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Piscine reovirus in wild and farmed salmonids in British Columbia, Canada: 1974–2013

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Abstract

Piscine reovirus (PRV) was common among wild and farmed salmonids in British Columbia, western Canada, from 1987 to 2013. Salmonid tissues tested for PRV by real-time rRT-PCR included sections from archived paraffin blocks from 1974 to 2008 ($n = 363$) and fresh-frozen hearts from 2013 ($n = 916$). The earliest PRV-positive sample was from a wild-source steelhead trout, *Oncorhynchus mykiss* (Walbaum), from 1977. By histopathology ($n = 404$), no fish had lesions diagnostic for heart and skeletal muscle inflammation (HSMI). In some groups, lymphohistiocytic endocarditis affected a greater proportion of fish with PRV than fish without PRV, but the range of Ct values among affected fish was within the range of Ct values among unaffected fish. Also, fish with the lowest PRV Ct values (18.4–21.7) lacked endocarditis or any other consistent lesion. From 1987 to 1994, the proportion of PRV positives was not significantly different between farmed Atlantic salmon, *Salmo salar* L. (44% of 48), and wild-source salmonids (31% of 45). In 2013, the proportion of PRV positives was not significantly different between wild coho salmon, *Oncorhynchus kisutch* (Walbaum), sampled from British Columbia (5.0% of 60) or the reference region, Alaska, USA (10% of 58).

Keywords: histopathology, piscine reovirus, real-time RT-PCR, retrospective study, wild fish.

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Introduction

Piscine reovirus (PRV) was first reported in association with the syndrome heart and skeletal muscle inflammation (HSMI) among farmed Atlantic salmon, *Salmo salar* L., in Norway (Palacios *et al.* 2010). HSMI is an emerging disease in Norway, first diagnosed in 1999 and now commonly diagnosed throughout the country (Finstad *et al.* 2012; Løvoll *et al.* 2012; Kristoffersen, Jensen & Jansen 2013). Differential diagnostic features of HSMI include inflammation and necrosis of the heart and red skeletal muscle in the absence of pancreatic lesions (Mcloughlin & Graham 2007). The primary microscopic lesions associated with HSMI include (*i*) a highly cellular epicarditis, endocarditis and myocarditis of the compact layer of the ventricle and (*ii*) myocardial necrosis; skeletal muscle lesions include necrosis and mononuclear cell infiltrates that are most common in red muscle fibres (Kongtorp, Taksdal & Lyngøy 2004).

Although many scientists hypothesize that PRV is the cause of HSMI, much remains unknown about the relation between PRV and HSMI. For example, PRV occurs in about 15% of wild Atlantic salmon in Norway, some of which have abundant virus (Ct values as low as 17), but these fish have no microscopic lesions diagnostic for HSMI (Garseth *et al.* 2013). Also, farmed Atlantic salmon in both fresh and salt water are sometimes infected with PRV, but HSMI occurs only in fish exposed to salt water (Biering & Garseth 2012), and the PRV status of fish while in fresh water is not correlated with the development of HSMI

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after they are moved to salt water (Løvoll *et al.* 2012). Tissues from fish with HSMI have had four virus-like particles identified by electron microscopy (Watanabe *et al.* 2006), but the relation of these particles to HSMI is unknown. In Norway, PRV infects nearly all populations of farmed Atlantic salmon at some time during their marine phase of growth, but only a fraction of these fish develop HSMI (Løvoll *et al.* 2012). PCR surveys have identified PRV among wild marine fish in Europe (Wiik-Nielsen *et al.* 2012; Garseth *et al.* 2013), but information about the relation of PRV to microscopic lesions in wild salmonids is limited to histopathology of hearts from 21 PRV-positive Atlantic salmon sampled in Norway from 2007 to 2009, none of which had lesions diagnostic for HSMI (Garseth *et al.* 2013).

In British Columbia, the westernmost province of Canada, HSMI is not known to occur, and little is known about the status of PRV. Among 200 wild juvenile pink salmon, *Oncorhynchus gorbuscha* (Walbaum), sampled in 2008 from the Broughton Archipelago region, all were negative for PRV (Saksida *et al.* 2012). Positive PCR results for PRV were reported for two wild cutthroat trout, *Oncorhynchus clarkii* Richardson, and one wild chum salmon smolt, *Oncorhynchus keta* (Walbaum), sampled in British Columbia in 2012 (Kibenge *et al.* 2013) but no details were provided about the sample location, sample size analysed, or whether the infected fish had lesions. The same study reported PRV in 10 Atlantic salmon and one steelhead trout, *Oncorhynchus mykiss* (Walbaum), that had been sampled from consumer markets in British Columbia, but details were not provided about where these fish had been farmed or the sample size from which the results were derived.

In April 2013, Marine Harvest Canada learned that smolts from one of their hatcheries had tested positive for PRV. To learn more about the significance of this finding, we developed a study plan with two main objectives. Our first objective was to determine whether the fish at a marine site that received smolts from the same hatchery were infected with PRV, whether that infection was related to disease and whether fish from the hatchery had been infected with PRV in the past. Because of the potential for transfer of PRV between farm fish and wild fish, our second objective was to broaden our understanding of the occurrence of PRV and HSMI among wild salmonids in British Columbia. Our research focused on determining whether PRV occurred in two groups of fish: (i) fresh tissues collected in 2013 from British Columbia, Canada, specifically for this study (n = 532 wild salmonids, 13 wild non-salmonids and 20 Atlantic salmon from the marine site) and from a reference region, Alaska, USA (n = 295 wild-caught salmonids; Atlantic salmon are not farmed in Alaska); and (ii) archived paraffin-embedded tissues from salmonids originally collected in British Columbia from 1974 to 2008 for other reasons.

Material and methods

Sample collection

Samples from farmed Atlantic salmon were collected 16 April 2013 from a marine net cage site operated by Marine Harvest Canada in Fisheries and Oceans Canada (DFO) management area 12 (map in online Dataset); the fish from Pen #5 sampled for this study had been transferred from the source hatchery on 12 March 2013. Opportunistic samples from wild fish, mostly salmonids, were collected from May through September 2013 (Table 1). Live fish were killed with an overdose of tricaine methane sulfonate or by percussion. Tissues were harvested using tools that were cleaned with 1% Virkon® and 10% bleach and then rinsed with sterile saline before opening each fish; the same cleaning protocol was repeated before harvesting the sample for the PRV analysis. The PRV sample for juvenile fish was the body caudal to the anus; for other fish sampled in 2013, the heart was sampled. For one group of 48 fish received from recreational fishers in 2013, the fishers had killed the fish by percussion, removed the viscera and gills, placed them in a plastic bag and later transferred the bags to our sampling personnel, who harvested the gill, heart, liver, kidney, spleen, intestinal ceca and surrounding mesenteries using the standard cleaning protocol. From 76 fish that had been sampled and frozen in 2007 or 2011, kidney or gill were tested by real-time RT-PCR (rRT-PCR) for PRV (details in online Dataset).

Samples for histopathology were immersed in 10% neutral buffered formalin for ≥24 h and then transferred to tap water or 70% isopropyl alcohol for shipment. Heart was sampled from nearly all fish for histopathology; other sampled

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Table 1 Test results and sample date, location, life stage and species tested by rRT-PCR for piscine reovirus (PRV) and by histopathology for heart and skeletal muscle inflammation (HSMI). Except for the first two rows, rRT-PCR tests were conducted on all fish by the BC Centre for Aquatic Health Sciences (CAHS); confirmatory rRT-PCR tests and all histopathology were conducted at the British Columbia Animal Health Centre (AHC) on tissues from the same fish analysed by CAHS

						No. of fish analysed			No. of fish positive				
						AHC		CAHS	AHC		CAHS	Total PRV	
Sample date	Sample location[a]	Habitat	Type of sample	Life stage	Salmon species	PRV	HSMI	PRV	PRV	HSMI	PRV	*n*	%
British Columbia, Canada, samples from 2013													
April 2013	DFO area 12	Marine	Farmed fish (sampled)	Smolts	Atlantic	20	20	0	20	0	NA	20	100
May 2013	N. Vancouver Island, Port Hardy area	Marine	Wild fish (sampled)	Fry and juveniles	Pink	76	76	0	0	0	NA	79	0
July 2013	DFO Area 6	Marine	Wild fish (commercial fishery)	Adult, maturing	Pink	49	49	49	0	0	0	49	0
July 2013	DFO Area 6	Marine	Wild fish (commercial fishery)	Adult, maturing	Pink	40	16	51	0	0	0	51	0
July 2013	DFO Area 6	Marine	Wild fish (commercial fishery)	Adult, maturing	Chum	4	4	101	0	0	0	101	0
July 2013	DFO Area 6	Marine	Wild fish (commercial fishery)	Adult, maturing	Chinook[b]	0	0	1	NA	NA	0	1	0
July 2013	DFO Area 27	Marine	Wild fish (recreational fishing)	Adults, maturing	Coho	0	0	2	NA	NA	0	2	0
July & August 2013	DFO Area 27	Marine	Wild fish (recreational fishing)	Adults, maturing	Chinook	0	0	7	NA	NA	0	7	0
August 2013	Carter River, Klemtu	Fresh	Wild fish (sampled)	Adult, mature	Pink	0	0	20	NA	NA	0	20	0
August 2013	Quatsino Lodge, DFO Areas 127 and 27	Marine	Wild fish (recreational fishing)	Adult, maturing	Chinook	6	6	32	2	0	2	32	6.3
August 2013	Quatsino Lodge, DFO Areas 127 and 27	Marine	Wild fish (recreational fishing)	Adult, maturing	Coho	4	4	10	0	0	0	10	0
August 2013	Ocean Falls, DFO Area 8	Marine	Wild fish (recreational fishing)	Adult, maturing	Chinook	0	0	7	NA	NA	0	7	0
August 2013	Ocean Falls, DFO Area 8	Marine	Wild fish (recreational fishing)	Adult, maturing	Coho	6	6	33	1	0	1	33	3.0
August 2013	Quatsino Lodge, DFO Areas 127 and 27	Marine	Wild fish (recreational fishing)	Adult, maturing	Chinook	6	6	19	4	0	4	19	21.1
August 2013	Quatsino Lodge, DFO Areas 127 and 27	Marine	Wild fish (recreational fishing)	Adult, maturing	Coho	4	4	15	2	0	2	15	13.3
September 2013	Fraser River	Fresh	Wild fish (sampled)	Adult, mature	Sockeye[b]	0	10	60	NA	0	1[c]	60	1.7
September 2013	Skeena River	Fresh	Wild fish (sampled)	Adult, mature	Sockeye	0	23	120	NA	0	0	120	0
British Columbia salmonid totals for 2013						215	224	527	29	0	10	626	4.6
August 2013	Quatsino Lodge, DFO Areas 127 and 27	Marine	Wild fish (recreational fishing)	Adult, maturing	Non-salmonids[d]	0	0	14	NA	NA	0	14	0.0
Alaska, USA, samples from 2013													
July 2013	Tutka Lake, Alaska, USA	Fresh	Wild fish (sampled from hatchery)	Adult, spawners	Pink	0	0	117	NA	NA	0	117	10.3
August 2013	Bear Lake, Alaska, USA	Fresh	Wild fish (sampled from hatchery)	Post-spawning	Sockeye	0	12	120	NA	0	0	120	0
August 2013	Copper River, Alaska, USA	Marine	Wild fish (commercial fishery)	Adult, maturing	Coho	15	15	58	6	0	6	58	10.3
British Columbia, Canada, Archived samples (rRT-PCR only)													
June 2007	Skeena River	Fresh	Wild fish (sampled)	Smolts	Sockeye	0	0	30	NA	NA	0	30	0
October 2007	Ogden, Beaver Pass	Fresh	Wild fish (sampled)	Adult, mature	Sockeye	0	0	30	NA	NA	0	30	0

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Table 1 *Continued*

Sample date	Sample location[a]	Habitat	Type of sample	Life stage	Salmon species	No. of fish analysed			No. of fish positive			Total PRV	
						AHC		CAHS	AHC		CAHS		
						PRV	HSMI	PRV	PRV	HSMI	PRV	*n*	%
September 2011	Gates Creek	Fresh	Wild fish (sampled)	Adult, mature	Sockeye	0	0	10	NA	NA	0	10	0
October 2011	Harrison River	Fresh	Wild fish (sampled)	Adult, mature	Chinook	0	0	2	NA	NA	0	2	0
October 2011	Harrison River	Fresh	Wild fish (sampled)	Adult, mature	Sockeye	0	0	1	NA	NA	0	1	0

[a]Areas delineated by Canadian Fisheries and Oceans (DFO) are included in a map in the online Dataset.
[b]Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum); sockeye salmon, *Oncorhynchus nerka* (Walbaum).
[c]This result is considered suspect because the replicates yielded Ct values (37.1, 37.9) that were >35.0, and tissues had not been sent to the AHC for confirmation.
[d]Nonsalmonids include Canary rockfish (*Sebastes pinniger* Gill; $n = 4$), lingcod (*Ophiodon elongatus* Girard; $n = 3$) and Pacific halibut (*Hippoglossus stenolepis* Schmidt; $n = 7$).

organs for some fish included skin with skeletal muscle (sampled near the lateral line, but not available from commercial or recreational fisheries), head kidney, trunk kidney, liver, spleen, gill, distal intestine, intestinal ceca and mesenteric fat, and sometimes brain (details in online Dataset).

Archived paraffin samples from 2000 to 2008 were selected from cases that had been submitted by Marine Harvest Canada to the Animal Health Centre for diagnostic evaluation. All samples had been collected directly from their hatchery or were from marine net cages that included fish that had been reared at their hatchery. All of these samples had been preserved in 10% neutral buffered formalin.

Archived paraffin samples from 1974 to 1994 were from the histology laboratory of the Pacific Biological Station, Fisheries and Oceans Canada, Nanaimo, British Columbia, Canada. Blocks were selected to include a range of years and a mixture of farmed salmon and salmonids sampled from the wild or from enhancement hatcheries. Records related to many of these samples are incomplete, but in all cases, the year of sample collection is known. In some cases, tissues from a single fish seem to be distributed in more than one paraffin block, but these records are also unclear; therefore, prevalence for these samples is based on the known number of paraffin blocks rather than the unknown number of fish. As an estimate of the preservative that was used, tissue colouration was recorded when the paraffin sections were processed for rRT-PCR analysis: yellow (probably Bouin's fixative) or normal (probably Davidson's fixative); during these years, the Pacific Biological Station did not use 10% neutral buffered formalin (W. Bennett, DFO, personal communication).

Polymerase Chain Reaction (rRT-PCR) and sequence confirmation

All samples analysed at the BC Animal Health Centre were tested for a 59-bp fragment of PRV segment L1 as previously described (Saksida *et al.* 2012). All archived paraffin samples were tested for segment L1 and also tested for a 180-bp fragment of segment L2 (Table 2). The second test was added for two reasons: first, to increase the chance of detecting Pacific PRV genotypes that might have differed from the Atlantic PRV genotypes that had been used to develop the tests, and second, the 180-bp fragment amplified by the test

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Table 2 Reverse transcriptase polymerase chain reaction (rRT-PCR) tests used to detect fragments of the piscine reovirus (PRV) genome

Segment	L1[a]	L2	M2
Forward primer	2671F (5′- TGC TAA CAC TCC AGG AGT CAT TG -3′)	SRV-L2-F (5′- TTG GCG TGG TTG CAC CTT TGA TAC -3′)	Proprietary
Reverse primer	2729R (5′- TGA ATC CGC TGC AGA TGA GTA - 3′)	SRV-L2-R (5′- ACA CCA GTG GGC TTG GTC ATT AGA -3′)	Proprietary
Probe	5′- 6FAM/CGC CGG TAG CTC T/MGBNFQ -3′	SRV-L2 (5′- 6FAM/TCT TGA AAG/ZEN/ACG CCG GTT GTA CTC GA/3IABkFQ -3)	Proprietary
Fragment size	59 bp	180 bp	59 bp
Analytical sensitivity with purified clone	3 copies	30 copies	3 copies

[a]The primers and probe from Palacios *et al.* (2010) were used by Saksida *et al.* (2012); the analytical sensitivity is new information.

for the L2 segment was sufficiently long for sequence confirmation that was not possible with the 59-bp fragment of the L1 test. Because the fixatives used for preservation of archived tissues for histopathology – Bouin's, Davidson's and 10% neutral buffered formalin – cause fragmentation of RNA (Cox *et al.* 2006), a subset of archived Atlantic salmon samples (six from 1977, 31 from 1986, 18 from 1987 and six from 2001) were also tested by rRT-PCR for a 59-bp fragment of the reference gene elongation factor 1A ($EF1A_B$) (Olsvik *et al.* 2005). A sample was considered to be degraded if the $EF1A_B$ PCR yielded a Ct value ≥25.0.

For RNA extraction from paraffin-embedded tissues, sequential 10-μm-thick sections were cut using a clean microtome blade. Nucleic acid was extracted from one of the sections for the segment L1 rRT-PCR and independently extracted from the other section for the segment L2 rRT-PCR. All extractions from paraffin-embedded tissues were carried out using the RNeasy FFPE kit (Qiagen Sciences) according to manufacturer's instructions.

rRT-PCR for segment L2 was carried out using the Agpath-IDTM One-Step rRT-PCR Kit (Life Technologies). Each 25-μL reaction contained 12.5 μL of 2 × rRT-PCR buffer, 1 μL of 25 × RT-PCR enzyme, 1 μL (20 μM) each of forward and reverse primers, 0.5 μL (5 μM) probe, 4 μL nuclease-free water and 5 μL of RNA template. rRT-PCR amplifications were performed on an ABI7500 Fast rRT-PCR System (Applied Biosystems) under the following conditions: 30 min at 50 °C for reverse transcription, 94 °C for 15 min for initial denaturation, followed by 45 cycles of amplification with denaturation at 94 °C for 15 s, and annealing and extension at 60 °C for 1 min. Samples were analysed using the SDS software version 1.4. For sequence confirmation of the amplified product (one fish from 1992 and 2 fish each from 2000, 2001 and 2005), segment L2 rRT-PCR fragments were purified using Amicon Ultra centrifugal filters, 30K (Fisher Scientific, Canada) following the manufacturer's instructions. Sequencing was carried out using the Big Dye Terminator version 3.1 Cycle sequencing kit (Applied Biosystems) and an ABI 3130 Genetic Analyzer (Applied Biosystems). The DNASTAR Lasergene 10 was used for the editing, assembly and analysis of nucleotide sequence data.

All samples analysed at the BC Centre for Aquatic Health Sciences were tested for a fragment of PRV segment M2 using primers and probe designed by A. Nylund (University of Bergen, Norway, personal communication). Approximately 30 mg of each heart sample was collected, and RNA was extracted using Qiagen RNeasy Mini Kit according to the manufacturer's protocol (Qiagen). Total RNA was eluted with 50 μL of RNAse-free water. rRT-PCR was performed using AgPath-IDTM One-Step RT-PCR kit according to the manufacturer's recommendations (Life Technologies). Analysis was performed using a total reaction final volume of 12.5 μL comprising 200 nM final concentration of PRV forward and reverse primers and 250 nM final concentration of the FAM probe. Two μL of RNA was added to each reaction.

For an interlaboratory comparison, the Animal Health Centre sent 10 coded samples that had been tested by their rRT-PCR assay targeting segment L1 to the Centre for Aquatic Health Sciences for analysis using their rRT-PCR assay targeting segment M2. Both laboratories agreed on six positive results and four negative results,

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and variability of the Ct values between the two laboratories testing the same sample was ±2 for all six positive results (online Dataset). Thereafter, the rRT-PCR for segment M2 was used as a screening assay for fresh tissues from wild fish collected in 2013; all positive tests were confirmed using the rRT-PCR test targeting segment L1 on different samples from the same fish, except for one fish from which different tissues for confirmation had not been collected. When PRV results from fresh-frozen samples differed between the two laboratories conducting the PRV rRT-PCR tests ($n = 14$ fish), the rRT-PCR for reference gene $EF1A_B$ was run on fresh-frozen and formalin-fixed samples submitted to the Animal Health Centre.

The retrospective part of the study with paraffin-embedded tissues included rRT-PCR analysis of PRV in archived samples from (i) wild or enhancement-hatchery fish (1974–1994, $n = 67$; these hatcheries spawned wild-caught fish for release of progeny back into the wild); (ii) commercially farmed fish (1986–2008, $n = 256$); (iii) Pacific salmonids for which the farm status (i.e. whether they were from a commercial farm or wild-source) is not in available records (1986–1993, $n = 24$); and (iv) salmonids sampled from enhancement hatcheries for which neither the species nor farm status is in available records (1994, $n = 24$). Results with a Ct value <35.0 were considered positive, with or without replication. Results with a Ct value ≥ 35.0 were considered positive if replicated and suspect if not replicated or if replication was not attempted.

For the entire project, 1368 samples that were collected from 1974 to 2013 were analysed by rRT-PCR for evidence of PRV. This included archived frozen samples from 73 salmonids captured from freshwater in British Columbia in 2007 and 2011.

Histopathology

Histopathology for evidence of HSMI focused on fish that were positive for PRV and a subset of fish that were negative for PRV. For the first samples collected in 2013, all fish analysed by rRT-PCR for PRV were subjected to histopathology. For later samples, fish tissues were first screened by rRT-PCR for PRV. Fish that were positive by rRT-PCR for PRV were subjected to histopathology along with a subset of sample cohorts that were negative for PRV. Histopathology was not carried out for some sample groups that were all negative by rRT-PCR for PRV (Table 1). The proportion of the fish examined that were positive by rRT-PCR for PRV included: 13% (29 of 227) of the fish sampled in 2013 from British Columbia, 22% (6 of 27) of the fish sampled in 2013 from Alaska and 67% (12 of 19) of the sections of paraffin blocks from 1977 to 1994. Sections from all paraffin blocks from 2000 to 2008 that were tested for PRV were also examined by histopathology for evidence of HSMI.

For formalin-fixed samples collected in 2013, those containing bone were decalcified using size-specific protocols: 79 fry and juveniles were immersed whole overnight in a 10% EDTA solution; organs from 175 larger fish were immersed 1–3 h (depending on size) in Protocol® Decalcifier B (10% hydrochloric acid solution). After decalcification, all tissues were processed routinely into paraffin, sectioned at 3 μm and stained with haematoxylin and eosin (H&E). Complete histopathology was carried out on all sampled organs by one pathologist (GDM). Microscopic features were categorized and scored as none (0), mild/small amounts (1), moderate (2), or severe/abundant (3) as previously described (Marty, Heintz & Hinton 1997; Marty & Heintz 2010).

For archived formalin-fixed paraffin-embedded samples that had been collected from 2000 to 2008 ($n = 164$ fish plus 4 sections that each contained 2–4 alevins), the type of processing had varied by fish size. Alevins and young fry had been embedded in paraffin whole in lateral recumbency; 3 step-sections had been cut through each block at approximately 250-μm intervals. For larger fry, the body had been transected midsagittally and embedded so that the resultant slide contained both cut faces. Organs from larger fish had been processed, embedded and sectioned individually. Sections had been cut at 3–6 μm, and slides were stained routinely with H&E. Original slides from fish sampled in 2000 and 2001 had not been retained; therefore, new 3-μm-thick sections were cut from the remaining tissues in the paraffin blocks and stained with H&E. Original slides from fish sampled from 2005 to 2008 were retrieved and re-examined for this study. All sections of heart and skeletal muscle in these slides were examined for inflammation by a single pathologist (GDM) using the same methods as for samples collected in 2013.

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For archived paraffin-embedded samples that had been collected from 1974 to 1994, histopathology was focused on recuts or original slides from paraffin block groups that tested positive by rRT-PCR for PRV (slides from 45 paraffin blocks were examined). All sections of heart (n = 19) and skeletal muscle (n = 20) in these slides were examined for inflammation by a single pathologist (GDM) using the same methods as for samples collected in 2013.

For the entire project, 404 samples that were collected from 1977 to 2013 were analysed by histopathology for evidence of HSMI. All 404 samples included heart: 214 also included skeletal muscle, 173 also included other organs but not skeletal muscle and 17 included only heart. Differences in Ct values were used to identify trends in the data. For statistical analysis, proportions were compared using 2 × 2 contingency tables and Fisher's exact test; results with $P < 0.05$ were considered significant.

Results

Farmed Atlantic salmon from 2013

All 20 of the Atlantic salmon sampled from the index marine farm in 2013 were positive by rRT-PCR for PRV (Ct range = 25.5–37.9; Table 1), but the PRV status of the fish was not associated with microscopic lesions. Seven of the 20 fish had lymphohistiocytic endocarditis, but the median Ct value for the affected fish was greater than the median Ct value for the unaffected fish (Fig. 1), meaning that samples from fish with endocarditis tended to have less PRV than samples from fish without endocarditis. In only one fish was epicarditis or endocarditis of greater than mild severity (severity in the affected fish was moderate); however, this fish had no skeletal muscle lesions, and its PRV Ct value (32.5) was slightly greater than the group median Ct = 31.6 (range = 25.5–37.9): evidence that samples from the affected fish had less PRV than the majority of its cohorts. Among other common microscopic changes, the only change with a trend related to PRV Ct value was renal tubular epithelial protein droplets: the median Ct value was greater for fish with the droplets (Ct = 32.5) than for fish without the droplets (Ct = 29.1). Also, body weight among the 20 fish in this group varied from 81.5 to 218.8 g, but the median Ct values were similar for the heaviest 10 fish (Ct = 31.3) and the lightest 10 fish (Ct = 31.9): evidence that PRV Ct value was not related to fish size.

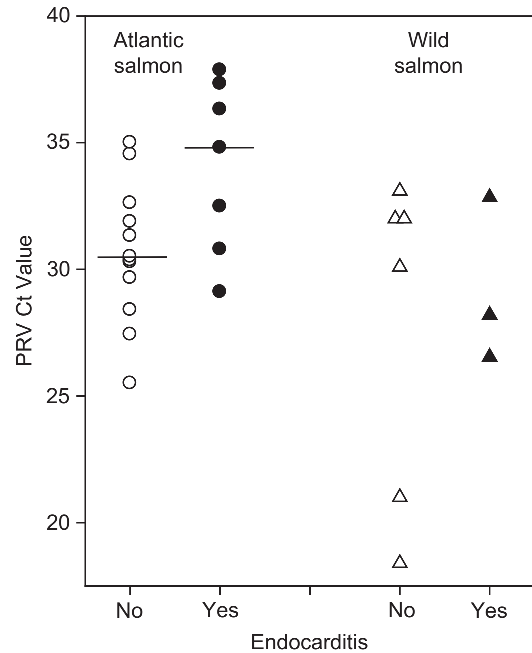

Figure 1 real-time RT-PCR (rRT-PCR) Ct values for farmed Atlantic salmon and wild Chinook or coho salmon, sampled in British Columbia in 2013, that tested positive for piscine reovirus (PRV). Lymphohistiocytic endocarditis occurred in some fish (closed symbols) but did not occur in other fish (open symbols). Horizontal bars designate the median value within the Atlantic salmon categories; too few wild fish were PRV positive for the median to provide an informative summary.

Wild Pacific salmon from 2013

Trends in the species-specific occurrence of PRV in 2013 were similar in British Columbia and the reference region, Alaska. rRT-PCR results were positive for PRV in 10 of 626 (4.6%) fish from British Columbia and 6 of 295 (2.0%) fish from Alaska (Table 1). Among coho salmon, *Oncorhynchus kisutch* (Walbaum), the proportion of PRV positives was not significantly different between fish from British Columbia (5.0%, 3 of 60; range of Ct values = 14.7–37.1) and Alaska (10%, 6 of 58; range of Ct values = 29.2–38.4) (Table 1; 2 × 2 contingency table, Fisher's exact test, $P = 0.32$). The coho salmon from British Columbia with PRV Ct = 14.7 had no microscopic lesions in the heart, and the only microscopic finding in any organ involved a few renal tubules

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that had small numbers of epithelial cytoplasmic protein droplets. Among sockeye salmon, *Oncorhynchus nerka* (Walbaum), one of the 180 fish sampled in British Columbia was PRV suspect (Ct = 37.5), and none of the 120 fish sampled in Alaska was PRV positive. All pink salmon, *Oncorhynchus gorbuscha* (Walbaum), samples from both regions were PRV negative (Table 1). The greatest proportion of PRV positives in any sample group of wild salmon sampled in 2013 was among British Columbia Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum) (21%, range of Ct values = 26.5–35.5); Chinook salmon in Alaska were not examined.

Among the 204 wild salmon sampled in 2013 from British Columbia for histopathology, none of the fish had microscopic lesions diagnostic for HSMI. Twelve (5.9%) of the fish had lymphohistiocytic endocarditis, and all cases were mild (Fig. 2). None of the fish had lymphohistiocytic epicarditis. A majority of the PRV-positive fish (6 of 9 = 67%) had no inflammation in the heart, but the proportion of hearts with endocarditis was significantly greater among PRV-positive fish (3 of 9 = 33%) than among PRV-negative fish (9 of 195 = 4.6%) (2 × 2 contingency table, Fisher's exact test, $P = 0.0108$). However, the range of Ct values for wild salmon with endocarditis was entirely contained within the range of Ct values for wild salmon without endocarditis (Fig. 1), and the two samples with the lowest Ct values (18.4 and 21.0) did not have endocarditis. The PRV Ct = 18.4 was our study's lowest segment L1 Ct value, but this fish had no heart lesions (Fig. 2); the only microscopic lesion in this fish was splenic peritonitis, chronic, mild.

In nearly all cases, the cause of inflammation in the heart was not determined. However, two of the six wild coho salmon sampled from the Ocean Falls area of British Columbia each had a single focus of acute myocardial necrosis and several foci of fibrin and lymphohistiocytic inflammation that were associated with scattered *Loma salmonae* spores. One of these two fish was rRT-PCR positive for PRV, but the relatively high Ct value (32.0) is evidence of a low copy number of viral genome in the sample.

For samples of fresh-frozen heart from 14 fish, initial results differed between the rRT-PCR tests for PRV segment M2 (conducted at the BC Centre for Aquatic Health Sciences) and rRT-PCR tests for segment L1 (conducted on different samples from the same fish at the British Columbia Animal Health Centre). From these 14 fish, samples from all 14 hearts tested positive for segment M2, but only two of the 14 different fresh heart samples were positive for segment L1, and segment L1 Ct values for those two samples (32.3 and 33.3) were many orders of magnitude greater than the Ct values for segment M2 on the matched samples (14.7 and 21.8). In contrast, formalin-fixed paraffin-embedded tissues from the same 14 fish were all positive for segment L1; this included the lowest segment L1 Ct value (18.4) of the entire study; this Ct value might be even more significant than it appears, because the reference gene $EF1A_B$ Ct value = 32.5 for this extract is evidence that significant RNA degradation decreased our ability to detect PRV in this sample. For the different samples tested for segment L1, the reference gene $EF1A_B$ Ct value tended to be greater for extracts from the fresh hearts (median Ct = 32.7; range = 23.9–37.9) than for the extracts from the paraffin-embedded sections (median Ct = 26.4; range = 23.4–32.5): evidence that sample degradation of the fresh hearts might have contributed to some of the initial negative results for the PRV segment L1. For two of the 14 samples, however, the $EF1A_B$ Ct was less in the extract from the fresh-frozen heart than in the extract from the paraffin sections, but the segment L1 Ct values for samples from these fish were substantially greater for the fresh-frozen hearts (32.3 and negative) than for the paraffin sections (21.0 and 32.8, respectively). In these two cases, the paraffin sections contained organs in addition to the heart (liver, kidney, spleen and intestinal ceca) that might have contributed to the lower Ct values in extracts from the paraffin sections.

Wild Pacific salmon from 2007 and 2011

None of the fresh-frozen archived tissues from 73 wild Pacific salmon, all sampled from fresh water in 2007 or 2011, were positive for PRV (Table 1).

Archived samples of farm salmon from 2000 to 2008

The proportion of PRV positives was 85% (134 of 168) among the analysed paraffin samples from 2000 to 2008. For the years 2000–2001, when both Chinook salmon and Atlantic salmon were tested, the proportion of PRV positives was nearly

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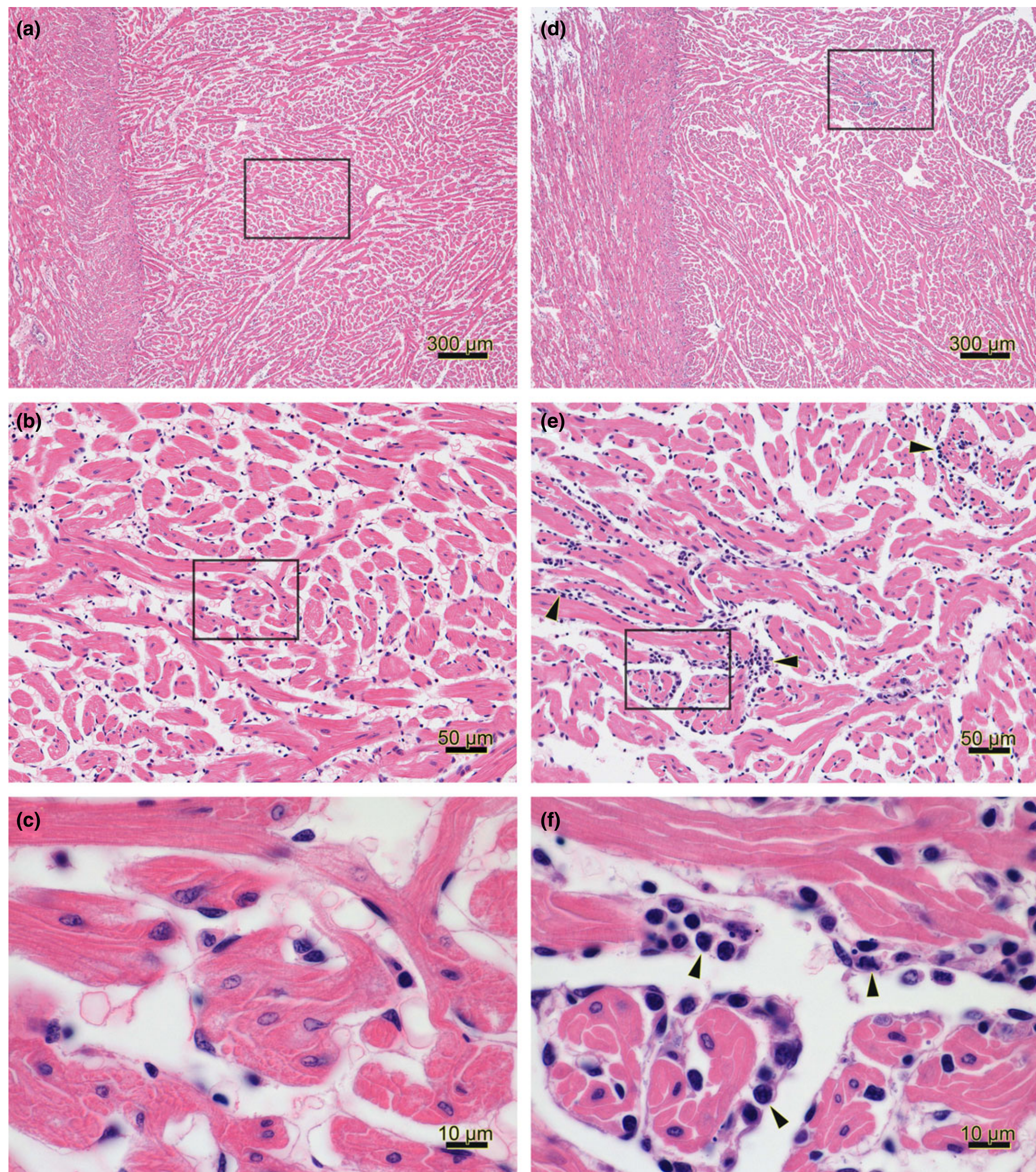

Figure 2 Heart sections (H&E stain) from two wild Chinook salmon sampled from British Columbia in August 2013. Figures (a, b, c) are progressively higher magnifications of a normal ventricle, from a fish with a PRV rRT-PCR segment L1 Ct value = 18.4. Figures (d, e, f) are progressively higher magnifications of a ventricle with endocarditis, mild, focal and lymphohistiocytic (arrowheads) in the spongy layer, from a fish with a PRV rRT-PCR segment L1 Ct value = 26.5. Black boxes outline the area shown at higher magnification in the next image.

identical between the 29 Chinook salmon (97%) and the 23 Atlantic salmon (96%). Among the six sequenced products from our segment L2 rRT-PCR – four from Atlantic salmon (one each from 2000, 2001, 2004 and 2005) and two from Chinook salmon (from 2000 and 2001) – five were identical to a PRV sequence posted in GenBank (Accession KC776257.1); the Chinook salmon from 2000 had a single base pair different from the other sequences, yielding a 99% match.

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PRV occurred in all 30 sample groups from 2000 to 2008, with the proportion of PRV positives within each case varying from 38 to 100% (Table 3). The range of Ct values included lower values for segment L1 rRT-PCR (19.6–39.9) than for segment L2 rRT-PCR (25.8–39.6). Three of the results for the PRV segment L1 rRT-PCR (Ct values from 19.6 to 20.9) are among the lowest PRV Ct values on record at the BC Animal Health Centre; all of these Atlantic salmon were from the same diagnostic case submission in 2001, and tissues from all seven fish from this case were strongly positive for PRV (segment L1 Ct range = 19.6–25.1; segment L2 Ct range = 26.4–33.0); hearts were included among the archived organs for only two of these fish, and they had no inflammation. For all seven fish in this case, the PRV Ct value for segment L1 (detects a 59 bp fragment) was less than for segment L2 (detects a 180 bp fragment). The differences in Ct values (range = 4.7–8.9) might be a result of (*i*) differences in sensitivity of the two tests (a 10-fold difference would account for a Ct difference of 3.3) and (*i*) RNA degradation that differentially impaired detection of the longer segment L2 fragment. Also, rRT-PCR test results from this case also provide two lines of evidence that post-mortem decomposition before formalin fixation did not greatly impair our ability to detect PRV. First, the range of Ct values for the reference gene $EF1A_B$ was only slightly greater for the four fish with moderate or severe hepatic autolysis (Ct = 25.0–26.7) than for the three fish with mild hepatic autolysis (Ct = 22.1–25.2). And second, the magnitude of differences between the PRV Ct values for segments L1 and L2 was

Table 3 Sample date, location and PRV test results (segments L1 and L2 rRT-PCR results combined) from analysis of sections of formalin-fixed tissues from commercially farmed salmon archived in paraffin blocks from 2000 to 2008

Sample date	Location[a]	No. of fish	% positive	Ct value range
Atlantic salmon				
2008-10-27	Hatchery	10	60	30.0, 39.2
2008-09-11	Hatchery	5	40	34.7, 38.5
2008-08-19	DFO area 12	3	67	33.3, 38.5
2008-07-07	DFO area 12	2	100	37.0, 38.6
2008-06-02	Hatchery	6	100	30.9, 39.6
2008-04-10	Hatchery	15	67	36.1, 39.8
2008-02-18	DFO area 12	3	67	36.4, 37.1
2008-02-08	Hatchery	4	100	36.5, 38.4
2007-12-06	DFO area 13	4	100	27.5, 35.2
2007-07-13	DFO area 13	5	100	25.1, 35.3
2007-06-14	Hatchery	10	70	34.8, 39.5
2007-05-23	DFO area 13	2	100	23.6, 29.2
2007-05-14	Hatchery	10	60	35.2, 39.8
2007-05-08	DFO area 7	1	100	34.1, 39.3
2007-05-07	DFO area 13	2	100	33.8, 38.2
2006-03-29	DFO area 12	2	100	23.7, 34.1
2006-03-24	Hatchery	4	75	28.2, 37.6
2006-02-08	Hatchery	6	100	29.1, 38.2
2005-07-27	Hatchery	3	100	28.4, 34.9
2005-04-14	Hatchery	16	38	33.8, 38.3
2005-03-24	DFO area 18	2	100[b]	24.0, 31.5
2005-03-06	DFO area 7	1	100[b]	22.5, 28.5
2001-08-29	DFO area 18	7	100[b]	19.6, 33.0
2001-05-14	Hatchery	5	80	31.0, 37.8
2000-09-29	DFO area 13	3	100	28.2, 37.3
2000-06-06	Hatchery	8	100[b]	30.9, 39.4
2000–2008	All Atlantic salmon	139	76	19.6, 39.8
Chinook salmon				
2001-07-01	Hatchery	3	100	35.6, 39.9
2001-05-29	DFO area 13	12	100[b]	22.5, 39.1
2000-10-19	Hatchery	4	75	30.4, 37.0
2000-10-18	DFO area 26	10	100[c]	28.2, 38.0
2000–2001	All Chinook salmon	29	97	22.5, 39.9

[a]Areas delineated by Canadian Fisheries and Oceans (DFO) are included in a map in the online Dataset.
[b]L2 rRT-PCR amplicon nucleic acid sequencing from one fish in this group; 132/132 bp = 100% match to GenBank accession No: KC776257.1.
[c]L2 rRT-PCR amplicon nucleic acid sequencing from one fish in this group; 131/132 bp = 99% match to GenBank accession No: KC776257.1.

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similar for the three fish with mild autolysis (Ct difference = 4.7–8.9) and the four fish with moderate or severe autolysis (Ct difference = 7.4–8.8).

Among the 134 farmed salmon sampled from 2000 to 2008 that included heart for histopathology, 81% were positive for PRV (Ct range = 20.9–39.8), but none of the fish had skeletal muscle inflammation and only four fish had inflammation in the heart that was greater than mild severity (all were moderate). The fish with moderate inflammation in the heart included (*i*) one Atlantic salmon sampled in 2000 and one in 2005 and (*ii*) two Chinook salmon that were part of a single diagnostic case submission in 2001. One of the Chinook salmon from 2001 (PRV segment L1 Ct = 29.2) had skeletal muscle with mild myodegeneration and necrosis, one large intralesional *Kudoa thyrsites* pseudocyst, but no associated inflammation. The Atlantic salmon sampled in 2005 (PRV segment L1 Ct = 22.5) had a necrotic myofibre in the skeletal muscle, and the overlying skin was ulcerated and infiltrated by filamentous bacteria. The other two fish with moderate heart inflammation had no other remarkable lesions.

Among the Atlantic salmon samples from 2000 to 2008 that included heart for histopathology, most of the PRV-positive fish (75 of 83 = 90%) had no inflammation in the heart, but samples with lower PRV Ct values tended to have a greater proportion of hearts with lymphohistiocytic endocarditis (Table 4). The proportion of hearts with endocarditis was significantly greater among fish with PRV Ct values < 35.0 (6 of 40 = 15%) than among fish with PRV Ct values ≥35.0 (0 of 70 = 0%) (2 × 2 contingency table, Fisher's exact test, $P = 0.0018$). However, the range of Ct values for Atlantic salmon with endocarditis was entirely contained within the range of Ct values for Atlantic salmon without endocarditis, with only a slight trend towards lower Ct values among fish with endocarditis (Fig. 3). The two samples with the lowest Ct values (20.9 and 21.7) did not have endocarditis.

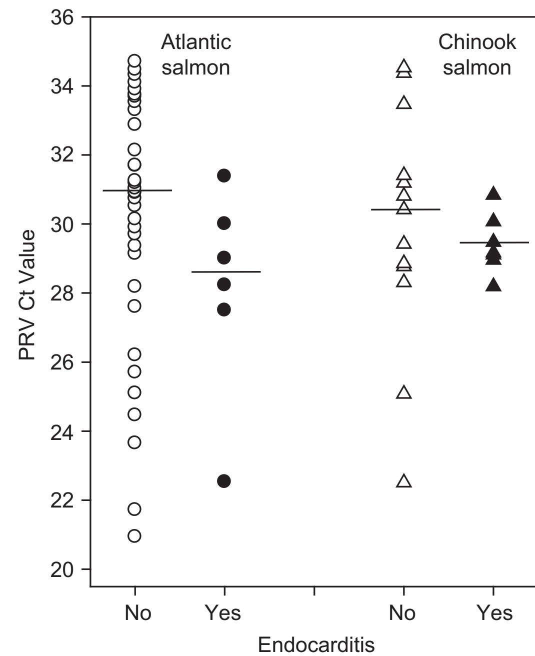

Figure 3 Piscine reovirus rRT-PCR Ct values in archived paraffin-embedded samples of farmed salmon without (open symbols) and with (closed symbols) lymphohistiocytic endocarditis. Only results with Ct value <35.0 are shown. The 40 Atlantic salmon were sampled from 2000 to 2008. The 20 Chinook salmon were sampled from 2000 to 2001. Horizontal bars designate the median value within a category.

Table 4 Sample prevalence of piscine reovirus (PRV) in relation to lymphohistiocytic inflammation in the heart. rRT-PCR analysis was carried out on sections of formalin-fixed tissues from commercially farmed salmon archived in paraffin blocks from 2000 to 2008

		Heart inflammation (%)			
Ct value	*n*	None	Endocarditis	Epicarditis	Endocarditis or epicarditis
Atlantic salmon 2000–2008					
None/suspect	27	96	0.0	3.7	3.7
35.0–39.9	43	100	0.0	0.0	0.0
30.0–34.9	23	87	8.7	8.7	13
<30.0	17	71	24	5.9	29
Chinook salmon 2000–2001					
None/suspect	1	0	0	100	100
35.0–39.9	3	67	0	33	33
30.0–34.9	9	56	22	33	44
<30.0	11	27	46	55	73

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Among the Chinook salmon samples from 2000 to 2001 that included heart for histopathology, a majority of the PRV-positive fish (13 of 23 = 57%) had inflammation in the heart, and samples with lower PRV Ct values tended to have a greater proportion of hearts with lymphohistiocytic endocarditis (Table 4). The proportion of hearts with endocarditis tended to be greater among fish with PRV Ct values < 35.0 (7 of 20 = 35%) than among fish with PRV Ct values ≥35.0 (0 of 4 = 0%), but these differences were not significant (2 × 2 contingency table, Fisher's exact test, $P = 0.2833$). The range of Ct values for Chinook salmon with endocarditis was entirely contained with the range of Ct values for Chinook salmon without endocarditis, with only a slight trend towards lower Ct values among fish with endocarditis (Fig. 3). The two samples with the lowest Ct values (22.5 and 25.1) did not have endocarditis.

Archived samples of farm and wild salmon from 1974 to 1994

The proportion of PRV positive or suspect was 25% (49 of 195) among the analysed paraffin blocks from 1974 to 1994, but none of the examined sections had lesions diagnostic for HSMI. The proportion of PRV positive or suspect within each sample group varied from 0 to 100% (Table 5). The range of Ct values included lower values for segment L1 (27.8–38.3) than for segment L2 (34.5–39.9). Thirty-five samples were positive or suspect for only segment L1, nine samples were positive or suspect for only segment L2, and five samples were positive for both segments. The earliest positive sample was a liver from a wild or hatchery sourced steelhead trout sampled from freshwater sometime between January and 6 April 1977. This 1977 sample was positive for only segment L1 at a high Ct value, but repeated positive test results were from independent extracts from two different sections of the same paraffin block (Ct = 38.3 from one section; Ct = 38.3 and 38.4 from a different section). The earliest sequence confirmation was from a Chinook salmon sampled as part of a marine anaemia survey on 7 February 1992; the 132-bp amplicon from segment L2 rRT-PCR was identical to a PRV sequence posted in GenBank (Accession KC776257.1). Sequencing was not attempted on

Table 5 Sample year, species, farm status and PRV test results (segments L1 and L2 rRT-PCR results combined) from analysis of sections of Bouin's- or Davidson's-fixed tissues from fish sampled in British Columbia, Canada, and archived in paraffin blocks from 1974 to 1994

Year sampled	Salmonid species	Commercially farmed?	No. of blocks	% Positive[a]	Ct range
1974–1977	Sockeye	No	9	0	NA
1975–1976	Chinook	No	6	0	NA
1977	Steelhead	No	6	17	38.3, 38.4
1986	Atlantic	Yes	31	0	NA
1986	Coho	Yes (3), ND (2)	5	0	NA
1986	Sockeye	No	1	0	NA
1987	Atlantic	Yes	18	22	37.2, 38.2
1987	Coho	ND	2	0	NA
1987	Sockeye	No	1	0	NA
1988	Atlantic	Yes	18	22	33.3, 38.2
1988	Sockeye	No	5	40	36.6, 38.0
1988	Steelhead	No	4	50	37.7, 37.8
1992	Atlantic	Yes	1	100	35.1
1992	Chinook	ND	9	56[b]	27.9, 38.3
1992	Chinook	No	10	40	36.9, 39.2
1992	Chinook	Yes	5	100	33.1, 38.0
1993	Atlantic	Yes	12	92	27.8, 39.9
1993	Chinook	No	10	30	34.4, 38.1
1993	Coho	ND	3	0	NA
1993	Sockeye	No	3	33	37.0
1994	ND	ND (hatchery)	24	13	36.2, 37.9
1994	Sockeye	No	12	17	36.6, 37.6
1987–1993	Atlantic	All	49	41	27.8, 39.9
1987–1994	Pacific	All	64	38	27.9, 39.2

[a]Because of nucleic acid degradation in many of the samples, the proportion of positive blocks includes suspect cases that had a single positive result with Ct ≥ 35.00. See online Dataset for details.
[b]L2 rRT-PCR amplicon nucleic acid sequencing from one fish in this group; 132/132 bp = 100% match to GenBank accession No: KC776257.1.
NA, not applicable; ND, no data.

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earlier samples because all segment L2 Ct values for earlier samples were ≥ 36.6. Among the slides from 45 paraffin blocks that were examined for microscopic lesions, 2 of the 19 hearts (11%) had mild lymphohistiocytic endocarditis (Ct values 31.9 and 34.4), and one of these hearts also had mild lymphohistiocytic epicarditis. None of the 19 sections of skeletal muscle had inflammation.

The lack of PRV among Atlantic salmon from 1986 (0 of 31, 0%) compared with 1987 (4 of 18, 22%) was probably not due to RNA degradation of the 1986 samples, but some cases of PRV infection among the 1987 samples might have been missed because of sample degradation. As evidence, the rRT-PCR Ct values for the reference gene $EF1A_B$ indicated only minor degradation of the 1986 samples (median Ct = 24.8 for the first section and 25.8 for the second section; total range = 19.8–30.9) but significant degradation for many of the 1987 samples (median Ct = 29.4 for both sections; range = 25.8 – undetected in six extracts). Sample degradation might also partly explain the high Ct values of the 1987 samples (Table 5).

For samples from 1974 to 1994, the proportion of PRV positives is significantly less among Bouin's preserved samples (19%, 12 of 64) than among Davidson's preserved samples (37%, 37 of 100) (2 × 2 contingency table, Fisher's exact test, $P = 0.0145$). This calculation does not include Bouin's preserved Atlantic salmon samples from 1986 because they were from fish imported to British Columbia within the previous year, and they might not have been exposed to PRV. Also, among samples collected from 1987 to 1994, the proportion of PRV positives was not significantly different between farmed Atlantic salmon (44%, 21 of 48) and wild-source salmonids (31%, 14 of 45) (2 × 2 contingency table, Fisher's exact test, $P = 0.2844$).

Discussion

Our results provide evidence that wild and farmed salmonids have been commonly infected with PRV since 1987, and a repeated weak positive rRT-PCR result provides evidence that PRV occurred in wild-source steelhead trout as early as 1977. Sample size was not sufficient before 1986 (21 samples collected from 1974 to 1977) to support strong conclusions about PRV in British Columbia during this time. We confirmed that the fish at the marine site that had received smolts from the source hatchery in 2013 were infected with PRV, and analysis of archived tissues back to 2000 confirmed that the source hatchery and all tested fish groups with links to the same hatchery were also PRV positive. The trend of a greater proportion of PRV positives in 2013 among farm salmon than wild salmon is consistent with PRV findings in Norwegian salmonids (Garseth *et al.* 2013). These data are consistent with the hypothesis that PRV transfers readily among salmon within a farm but does not transfer as readily from infected farm or wild salmon to other wild salmon. Further, in DFO area 12 in 2008, fish at both net cage Atlantic salmon marine farms that we studied were positive for PRV, but 200 wild juvenile pink salmon migrating in the same area were negative for PRV (Saksida *et al.* 2012). Additional work is needed to determine the relative role of salmon farms and hatcheries in the spread of PRV from cultured to wild salmon.

Our results do not support the hypothesis that PRV entered western Canada recently, nor do they provide evidence that the first Atlantic salmon imported to western Canada for aquaculture were infected with PRV. The only other study that reported PRV in British Columbia concluded that PRV entered western Canada recently and that Canadian isolates diverged from Norwegian isolates between 2006 and 2008 (Kibenge *et al.* 2013); this conclusion was based on analysis of 14 salmon collected in British Columbia from February through June 2012. Our nucleic acid sequencing was sufficient to confirm that our test was amplifying PRV, but the amount of sequencing that would be needed to clarify differences in British Columbia and Norwegian PRV genotypes was beyond the scope of our study. Phylogenetic analysis is best done on long sequences of intact genes (usually > 1000 bp) in fresh-frozen samples (e.g. Kibenge *et al.* 2013) rather than the short genome segments (often < 180 bp) in the paraffin-embedded tissues available to our study before 2013. From 2000 to 2013, no changes occurred in the proportion of PRV positives or PRV-associated lesions among farmed salmon that would suggest a change in PRV genotype between 2006 and 2008. In Norway, differences in PRV genotype have not been helpful in determining whether fish with PRV develop HSMI (Løvoll *et al.* 2012), and in British Columbia, we cannot rule out historic changes in genotype that had no

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effect on infectivity or pathogenicity. Atlantic salmon were first imported into western Canada for stock enhancement in 1905 (Maccrimmon & Gots 1979), but they were not imported for aquaculture purposes until 1985 (Castledine 1991). We did not identify samples from farmed Atlantic salmon in BC that had been collected in 1985, but no positive or suspect cases in 1986 (31 paraffin blocks from seven different case numbers) is clearly different from the pattern in the paraffin samples that we tested from 2000 to 2013, where all cases included fish positive for PRV. The lack of PRV-positive samples in 1986 changed in 1987, when two of five groups included samples suspect for PRV, despite evidence of significant nucleic acid degradation among the 1987 samples. With salmonids widely transferred within and between Northern Europe and North America since the early 1800s (Maccrimmon & Gots 1979; Savini *et al.* 2010), and with coho salmon, Chinook salmon, and rainbow trout imported into Chile since the first decade of the 1900s (Crawford & Muir 2008), substantial additional work is needed to determine the original source of PRV.

Because of limitations of rRT-PCR analysis using tissues originally preserved for histopathology, our results might underestimate the true prevalence of PRV in the archived paraffin samples. When analysing paraffin sections by rRT-PCR, the amount of nucleic acid in each test is highly variable because of differences in (i) fixation time (fixatives for optimal histopathology can render segments of nucleic acid undetectable) (Cox *et al.* 2006), (ii) decalcification (the acids used for tissue decalcification can render segments of nucleic acid undetectable) and (iii) initial tissue volume (tissues in this study varied from small fractions of alevins that did not render sufficient material for a visible pellet for nucleic acid extraction, to sections of multiple organs from large fish that nearly filled the 35 × 25 mm block). A positive result is evidence that PRV was present in the sample tested, but a negative result does not rule out the presence of PRV in the fish when it was alive. Also, because some sections contained skin, gills and gastrointestinal contents, we cannot differentiate whether a positive result represents an infected fish, an infected environment (on skin, gills or feed) or infected food. Finally, because of the potential for cross-contamination during sampling and initial paraffin processing, a weak positive rRT-PCR result in one fish might be a result of contamination from other fish in the case or block.

None of the fish in our study had microscopic lesions diagnostic for HSMI, and the relation of lymphohistiocytic endocarditis and PRV was variable. Our data best support the hypothesis that PRV among salmonids in the eastern Pacific Ocean occurs primarily as a subclinical infection rather than as a persistent infection after disease. If PRV was causing disease, at least some of the farm fish tested during our study should have had significant lesions associated with PRV, but that was not the case. The only infectious agent clearly related to heart inflammation in our study was *Loma salmonae* in two wild coho salmon, one of which was also rRT-PCR positive for PRV. The lesions in these two fish seem sufficient to have impaired cardiac function and, thereby, upstream-spawning migrations. If tests for infectious agents in these fish had been limited to PRV, impaired cardiac function in the PRV-infected fish might have been falsely attributed to PRV. Because fish with cardiac *Loma salmonae* infection included both PRV-positive and PRV-negative fish, histopathology provides better evidence that any impairment in cardiac function was due to *Loma salmonae* infection rather than PRV.

Our finding of low PRV Ct values in fish without heart lesions supports the hypothesis that fish can have high levels of PRV without HSMI, consistent with the occurrence of abundant PRV in some wild Atlantic salmon in Norway with few heart lesions (Garseth *et al.* 2013). This finding is also contrary to the relation of PRV and HSMI in one experiment in Norway, where the peak level of virus replication coincided with the peak of heart lesions (Mikalsen *et al.* 2012). Among the 20 farm fish sampled in 2013 in our study, PRV Ct values tended to be greater in fish with endocarditis than in fish without endocarditis. In contrast, several other sample groups had a consistent pattern in which the fish with the lowest PRV Ct values had no endocarditis, but the proportion of hearts with endocarditis was greater among fish with lower PRV Ct values than among fish that had higher PRV Ct values (or were negative for PRV). In all of these other groups, the range of PRV Ct values among fish with endocarditis was included within the range of PRV Ct values of fish without endocarditis. This overlap is similar to the relation of PRV and HSMI-associated lesions in Norway (Løvoll *et al.* 2012). Further

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work is needed to differentiate whether PRV has a direct role in the pathogenesis of some cases of lymphohistiocytic endocarditis or whether the presence of endocarditis sometimes enhances replication of PRV. Also, further work in Norway to determine whether PRV predated the first diagnosis of HSMI in 1999 might provide insights on the role of PRV in HSMI.

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Conflict of interest

Coauthor D.B. Morrison is an employee of Marine Harvest Canada; she designed the sampling study plan for the entire study. The other coauthors have no conflict of interest to declare.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1. Sample data and interlaboratory comparison.

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