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Piscine reovirus, but not Jaundice Syndrome, was transmissible to Chinook Salmon, *Oncorhynchus tshawytscha* (Walbaum), Sockeye Salmon, *Oncorhynchus nerka* (Walbaum), and Atlantic Salmon, *Salmo salar* L.

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Abstract

A Jaundice Syndrome occurs sporadically among sea-pen-farmed Chinook Salmon in British Columbia, the westernmost province of Canada. Affected salmon are easily identified by a distinctive yellow discolouration of the abdominal and periorbital regions. Through traditional diagnostics, no bacterial or viral agents were cultured from tissues of jaundiced Chinook Salmon; however, piscine reovirus (PRV) was identified via RT-rPCR in all 10 affected fish sampled. By histopathology, Jaundice Syndrome is an acute to peracute systemic disease, and the time from first clinical signs to death is likely <48 h; renal tubular epithelial cell necrosis is the most consistent lesion. In an infectivity trial, Chinook Salmon, Sockeye Salmon and Atlantic Salmon, intraperitoneally inoculated with a PRV-positive organ homogenate from jaundiced Chinook Salmon, developed no gross or microscopic evidence of jaundice despite persistence of PRV for the 5-month holding period. The results from this study demonstrate that the Jaundice Syndrome was not transmissible by injection of material from infected fish and that PRV was not the sole aetiological factor for the condition. Additionally, these findings showed the Pacific coast strain of PRV, while transmissible, was of low pathogenicity for Atlantic Salmon, Chinook Salmon and Sockeye Salmon.

Keywords: Atlantic Salmon, Chinook Salmon, Jaundice Syndrome, piscine reovirus, Sockeye Salmon.

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Introduction

Jaundice occurs sporadically in numerous teleost species throughout the world. Fish suffering from this syndrome often have a mild to severe yellow discolouration of the skin, and this distinctive clinical presentation likely accounts for its global recognition. From the multitude of reports describing this condition, the aetiology associated with jaundice in fish is probably varied and may involve infectious agents, nutritional and toxic factors, as well as genetic abnormalities (Jones & Smith 2014).

Among salmonid species, jaundice occurs in Atlantic Salmon, Chinook Salmon, coho salmon, *Oncorhynchus kisutch* (Walbaum), and Rainbow Trout *O. mykiss* (Walbaum), with cause attributed to either erythromycin toxicity (Haukenes & Moffitt 2002), exposure to industrial effluents (Everall, Nitchell & Robson 1992) or potentially due to a viral infection (Sakai *et al.* 1994). There has also been the sporadic occurrence of a jaundice condition in seawater-cultured coho salmon in Chile,

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where the cause of these outbreaks remains unresolved. Initially, infectious salmon anaemia virus (ISAV) was suspected as the virus was associated with one case of jaundiced coho salmon in Chile; however, Atlantic Salmon held in the same sea net-pens did not develop disease despite being a susceptible host to ISAV (Kibenge *et al.* 2001). Furthermore, under controlled laboratory conditions, coho salmon are largely refractory to ISAV (Rolland & Winton 2003), and the Chilean jaundice disease (denoted infectious haemolytic anaemia of salmon, IHAS) is transmissible in the absence of ISAV (Smith *et al.* 2006). Because IHAS was transmitted after experimental inoculation with filtered organ homogenate from an IHAS fish, a viral aetiology is suspected (Smith *et al.* 2006).

In British Columbia (BC), the westernmost province of Canada, a recurrent Jaundice Syndrome sporadically affects cultured Chinook Salmon. Fish with clinical signs of this syndrome are commonly referred to as 'yellow fish' with discoloration of the skin of the abdominal and periorbital region. Because yellow fish are not observed among fish identified as 'slow swimmers' or during dives that occur at least once a week, the syndrome is thought to be acute: probably no more than a few days from onset of clinical signs until death. Cumulative mortality associated with this syndrome, albeit low (<1.5% per production cycle), is sometimes the most significant cause of death in a pen. In this study, we investigated Jaundice Syndrome using field observations, a series of laboratory diagnostics and experimental infectivity trials."

Material and methods

Jaundice fish sampling

Over two separate collection periods, a total of 10 moribund or dead Chinook Salmon with clinical signs of jaundice were sampled from a seawater net-pen farm in Clayoquot Sound, BC (Table 1). The first four fish were collected in January 2009, at which time fish were examined by necropsy and specimens of brain, liver, anterior kidney, and spleen were aseptically sampled for bacterial and viral analyses via culture and molecular methods as described below. Additionally, kidney and liver imprints were fixed in pure methanol and stained with Diff-Quick (Fisher Scientific, Kalamazoo, MI, USA) following manufacturer's instructions.

Table 1 Summary of Chinook Salmon with Jaundice Syndrome and PRV screening results

Collection date	Fish #	Tissue	PRV RT-qPCR Ct value[a]
January 2009	1	Kidney	20.4
	2	Spleen	16.7
	3	Spleen	15.6
	4	Liver	19.4
		Brain	19.0
April 2012	5[b]	Kidney	22.5
		Liver	19.7
	6[b]	Kidney	22.9
		Liver	17.8
	7[b]	Kidney	22.2
		Liver	19.4
	8	Kidney	21.5
		Liver	19.7
	9	Kidney	21.5
		Liver	19.9
	10[b]	Kidney	20.6
		Liver	25.1
	Challenge inoculum	Kidney + Liver	26.2

[a]Ct value is an average of duplicate or triplicate runs.
[b]Fish contributed tissue to the challenge inoculum.

The second sampling occurred in April 2012, when six Chinook Salmon with clinical signs of jaundice were bled for haematocrit and blood smear. Blood smears were stained using Diff-Quick. Samples of anterior and posterior kidney, spleen, liver, heart, gills and skeletal muscle with skin from each fish were preserved in 10% (v/v) neutral buffered formalin, processed by routine methods, sectioned at 5 μm and stained with haematoxylin and eosin (H&E). Samples of liver and anterior kidney from each fish were also harvested for viral screening via reverse-transcriptase real-time PCR (RT-rPCR) and for generating a pooled tissue homogenate inoculum to be used in infectivity trials. Tissue samples destined for molecular diagnostics were placed directly into RNAlater, while larger tissue pieces used in the infectivity trials were placed into Hanks' balanced salt solution (HBSS) and immediately placed on ice until further use."

Bacteriology and virology diagnostics

Kidney tissues from the four fish collected in 2009 were aseptically streaked onto trypticase soy agar (TSA), TSA + 1% NaCl (TSA/s), and Shieh's medium. The TSA and TSA/s plates were incubated at 20 °C and Shieh's at 16 °C for 13 days. For virological culture, brain, liver, kidney and spleen from each of the four fish sampled in 2009

were individually homogenized with a stomacher (Seward Stomacher 80 Biomaster Labsystem, Bohemia, NY, USA) and diluted with MEM-4+ antibiotics (minimum essential medium supplemented with 4% foetal bovine serum and buffered with HEPES; Gibco, Grand Island, NY, USA) to yield a 2% tissue homogenate which was centrifuged at 2500 g for 15 min at 4 °C. Clarified supernatant was passed through a 0.45-μm low-protein binding membrane filter (Nalgene, Rochester, NY, USA) and inoculated onto duplicate monolayers of epithelioma papulosum cyprini (EPC) (Fijan *et al.* 1983; Winton *et al.* 2010), salmon head kidney (SHK-1)(Dannevig, Falk & Namork 1995), Rainbow Trout gonad (RTG-2) (Wolf & Quimby 1962), and Chinook Salmon embryo (CHSE-214) cells (Fryer, Yusha & Pilcher 1965) and incubated at both 10 and 15 °C. Virus screening was also conducted on the organ homogenate used in the infectivity trial with inoculations performed on common carp brain (CCB) (Neukirch, Bottcher & Bunnajirakul 1999), Atlantic Salmon kidney (ASK) (Devold *et al.* 2000) and orange-spotted grouper fin (GF-1) (Chi, Hu & Lo 1999) cell lines in addition to those lines mentioned previously. All field-collected jaundice fish and the pooled organ homogenate used in the infectivity trial were screened by PCR, using published primers and probe sequences, for the presence of *Renibacterium salmoninarum* (Brown *et al.* 1994), infectious hematopoietic necrosis virus (IHNV) (Purcell *et al.* 2013), viral haemorrhagic septicaemia virus (VHSV) (Garver *et al.* 2012) and piscine reovirus (PRV) (Løvoll *et al.* 2012)."

Jaundice transmission trial

Inoculum. Pooled kidney and liver tissue homogenate was prepared from four Chinook Salmon with clinical signs of the Jaundice Syndrome (Table 1). From each fish, 2 g of kidney and 3 g of liver were collected and amalgamated into a sterile container, diluted 1:5 (w/v) in HBSS and homogenized using a Polytron. The homogenate was clarified by centrifugation at 2500 g at 4 °C for 12 min. The resulting supernatant along with an aliquot which was filtered at 0.2 μm were used as inocula in the infectivity trial and were denoted as either the unfiltered or filtered homogenate."

Fish and experimental inoculum. Chinook Salmon Jaundice Syndrome has only been observed during the fish's saltwater phase, with signs of jaundice first occurring in the late fall (November) and subsiding in the late spring (June) (authors' observations). Therefore, experimental exposures were conducted in marine life-stage fish in partial sea water at 10 °C to reflect the winter/spring temperature and salinity conditions experienced at the farm site where jaundice occurs. Chinook Salmon, Sockeye Salmon and Atlantic Salmon of mean length 50.4, 15.6 and 24.7 cm, respectively, were used in infectivity trials to investigate the potential for jaundice transmission. The Chinook Salmon were adult fish maintained in sea water, while the Sockeye Salmon and Atlantic Salmon were smolts that were transitioned to sea water on the day of the injection challenge. Chinook Salmon (Quinsum stock, $n = 16$) were injected intraperitoneally (ip) with 1 mL unfiltered homogenate and held in partial sea water (21 ppt) at 10 °C. Sockeye salmon (Sakinaw stock, $n = 22$) and Atlantic Salmon (Mowi, $n = 25$) were ip injected with 0.2 or 0.5 mL unfiltered homogenate, respectively, and held in sea water (31 ppt) at 10 °C. Additionally, one group of Atlantic Salmon ($n = 25$) was ip injected with 0.5 mL filtered (0.2 μm) homogenate and held in sea water (31 ppt) at 10 °C. For each species, a control group of equivalent size and number remained untreated. After initiation of the infectivity trial, fish were monitored daily for clinical signs of disease and all data related to mortality, feed, water temperature and salinity were recorded for 22 weeks. At the conclusion of the study, a subset of each fish species was necropsied and tissue samples of liver, kidney, heart and skeletal muscle were collected for histological examination, while liver and brain specimens were sampled for the RT-rPCR screening of PRV and for quantification of *Mx-1* expression as described below. Histopathology was performed by a single pathologist (GDM) that was not provided information about the PRV status of the fish, that is blinded analysis. Photomicrograph illumination and colour balance were optimized with blank-field correction (Marty 2007)."

PRV detection and quantification of Mx-1 gene expression. Samples of liver and brain from fish experimentally injected or left untreated (controls) were individually homogenized in 1 mL TRIzol® (Life Technologies™, Carlsbad, CA, USA) using one stainless steel bead (5 mm; Qiagen, Mississauga, ON, Canada) and a TissueLyser II (Retsch

Inc., New Town, PA, USA) for 2 min at 25 Hz at room temperature. Total RNA was extracted following the TRIzol® protocol using 1-bromo-3-chloro-propane in place of chloroform. Total RNA (1.5 μg) from liver and brain was individually screened using RT-rPCR targeting the L1 segment of PRV using primer and probe sequences described in Løvoll *et al.* 2012;. The PRV RT-rPCR was conducted as a two-step reaction such that cDNA was first generated using a High Capacity cDNA reverse transcription kit (Life Technologies™) of which 2.5 μL of this product was subsequently used in the real-time PCR (rPCR) with 0.4 μM each of forward and reverse primer, 0.3 μM probe and 1× Taq Man 2× Universal Reaction Mix (Life Technologies™). Cycling conditions for PRV amplification were 1 cycle of 94 °C for 15 min and 45 cycles of 94 °C for 15 s, 54 °C for 30 s and 72 °C for 15 s. To evaluate whether presence of PRV was associated with an antiviral response, the *Mx-1* gene expression in the liver of inoculated and control fish was analysed using RT-qPCR. Prior to *Mx-1* gene RT-qPCR, genomic DNA was removed from the total RNA from liver specimens through DNase I treatments using the TURBO DNA-*free*™ Kit (Life Technologies™) following manufacturer's instructions. The RNA quantity and quality was determined by spectrophotometry (NanoDrop-1000). The cDNA was synthesized using a high capacity cDNA reverse transcription kit and qPCR consisted of 2.5 μL cDNA (input of 187.5 ng of total RNA), 0.9 μM each of forward and reverse primer, 0.25 μM probe and 1× Taq Man 2× Universal Reaction Mix (Life Technologies™). The cycling programme consisted of 1 cycle of 50 °C for 2 min, 1 cycle of 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. For each sample, the target transcript and reference gene were run in triplicates. Both negative and positive controls were included on every plate. For analysis of the reference gene *acidic ribosomal phosphoprotein P0* (*ARP*), the primers and probe were used as described previously (Pierce *et al.* 2004). For *Mx-1*, the primers were consistent among three salmon species; however, a different probe was used for Atlantic Salmon than used for Chinook and Sockeye. The primers and probe used for Atlantic Salmon were as described in Purcell *et al.* (2006), while *Mx-1* probes for Chinook and Sockeye Salmon were designed as in Purcell *et al.* (2009). Primer efficiencies were determined using a serial dilution (fivefold, 5-point) of undiluted cDNA of liver from Sockeye, Atlantic or Chinook Salmon. Relative expression levels were calculated using the comparative Ct method for relative quantification including primer efficiencies (Pfaffl 2001). One control fish for each species served as a calibrator, and *ARP* was used as a reference gene. Each control group consisted of four fish, while 10, 10 and 15 fish were used for Atlantic, Sockeye Salmon and Chinook Salmon, respectively. To find significant differences between control and injected fish within each species, t-tests ($\alpha = 0.05$) were carried out using $\log_2$-transformed fold changes of injected and control fish. To determine whether viral load correlates with *Mx-1* fold changes, Ct values of PRV L1 fragment and $\log_2$ fold changes of *Mx-1* expression were plotted, and R^2 was determined with SigmaPlot 10.0."

PRV sequence confirmation. The PCR detection of PRV from each of the four jaundice fish that contributed tissues to the challenge inoculum (#5, 6, 7, and 10; Table 1) and from a representative fish from each of the three salmon species sampled at the end of the transmission trial was further verified via nucleotide sequencing. Sequence confirmation was carried out on PCR-amplified fragments of PRV S1 gene using 2 overlapping sets of primers as described by Løvoll *et al.* (2012) with the exception of using the reverse complement sequence of primers S4_621R and S4_1036R. PCR products were purified using ExoSAP-IT kit (USB Affymetrix, Cleveland, OH, USA) according to the manufacturers' protocols. Purified PCR product (2 μL) was added to the BigDye Terminator (BDT) cycle sequencing kit (Applied Biosystems). Sequencing products were purified using the DyeEx spin kit (Qiagen) and run on a 3130xl Genetic Analyzer (Applied Biosystems). Sequencing analysis software (version 5.2, Applied Biosystems) was used for base calling, and sequences were edited with Sequencher software (version 4.7, Gene Code)."""

Results

Field observations

Fish collected from the net-pen sites with clinical signs of jaundice included external yellow discolouration of the abdominal and periorbital region (Fig. 1a). Internally, the fish had empty stomachs, and in some cases, the livers were pale (Fig. 1b).

Figure 1 Clinical presentation of Jaundice Syndrome in Chinook Salmon, *Oncorhynchus tshawytscha*. (a) yellow discolouration of the abdominal and periorbital region and (b) a pale liver.

From five of the six jaundice fish collected in 2012, histological examination revealed renal tubular epithelial cell necrosis as the most consistent lesion (Fig. 2). Consistent with Jaundice Syndrome being a systemic disease, all five fish also had at least one other significant lesion, including hepatocellular single cell necrosis, hepatocellular hydropic degeneration, renal interstitial cell necrosis, endocardial hypertrophy, splenic leucocytic karyorrhexis, splenic parenchymal fibrin and intestinal lamina propria congestion (Table S1 data set). All of these lesions are acute and might have developed <48 h before the fish died or became moribund."

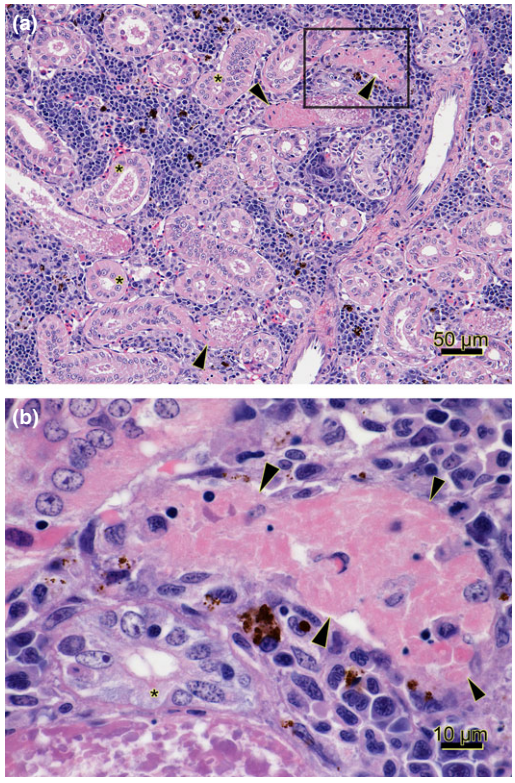

Figure 2 Trunk kidney section (H&E stain) from a jaundiced Chinook Salmon that was a source of inoculum for the transmission trial. Morphology of tubular epithelium varies from acute coagulative necrosis (arrowheads) to relatively normal (*). Black box in (a) outlines the area shown at higher magnification in (b).

Laboratory diagnostics

Bacteriology and haematology. No significant bacteria growth occurred on any of the culture media. Also, Gram-stained kidney smears had no bacteria, and *Renibacterium salmoninarum*-specific PCR screening of individual jaundice fish and pooled organ homogenate used in the infectivity trial were negative. Kidney imprints had no parasites, haematocrit varied from 18 to 39% with a mean of 29% (SD 9.1), and blood smears had no definitive inclusion bodies."

Virology. All 10 jaundice fish were positive for PRV, with Ct values ranging from 15.6 to 25.1 (Table 1), while RT-qPCR assays for IHNV and VHSV were negative. Cell cultures inoculated with

individual organ tissues or pooled tissues from jaundiced fish did not have sustained cytopathic effects (CPE). A non-conspicuous CPE occurred in EPC, CCB and GF-1 cells. The cellular changes appeared as early as 2 days post-inoculation and began with foci of rounded cells, some of which ultimately became detached. These cellular changes occurred at most in two passages and were not sustained through continual passage. The most extensive CPE occurred in GF-1 cells, but these changes could not be passaged. Additionally, PRV RNA copy number did not increase in the cell culture supernatant using RT-qPCR analysis from day 0 to day 6, despite evidence of CPE at day 6.""

Jaundice transmission trial

Mortality and histopathology. No disease or jaundice was reproduced in any of the species over the 155-day (22 week) period post-inoculation. One Atlantic Salmon died 3 days post-injection in the group receiving the filtered homogenate, and one Chinook Salmon was discovered dead outside of the tank 70 days post-inoculation. No other mortality occurred in any of the other tanks during the 22-week holding period. Similarly, no mortality occurred in the untreated control tanks.

By histopathology, fish surviving 22 weeks post-inoculation had no lesions consistent with Jaundice Syndrome and no evidence of infectious disease that would threaten their survival (details in Table S1 dataset). Among the Chinook Salmon, however, several microscopic lesions clearly separated the challenged fish from the non-injected controls. The most distinctive lesions in the challenged fish were hepatocellular cytoplasmic iron-rich pigment granules (87% affected, Fig. 3) and renal erythrophagocytosis (also 87% affected,

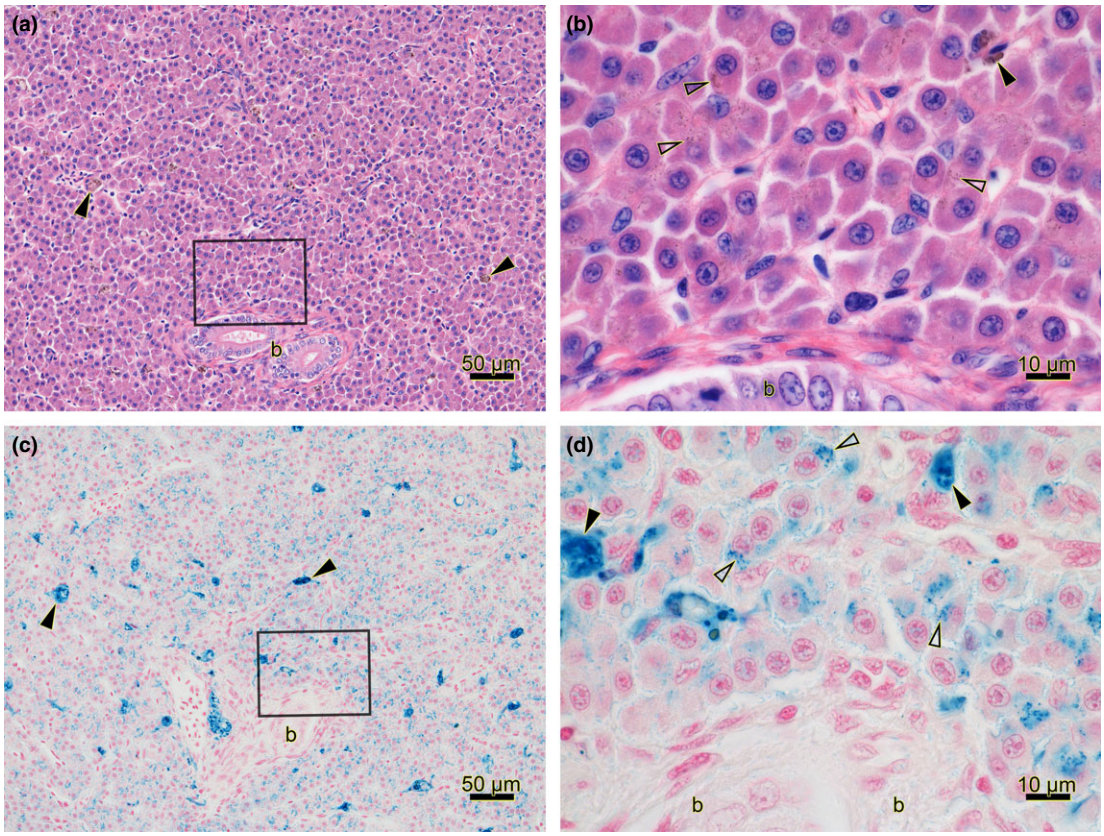

Figure 3 Liver sections from a Chinook Salmon sampled 5 months after receiving an ip injection of unfiltered inoculum from jaundiced Chinook Salmon. Scattered macrophages contain globular iron-rich cytoplasmic pigment (closed arrowheads). Hepatocyte cytoplasm contains iron-rich granules (open arrowheads). Biliary epithelium (b) does not contain pigment. Black boxes in (a, c) outline the area shown at higher magnification in (b, d). Images are from the same location within step sections cut from one paraffin block: top images (H&E stain); bottom images (Perl's iron stain).

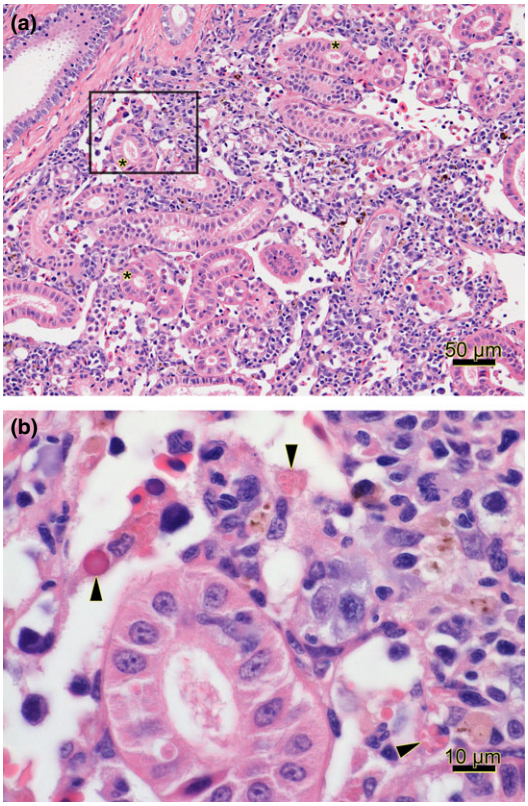

Figure 4 Trunk kidney section (H&E stain) from the same Chinook Salmon as in Fig. 3. The interstitium is expanded by macrophages, some of which contain cytoplasmic eosinophilic inclusions (arrowheads) that are roughly spherical, 1–6 µm in diameter, and might be a result of erythrophagocytosis. Morphology of tubular epithelium is relatively normal (*). Black box in (a) outlines the area shown at higher magnification in (b).

Fig. 4); neither of these lesions occurred among the control fish. Other lesions that affected only challenged fish – all of mild severity – included hepatocellular cytoplasmic vacuoles (33%), leucocytic hepatitis (33%), renal tubular cytoplasmic protein droplets (20%), renal glomerular protein deposits (20%), myocardial karyomegaly (20%) and lymphohistiocytic endocarditis (60%). Lesions that occurred in only the control fish included mild hepatocellular hydropic degeneration (50%) and mild renal mineralization (75%). Because the study included only four control fish, confidence intervals around control fish prevalence values are large.

Among the Sockeye Salmon, mild lymphohistiocytic endocarditis affected seven of 10 challenged fish but none of the five controls; these differences are probably real (i.e. not the result of chance alone). Also, small foci of hepatocellular

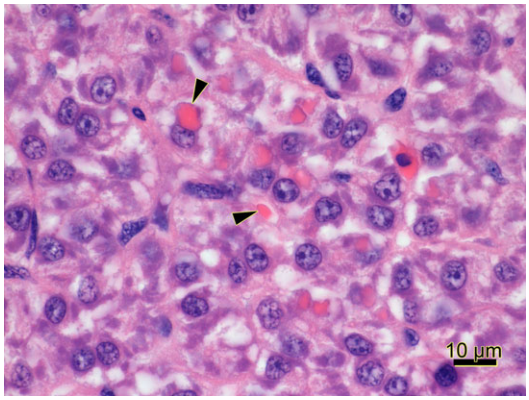

Figure 5 Liver section (H&E stain) from a Sockeye Salmon sampled 5 months after receiving an ip injection of unfiltered inoculum from jaundiced Chinook Salmon. The sections contain a single focus of hepatocytes with brightly eosinophilic, angular, cytoplasmic protein inclusions (arrowheads) that have tinctorial properties similar to erythrocyte cytoplasm. Hepatocytes without these inclusions are relatively normal, with wispy cytoplasmic vacuoles that are consistent with glycogen.

cytoplasmic protein occurred in three of 10 challenged fish (Fig. 5) compared with none of the five controls. Both of these lesions might be sequelae to injected foreign material, and the lymphohistiocytic endocarditis might also be the result of a low-grade or resolving viral infection.

Among the Atlantic Salmon, the 10 challenged fish included two fish with mild lymphohistiocytic endocarditis and no fish with lymphohistiocytic epicarditis. The five controls included no fish with lymphohistiocytic endocarditis and two fish with mild lymphohistiocytic epicarditis."

PRV detection. All fish given the jaundice inoculum tested positive for PRV nucleic acid by RT-rPCR at the conclusion of the infectivity trial (155 dpi). Positive amplification, identified by a repeatable cycle threshold (Ct) value, occurred in 100% of the liver and brain tissues sampled from 15 Chinook Salmon and 10 Atlantic Salmon injected with the unfiltered homogenate as well as in 10 Atlantic Salmon receiving the filtered homogenate. Among the 10 Sockeye Salmon that were sampled for PRV, all brain samples were positive, while only nine of the 10 liver samples were positive. Among the PRV-positive brain and liver specimens of the different salmon species, the lowest Ct value occurred in Atlantic Salmon brain with a median Ct at 31.5, while median Ct values for Chinook Salmon and Sockeye Salmon were

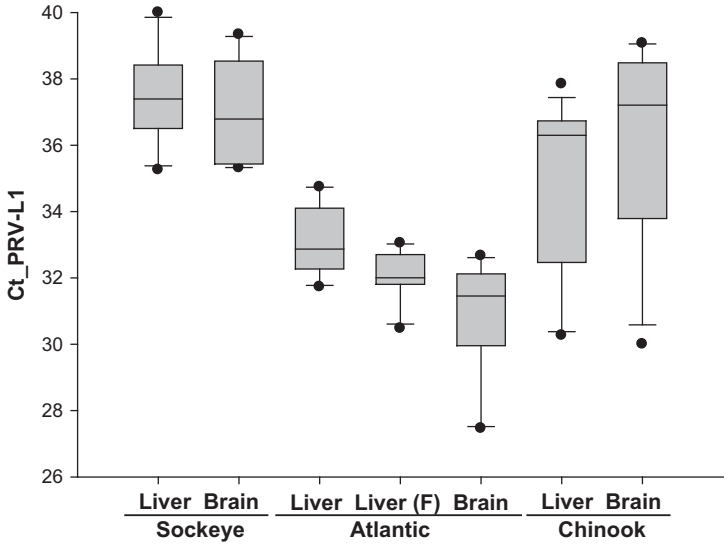

Figure 6 Ct values of the PRV-L1 fragment in liver and brain 5 months after salmon were injected with tissue homogenate of Chinook Salmon with Jaundice Syndrome. Inoculation volumes vary between species. Boxplots display median and quartiles, and circles are outliers. Error bars above and below the box indicate the 90 and 10th percentiles. (F) denotes filtered homogenate.

nearly equivalent (Fig. 6). No positive PRV amplification occurred in any of the unhandled control fish."

Quantification of Mx-1 expression changes. As evidence of an antiviral response, injected Atlantic Salmon and Chinook Salmon had a significant increase in *Mx-1* expression compared to their controls (Fig. 7a, b). In contrast, injected and non-injected Sockeye Salmon had no significant differences in *Mx-1* gene expression (Fig. 7c). For Atlantic Salmon, *Mx-1* expression ranged from 1.1 to 3.3 $\log_2$ fold change with an average of 2.5. For Chinook Salmon, *Mx-1* expression was considerably more variable among individuals, ranging from 0.6 to 9.5 $\log_2$ fold change with an average of 4.2. No clear correlation occurred between PRV loads and level of *Mx-1* expression (Fig. 8)."

Sequence results. PRV-specific sequences were confirmed in all seven of the PRV-positive PCR samples that underwent nucleotide sequence confirmation. Among the four jaundice fish whose tissues made up the challenge inoculum, two PRV sequence types were revealed. The two sequence types only differed by one base over the 977-bp fragment of the S4 gene: one sequence type occurred in fish #5 and #7, while the other type occurred in fish #6 and #10. Both of these sequence types were 99% similar to other PRV sequences identified in fish from western Canada (GenBank Accession KC795600.1). In samples from each of the three fish (one of each species studied) at the conclusion of the transmission trial, PRV-specific sequence was confirmed and each fish contained the identical sequence type as in fish #5 and #7 from the challenge inoculum. The sequence that occurred in fish #6 and #10 and used in the inoculum did not occur in samples from the three fish tested at the end of the experiment."""

Discussion

A recurrent Jaundice Syndrome observed in sea-pen-reared Chinook Salmon in British Columbia was not transmissible through laboratory exposure. This lack of experimental reproduction of the Jaundice Syndrome despite conducting exposures under environmental conditions mirroring those found in association with the jaundice suggests a low infectious nature of this syndrome. Such negligible transmissibility may in part explain the sporadic nature and limited prevalence of the Jaundice Syndrome on a farm. From histological examination of jaundice salmon, the syndrome is likely a systemic disease with lesions often occurring in multiple organs such as liver, kidney, spleen, heart and intestine. Because jaundice fish are not detected clinically until after they die, and microscopic lesions in dead fish are consistently acute to peracute, the time from first clinical signs to death might be <48 h.

Interestingly, PRV was detected in each of the field-collected jaundice Chinook and hence was

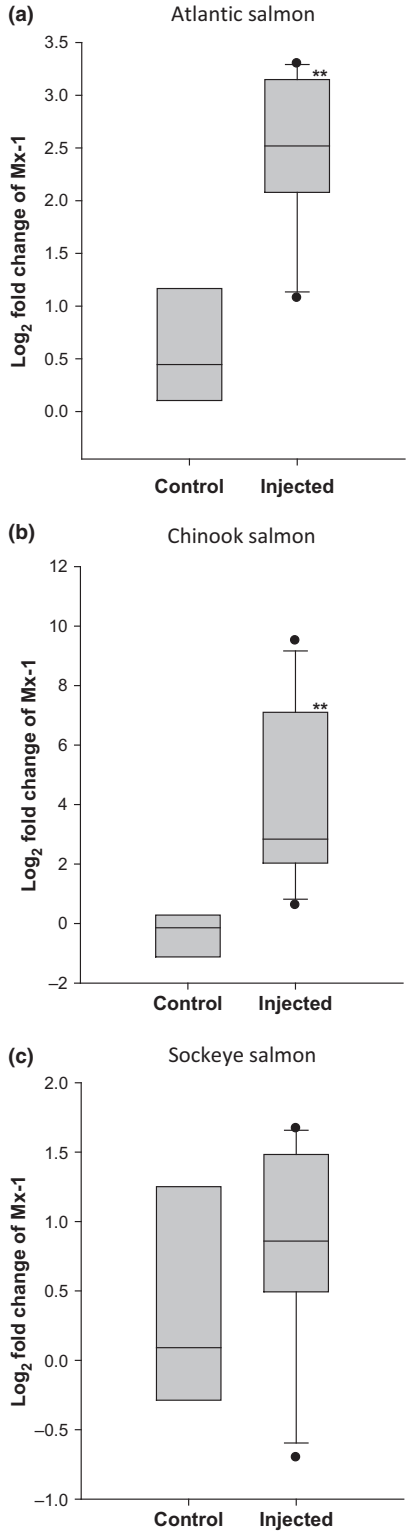

Figure 7 *Mx-1* gene expression in liver of (a) Atlantic Salmon, *Salmo salar* (b) Chinook Salmon and (c) Sockeye Salmon, *Oncorhynchus nerka* injected with tissue homogenate of Chinook with Jaundice Syndrome or left untreated (control). Log_2 fold changes in boxplots display median and quartiles, and circles are outliers. Error bars above and below the box indicate the 90 and 10th percentiles. ** denotes $P \leq 0.01$. Controls: $n = 4$ each; injected Atlantic Salmon, Sockeye Salmon: $n = 10$ each; injected Chinook Salmon: $n = 15$.

abundant in the challenge inoculum. PRV was first described in 2010 when its genomic sequence was recovered from high throughput pyrosequencing of Atlantic Salmon that had the syndrome heart and skeletal muscle inflammation (HSMI) (Palacios *et al.* 2010). Since this discovery, PRV has been hypothesized as the cause of HSMI; however, the true relationship between PRV and HSMI is confounded because PRV often occurs at low Ct values in wild Atlantic Salmon in Norway without signs of HSMI (Garseth *et al.* 2013).

In British Columbia, a retrospective study revealed PRV genetic material to be common among wild and farmed fish from 1987 to 2013 with no concomitant evidence of HSMI, which is not known to occur in BC (Marty *et al.* 2014). Nonetheless, the detections of PRV in the North Pacific raise concerns regarding the pathogenesis of this virus in native Pacific and farmed Atlantic Salmon that reside in these waters. In our controlled laboratory exposure study, we demonstrated that PRV persisted in each of the Chinook Salmon, Sockeye Salmon and Atlantic Salmon for 5 months after ip challenge without resulting in microscopic evidence of HSMI or any other disease. Among the fish receiving the ip injection of PRV-positive tissue homogenate, a few microscopic lesions were somewhat common and occurred only among challenged fish. Examples include lymphohistiocytic endocarditis in heart sections of all three salmon species, hepatocellular cytoplasmic iron pigment in Chinook Salmon and erythrophagocytosis in the renal interstitium of Chinook Salmon. Among wild Pacific salmon in BC (Marty *et al.* 2014), the proportion of fish with endocarditis was significantly greater among PRV-positive fish (3 of 9 = 33%) than among PRV-negative fish (9 of 195 = 4.6%). In our study, the suite of control fish was limited and we are unable to differentiate whether the lesions in challenged fish were a result of injection of foreign antigens, PRV infection, infection with some other agent or some combination of these variables. Consequently, further exposure studies are underway to better understand the relation of PRV and lesions.

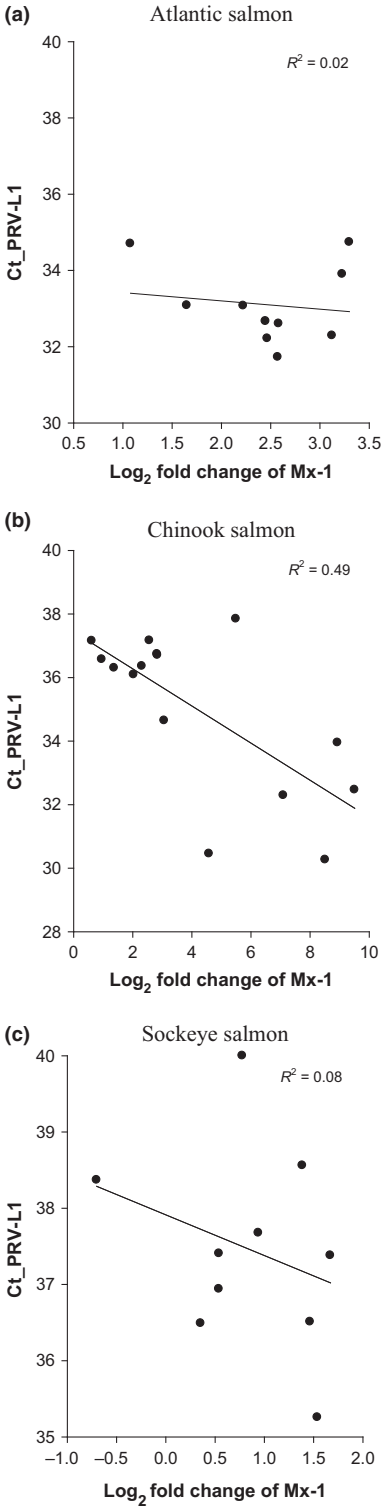

Figure 8 Correlation between $\log_2$ fold change of *Mx-1* gene expression and Ct values of the PRV L1 fragment in liver of (a) Atlantic Salmon, (b) Chinook Salmon and (c) Sockeye Salmon, injected with tissue homogenate of Chinook with Jaundice Syndrome.

Our study supports the hypothesis that exposure to PRV is not solely responsible for the development of Jaundice Syndrome. It may be possible that the presence of PRV is not contributory towards jaundice in Chinook but rather that its association is merely a reflection of the ubiquitous presence of PRV in wild and farmed salmon species of BC. Further research will be needed to better understand the relation of Jaundice Syndrome and PRV. It is noteworthy that in experiments based on cohabitant transfer of PRV in Atlantic Salmon, erythrocytes were revealed as a target cell for PRV infections (Finstad *et al.* 2014). In these virus-infected Atlantic Salmon, the proportion of PRV-positive erythrocytes reached 51% of the red blood cell population, and infections of erythrocytes often had cytoplasmic inclusion bodies. This tropism of PRV in Atlantic Salmon erythrocytes and its ability to form inclusions is of interest in the context of the Jaundice Syndrome described herein, because there have been reports describing viral inclusions in salmonid erythrocytes (collectively termed EIBS) that have been associated with jaundice (Sakai *et al.* 1994). In these previous reports on EIBS, electron microscopy, acridine orange staining and chloroform sensitivity measurements demonstrated the virus(es) causative of EIBS were likely enveloped particles (Leek 1987; Arakawa *et al.* 1989; Piacentini, Rohovec & Fryer 1989; Rodger *et al.* 1991) and, therefore, were not a result of the double-stranded, non-enveloped PRV. Nonetheless, studies are underway to evaluate relationships between PRV and EIBS.

The observation of PRV-persistent states in Pacific and Atlantic Salmon is curious in terms of how a virus modulates or escapes the host's antiviral responses. The significant upregulation of *Mx-1* expression in the Chinook and Atlantic Salmon that received the PRV-positive tissue homogenate suggests that an antiviral response was present in fish with persistent PRV infections. Mikalsen *et al.* (2012) demonstrated that in Atlantic Salmon experimentally exposed to PRV, *Mx-1* expression levels often tracked with viral load. At 9 weeks post-PRV exposure, induction of *Mx-1* expression coincided with peak viral load followed by a concurrent decline at 10 weeks, suggesting a possible involvement of *Mx-1* in controlling the virus replication during this stage of PRV infection (Mikalsen *et al.* 2012). However, in our study of *Mx-1* expression conducted at 5 months post-PRV exposure, no strong correlation was evident between level of *Mx-1* induction and

PRV load. Nevertheless, induction of *Mx-1* gene expression was most evident in those species (Atlantic Salmon and Chinook Salmon) with higher PRV load, while Sockeye Salmon – which had only a minor increase in *Mx-1* expression – also was the species with the lowest PRV load. Future studies will be conducted to better understand the antiviral responses and physiological consequences of PRV persistently infected fish. Particularly, it is of interest to evaluate whether susceptibility to secondary infections is altered in PRV persistently infected hosts.

The ability of PRV to establish persistent infections likely has implications towards the transmission, amplification and dissemination of PRV in fish and/or aquatic environments. For instance, if PRV persistently infected hosts are capable of continuously transmitting virus to a naïve hosts, then such an infection strategy would likely be advantageous for extending virus transmission time in comparison with infections that are rapidly resolved. Future work will be required to better understand the role of persistent infections in the epidemiology of PRV.

In summary, this is the first study to investigate the infectious potential of a Chinook Jaundice Syndrome. Our results suggest that the Jaundice Syndrome is of low transmission potential and likely not solely attributable to infection with PRV. Additionally, these experiments demonstrated that Atlantic Salmon and the Pacific salmon species, Chinook Salmon and Sockeye Salmon that were exposed to PRV all developed a sustained PRV infection without the occurrence of morbidity or evidence of disease. These initial findings suggest the Pacific coast strain of PRV is of low pathogenicity for Atlantic, Chinook and Sockeye Salmon."

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Supporting Information

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

Table S1. Tabulated histopathology scores.

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