for European source hosts to migrate to sea and transmit the virus to North American hosts, and for infected North American hosts to then survive their migration back to natal rivers, thereby completing the cycle of intercontinental exchange.

We detected and confirmed with genome sequencing the avirulent HPR0 strain of ISAV in a North American origin fish collected at sea near Greenland. ISAV is in the family *Orthomyxoviridae* and the virulent form of the virus (HPRΔ) can be highly pathogenic in aquaculture settings (Lovely et al. 1999). ISAV was first detected in Atlantic Canada in 1996, with sequence analysis showing three separate emergences in North America, including avirulent (HPR0) and virulent (HPRΔ) forms (Gagné and LeBlanc 2018). The virus has been observed in wild and cultured salmon in eastern Canada and the USA (Bouchard et al. 1999 2001; Ritchie et al. 2001; Olivier 2002) and detected at low prevalence (<1%) in escaped aquaculture fish in Norway (Madhun et al. 2017). In wild fish, most if not all detections have shown no evidence of disease (including challenged hosts); therefore, it is assumed to be the avirulent HRP0 strain that is affecting asymptomatic wild hosts (Plarre et al. 2005; Gustafson et al. 2018). It is not known whether wild fish have been affected by virulent strains of ISAV, which can develop spontaneously from the avirulent strain through a deletion in segment 6 (Nylund et al. 2003; Godoy et al. 2013). The virulent strain causes acute disease and is therefore unlikely to be detected in sampling of wild fish. No detections of ISAV were found in European origin fish; therefore, we could not compare ISAV sequences between hosts of different continental origins to characterize the potential for its intercontinental exchange at marine feeding areas.

Another virus, VERV, was detected in one North American origin fish at sea. This piscine nodavirus has a wide geographic range, including coastal waters of New Brunswick, Canada, where it has been described at extremely low prevalence in wild winter flounder (*Pleuronectes americanus*) (Barker et al. 2002). Susceptibility of Atlantic salmon to VERV infection and disease has been demonstrated following intraperitoneal challenge (Korsnes et al. 2005) but not via cohabitation (Korsnes et al. 2012). The detection of VERV in an Atlantic salmon in eastern Canada and at low prevalence and variable loads in wild and farmed salmon in western Canada (Tucker et al. 2018; Laurin et al. 2019) warrants its continued monitoring in wild fish to confirm low susceptibility and virulence.

Among fish collected in Greenland, overall infection profiles were quite similar between continental stock origin groups, generally including the same agents but at a higher richness in North American origin fish. Key differences between European and North American stocks were associated with the prevalence of *T. bryosalmonae* and *P. pseudobranchicola*. *Tetracapsuloides bryosalmonae* is a prevalent parasite endemic in eastern Canada (Khan 2009) that can cause proliferative kidney disease,

primarily at elevated water temperatures (Bettge et al. 2009). The absence of this agent in returning adult salmon in this study is interesting given its moderate prevalence in fish at sea. *Parvicapsula pseudobranchicola* is a prevalent myxosporean parasite originally characterized in Norway and newly detected in eastern Canada; it affects the pseudobranch of fishes as a generally low-virulence agent but can cause runting in cultured fish (Nylund et al. 2018). Both of these parasites cannot be horizontally transmitted at sea (Morris and Adams 2006; Nylund et al. 2018), so disparate relative prevalence of these two agents depending on continental origin (freshwater stage) is unsurprising.

Infection profiles of wild and escaped farm salmon in rivers

Among adult salmon in three New Brunswick rivers, the infection profile of a group of aquaculture escapees in the Magaguadavic River was unique relative to two wild salmon populations from the Restigouche and St. John rivers. Contrary to our expectation that proximity to aquaculture would enhance infection severity of wild populations through acquisition of agents that thrive in culture settings, infectious agent loads and richness were highest in the Restigouche population, which was furthest from aquaculture influence. St. John fish could only be nonlethally sampled for gill as opposed to gill, heart, and kidney from Restigouche and escapee fish. However, gill has been shown to have equal or greater infectious agent richness than multi-tissue pools (Teffer and Miller 2019). Only one virus, ASCV, was detected (in just one host) in the Restigouche River. The only other fish with ASCV detections in this study were aquaculture escapees. ASCV is common in Norwegian fish culture (Mikalsen et al. 2014) and is the most commonly detected virus in farmed salmon in western Canada (K. Miller, unpublished data). ASCV is often detected as a coinfection with other agents (e.g., with PRV), but studies to date have had variable and often inconclusive findings for its independent pathological effects (Mikalsen et al. 2014; Wiik-Nielsen et al. 2016). Interestingly, a related fish calcicivirus in baitfish was associated with clinical disease only when coinfected with a second virus (Mor et al. 2017). Given its widespread prevalence, future studies should evaluate sequence variation among ASCV isolates across geographic regions and examine the potential role ASCV plays in disease progression in coinfections.

Aquaculture escapees had the second highest overall infectious agent richness with few bacterial species and the highest prevalence of viruses of any group. Greater than half of escapees carried PRV-1 and ASCV, often as coinfections; for example, most fish positive for ASCV were also positive for PRV-1, and both SGPV-positive hosts were also positive for PRV-1 and ASCV. SGPV has previously been described in Norway and the Northeast Atlantic Ocean (Nylund et al. 2008; Garseth et al. 2018). In this study, we detected SGPV solely in escaped aquaculture fish, and its occurrence in eastern Canada has been reported to the ICES (ICES 2018). The composition of bacterial and microparasite species hosted by aquaculture escapees was more similar to marine-collected than river-sampled fish, despite hosts being collected in a freshwater environment (i.e., exposed to freshwater infectious agents). Closer alignment of infection profiles between escapees and marine-collected fish may be due to aquaculture practices that inhibit some infections (e.g., antibiotics for bacterial agents) as well as alternate dietary sources (e.g., fish feed versus wild, potentially infected prey) and extended coastal residence.

Microparasite species composition in wild Atlantic salmon in the Restigouche and St. John rivers was highly congruent with infection profiles described in adult Pacific salmon in western Canadian rivers (Bass et al. 2017; Teffer et al. 2017). Exceptions to this included *T. bryosalmonae* and *S. destruens*, which were absent in river-collected Atlantic salmon in this study. The consistent prevalence of *P. theridion* (aka *Desmozoon lepeophtherii*, a candidate causative agent of proliferative gill inflammation) across sampling locations in this study aligns with the widespread occurrence of its alternate sea louse host (*Lepeophtheirus salmonis*) in eastern Canada (Carr and Whoriskey 2004; Sveen et al. 2012). High prevalence among wild Atlantic salmon in this study suggests that *P. theridion* is not highly

virulent in this system. Lower prevalence of this agent among marine-collected fish versus freshwater adults supports adult infections as enhanced in the nearshore environment (Hendricks 1972; Rand 1992). The relatively low prevalence (one fish) of *P. theridion* in aquaculture escapees is interesting and potentially due to farm practices that inhibit exposure or spore development (e.g., low-temperature environment) (Sanchez et al. 2000; Sveen et al. 2012). We were unable to find any peer-reviewed literature describing the presence of *P. theridion* in eastern Canada despite records of its occurrence and association with sea lice in Scotland, Norway, and the eastern Pacific (Freeman and Sommerville 2011; Nylund et al. 2011; Jones et al. 2012; Sveen et al. 2012; Miller et al. 2014). *Ichthyophonus hoferi*, a protistan parasite, was detected at moderate prevalence among wild salmon in both rivers and is endemic in the Northwest Atlantic Ocean and coastal waters of eastern Canada (Hendricks 1972; Rand and Cone 1990; Rand 1992). Ecological impacts of this agent should be explored as *Ichthyophonus* spp. infections can affect host swimming ability, especially under suboptimal environmental conditions (e.g., high temperature) (Tierney and Farrell 2004; Kocan et al. 2009).

The Restigouche and St. John populations hosted two Chlamydiae species (*Ca.* P. salmonis and *Ca.* S. salmonis) that have not yet been described in eastern Canada. *Candidatus* Syngnamydia salmonis is a newly described species potentially associated with gill disease in Norway (Nylund et al. 2015) and has been detected intermittently among wild and aquaculture salmon on the west coast of Canada (Miller et al. 2014; Thakur et al. 2018). *Candidatus* Piscichlamydia salmonis is known to affect Atlantic salmon in aquaculture (Norway, Ireland) and farmed Arctic char (*Salvelinus alpinus*) in the USA and Canada (Draghi et al. 2004, 2010). The loads of these bacteria described in this study are unlikely to have been pathogenic, as the density of epitheliocysts would need to be extremely high to affect cell function and host respiration (Pawlikowska-Warych and Deptuła 2016). Other bacterial species detected in this study included *F. psychrophilum* and *A. salmonicida*. *Flavobacterium psychrophilum* is a common bacterial agent with a global distribution across temperate zones; its virulent strains can be pathogenic at low temperatures (<16 °C) (Holt 1987; Nilsen et al. 2014). *Aeromonas salmonicida* is the causative agent of furunculosis, endemic in eastern Canada (Foda 1973) and can be highly virulent. Preventative vaccination for *A. salmonicida* is generally applied in aquaculture (Mitchell and Rodger 2011), which may explain the absence of these bacteria in escapees in this study. Both of these bacterial agents can contribute to secondary infections following dermal injury (Svendsen and Bøgwald 1997; Janda and Abbott 2010; Starliper 2011; Teffer et al. 2017).

Conclusions

We present for the first time a quantitative molecular screening of dozens of infectious agents in wild and escaped Atlantic salmon in offshore feeding areas of the Northwest Atlantic Ocean and three eastern Canadian rivers. Our results offer baseline coinfection and viral phylogenic data that provide insight into potential transmission dynamics among wild Atlantic salmon stocks at sea and evidence to support natural incursion of infectious agents from Europe to North America. Continued study of marine infection dynamics is warranted to confirm this natural transatlantic transmission route for infectious agents, which would introduce a managerial challenge for infectious disease control if pathogenic effects result from this exchange.

This study was undertaken to improve baseline knowledge of infectious agents carried by wild and escaped cultured Atlantic salmon and investigate transmission potential through the use of phylogenetic analysis of viral isolates. We cannot assign pathology to any infectious agents detected in our study as host health and performance were not evaluated. As with any study of wild animals, it is also possible that heavily infected fish or those carrying highly pathogenic agents died or were predated prior to sampling (Miller et al. 2014). We were limited in our comparative analysis because of disparity in tissue types sampled across sites but included these informative results as a starting point for future hypothesis testing on transmission dynamics in eastern Canadian waters. Our understanding

of the mechanisms and frequency of infectious agent transmission among wild fishes is still in its infancy, especially for highly migratory and offshore marine hosts like Atlantic salmon and for pathogens that can cross continental borders via marine exchange. Molecular tools can be used to rapidly advance this knowledge and, combined with telemetry approaches and experimental studies, can improve our understanding of the disease ecology of Atlantic salmon throughout their range.

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Author contributions

JC, EBB, and KMM conceived and designed the study. JC, AT, AS, IB, DD, C-AG, and EBB performed the experiments/collected the data. AKT, JC, AS, GM, and KMM analyzed and interpreted the data. JC, IB, DD, C-AG, EBB, and KMM contributed resources. AKT, JC, DD, C-AG, EBB, GM, and KMM drafted or revised the manuscript.

Competing interests

The authors have declared that no competing interests exist.

Data availability statement

All relevant data are within the paper and Supplementary Material.

Supplementary materials

The following Supplementary Material is available with the article through the journal website at doi:10.1139/facets-2019-0048

Supplementary Material 1

Supplementary Material 2

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