Synergistic osmoregulatory dysfunction during salmon lice (*Lepeophtheirus salmonis*) and infectious hematopoietic necrosis virus co-infection in sockeye salmon (*Oncorhynchus nerka*) smolts

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1 | INTRODUCTION

Co-infections, defined as the infection of a host with two or more distinct pathogens, are common in both wild and cultured fish (Cox, 2001; Kotob, Menanteau-Ledouble, Kumar, Abdelzaher, & El-Matbouli, 2017). Co-infections are classified as either synergistic in which one pathogen increases host susceptibility to another, or antagonistic in which the first pathogen hinders growth or survival of the second. Synergistic co-infections can result in increased pathogen load, increased disease severity and increased mortality, while antagonistic co-infections can result in lower pathogen load and decreased host mortality. The frequent occurrence of disease outbreaks during co-infections in fish suggests that synergistic pathogen interactions are common. In addition, there is an interactive effect of multiple pathogens on host physiology, gene expression and immune response is limited. To evaluate the impact of co-infection on host survival, physiology and gene expression, sockeye salmon *Oncorhynchus nerka* smolts were infected with the salmon louse *Lepeophtheirus salmonis* (V−/SL+), infectious hematopoietic necrosis virus (IHNV; V+/SL−), both (V+/SL+), or neither (V−/SL−). Survival in the V+/SL+ group was significantly lower than the V−/SL− and V−/SL+ groups (p = 0.024). Co-infected salmon had elevated osmoregulatory indicators and lowered haematocrit values as compared to the uninfected control. Expression of 12 genes associated with the host immune response was analysed in anterior kidney and skin. The only evidence of *L. salmonis*-induced modulation of the host antiviral response was down-regulation of *mhc l* although the possibility of modulation cannot be ruled out for *mx-1* and *rsad2*. Co-infection did not influence the expression of genes associated with the host response to *L. salmonis*. Therefore, we conclude that the reduced survival in co-infected sockeye salmon resulted from the osmoregulatory consequences of the sea lice infections which were amplified due to infection with IHNV.

**KEYWORDS**
co-infection, gene expression, infectious hematopoietic necrosis virus, physiology, salmon lice, sockeye salmon
physiology, gene expression and immune response (Bandilla, Valtonen, Suomalainen, Aphalo, & Hakalahti, 2006; Barker et al., 2019).

In British Columbia (B.C.), infectious hematopoietic necrosis virus (IHNV) and Lepeophtheirus salmonis, the salmon louse, are enzootic pathogens of salmon and have overlapping host ranges. IHNV, a member of the genus Novirhabdovirus, is commonly isolated from Pacific salmon Oncorhynchus spp. (Wolf, 1988), and infections cause disease and mortality most often in the fry and juvenile life stages of wild and cultured salmonids (Dixon, Paley, Alegria-Moran, & Oidtmann, 2016). In B.C., IHNV is primarily associated with sockeye salmon O. nerka although outbreaks have occurred in Atlantic salmon Salmo salar in net-pen aquaculture operations (Saksida, 2006). Infection with IHNV induces a strong innate interferon response associated with a Th1-type immune response (Purcell, Laing, & Winton, 2012).

Sockeye and Atlantic salmon are also highly susceptible to L. salmonis (Braden, Koop, & Jones, 2015; Johnson, Blaylock, Elphick, & Hyatt, 1996). Infections with L. salmonis can have a significant impact on the host osmotic equilibrium with the most severe effects occurring when the adult stages of the parasite are present (Bowers et al., 2000; Grimm & Jakobsen, 1996; Long, Garver, & Jones, 2019). Changes in host gene expression resulting from L. salmonis infections include alterations in iron metabolism, carbohydrate metabolism and decreased expression of several antiviral genes (Braden et al., 2015; Krasnov, Skugor, Todorcevic, Glover, & Nilsen, 2012; Sutherland et al., 2014). Furthermore, initiation of a Th2-type regulatory pathway in response to L. salmonis infection has been reported in both Pacific and Atlantic salmon although the timing and/or magnitude of the response is modified in more susceptible species (Braden et al., 2015; Skugor, Glover, Nilsen, & Krasnov, 2008). In addition to disruptions in osmoregulation and gene expression, infections with L. salmonis or Caligus rogercresseyi, another species of sea lice, can negatively impact the host’s resistance to additional pathogens and facilitate entry of other pathogens into the host (Jakob, Barker, & Garver, 2011; Llorente, Gallardo, Villanueva, Carabaño, & Neira, 2014; Mustafa, Speare, Daley, Conboy, & Burka, 2000).

Co-infection studies involving L. salmonis and IHNV have not been conducted although differences in immune responses elicited by these pathogens as well as down-regulation of host antiviral genes upon infection with L. salmonis (Braden et al., 2015; Sutherland et al., 2014) suggests the interaction between the two will be synergistic. In the current study, we explore the hypothesis that primary infection with L. salmonis will increase host susceptibility to a secondary infection with IHNV in sockeye salmon. Using an adult female L. salmonis infection model previously validated in our laboratory, we evaluated the impact of co-infection on survival, physiology and gene expression in sockeye salmon smolts.

2 | MATERIALS AND METHODS

2.1 | Fish care

All procedures involving fish were carried out in accordance with the recommendations in the Canadian Council on Animal Care Guide to the Care and Use of Experimental Animals and approved by the Pacific Region Animal Care Committee, AUP 14-029. All experiments were conducted at the Pacific Biological Station (PBS; Nanaimo, B.C.).

Sockeye salmon (average body weight 152.2 g) from Pitt River stock were reared in brackish water and transferred to full sea water 10 days prior to initiation of the experiment. Fish were maintained at 9.06°C (±0.03°C) in 225-L tanks (stock density 15.4 kg/m³) with UV-treated flow-through sea water (flow rate 3.5 L/min; salinity 28.0 ± 0.1 ppt), and kept under a natural photoperiod. Fish were fed a commercial diet (EWOS Canada) at a rate of 0.5% total biomass/day during the first 7 days of the trial and a rate of 1% total biomass/day for the remainder of the trial.

2.2 | Experimental design

The experiment consisted of four treatment groups: uninfected control (V−/SL−); sea lice infection only (V−/SL+); virus infection only (V+/SL−); and co-infection (V+/SL+). All treatments were conducted in duplicate tanks each containing 20 fish.

2.3 | Sea lice collection and infection

Adult female L. salmonis were collected during harvest at an Atlantic salmon aquaculture site near mainland B.C. north of the Queen Charlotte Strait. Lice were rinsed in sea water and transported to PBS in chilled, aerated sea water. Upon arrival at PBS, lice were transferred to 10°C aerated static seawater baths and held up to 48 hr prior to experimentation.

Sockeye salmon were exposed to 6 sea lice/fish as previously described in Long et al. (2019). Fish in the V−/SL− and V+/SL− groups received the same handling treatment but were held in a mock 20-L exposure tank without sea lice for 15 min before transfer to the holding tank.

2.4 | Virus strain and infection

Infectious hematopoietic necrosis virus isolate BC93-057 was isolated from a net-pen reared Atlantic salmon during an epizootic in B.C. in 1993 (Garver et al., 2013). BC93-057 was amplified in Epithelioma papulosum cyprini (EPC; ATCC CRL-2872) cells and quantified using plaque assay as described previously (Battls & Winton, 1989). Plaques were enumerated and reported as plaque forming units per ml (pfu/ml).

Fish were exposed to IHNV by waterborne immersion challenge at 2 days post-louse infection (dpl). Water flow to the tanks was stopped, and a volume of virus stock (10⁸ pfu/ml) sufficient to produce a final virus concentration of 10⁵ pfu/ml was added to the V+/SL− and V+/SL+ tanks. The same volume of sterile Hank’s Balanced Salt Solution (Gibco) was added to the non-virus exposure tanks. Immediately after addition of the virus to the tank, water was briefly stirred and a 1 ml water sample collected to quantify the virus load in each tank. After 1 hr with supplemental
aeration and prior to resuming water flow to the tanks, a 1 ml water sample was collected to quantify the residual virus load in each tank. Fish were monitored daily for 30 days post-virus (dpv), 32 dpv. All mortalities were examined for sea lice and screened for the presence of IHNV by quantitative RT-PCR on anterior kidney samples.

2.5 | Sample collection

Tissue and blood samples were collected from 10 fish per group (five fish per tank) at 3, 5, and 7 dpv (1, 3, and 5 dpv). At 32 dpv (30 dpv), samples were collected from survivors: 10 fish each from the V−/SL− and V−/SL+ groups, nine fish from the V+/SL− group, and four fish from the V+/SL+ group. For sampling, water flow to each tank was temporarily stopped and 0.15 mg/L of metomidate hydrochloride (Aquacalm; Syndel Canada) was added. After 12 min, five fish were individually netted into separate buckets and killed in 400 mg/L of tricaine methanesulfonate (MS-222; Syndel Canada) in sea water. The total number of lice on the fish and in their individual buckets was used to determine mean parasite abundance according to Bush, Lafferty, Lotz, and Shostak (1997). Physical damage to skin was noted using a semi-quantitative scale from 0 to 4: (0) no skin damage, no haemorrhaging, no lesions; (1) minor petechial haemorrhaging and/or scale loss over 25% or less of body surface; (2) widespread petechial haemorrhaging and/or scale loss over 25%–50% of body surface; (3) subcutaneous oedema (raised scales), scale loss over 50%–75% of body surface and/or areas of blood; and (4) lesions present, erosion of the epidermis, ulcers and/or scale loss over 75% or greater of body surface (Long et al., 2019). Blood was collected for haematocrit and plasma analysis as described in Long et al. (2019). Anterior kidney and skin tissue were taken for gene expression and viral load determination. Tissue samples were immediately flash-frozen in liquid nitrogen and stored at −80°C. Skin samples 1 cm long and 1 cm wide were collected at a standardized location on the left mid-flank directly above the lateral line where a line drawn from the anterior end of the dorsal fin intersected with the lateral line (Fast et al., 2002). If a sea louse was attached to this site, then the sample was taken on the right side in the same location.

2.6 | RNA extraction and reverse transcription

Total RNA was extracted from anterior kidney and skin samples in TRIzol Reagent (Life Technologies) following manufacturer’s instructions using 5-mm stainless steel beads (Qiagen). Kidney tissue was mechanically homogenized in a TissueLyser II (Qiagen) for 2 min at 25 Hz, and skin was mechanically homogenized for 10 min at 30 Hz. RNA was stored at −80°C.

To prepare cDNA for viral load determination in anterior kidney samples, 1.5 µg of total RNA was reverse-transcribed using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) following the manufacturer’s instructions. cDNA was stored at −20°C until needed.

For gene expression analysis, RNA was DNase treated using a TURBO DNA-free™ Total kit (Ambion) prior to cDNA synthesis. RNA quality was confirmed by agarose gel electrophoresis with a subset of samples from both tissues. To prepare cDNA, 1 µg of DNase-treated RNA was reverse-transcribed in a 40-µl reaction using a High Capacity cDNA Reverse Transcription kit (Applied Biosystems) with equal concentrations of random hexamers and Oligo d(T)$_{18}$ primer (Thermo Scientific). cDNA samples were diluted 1:4 in nuclease-free water and stored at −20°C until needed.

2.7 | IHNV quantitative RT-PCR

Quantification of IHNV in kidney tissue was carried out using published primer and probe sequences targeting the IHNV N gene (Purcell et al., 2013). An individual reaction was comprised of 1X TaqMan™ Universal PCR Master Mix (Applied Biosystems), 900 nm each of the forward and reverse primer, 250 nm each of the probe and artificial positive control, 2.5 µl cDNA template, and nuclease-free water for a final reaction volume of 25 µl. Reactions were run on a Stratagene Mx3000P qPCR system following the manufacturer’s protocol. To determine the number of virus copies per µg total RNA, a double-stranded DNA gBLOCK fragment (IDT Technologies) consisting of the sequence targeted by the IHNV primers was used. An 8-step serial dilution of the gBLOCK spanning 10$^7$ to 50 copies per reaction was used as a standard curve for each run. All samples and standard controls were tested in duplicate and considered positive if at least one replicate had a Ct value <40.

2.8 | Host gene expression using quantitative real-time PCR

Gene expression in anterior kidney and skin samples wasanalysed at 3 and 7 dpv (1 and 5 dpv). See Supporting Information Table S1 for primer concentrations, primer efficiency values, standard curve dilution, primer sequences, and source. To prepare the standard curve, equal volumes of DNase-treated RNA from all samples were combined and cDNA prepared. Standard material was then diluted accordingly (Supporting Information Table S1). To confirm absence of genomic DNA, the standard control RNA was used in a no-reverse transcriptase reaction for each primer set. If amplification occurred in the no-RT reaction, there had to be a difference of at least five cycles between the no-RT reaction and sample reactions. All reactions were carried out on a StepOne-Plus machine (Applied Biosystems). An individual PCR mixture was comprised of 1X Power SYBR® Green PCR Master Mix (Applied Biosystems), 1 µl of diluted cDNA template, forward and reverse primers (concentrations given in Supporting Information Table S1), and nuclease-free water for a final reaction volume of 15 µl. Cycling conditions were 95°C for 10 min and 40 cycles of 95°C for 5 s, 60°C for 20 s, and 72°C for 10 s. A dissociation curve was performed with each run to confirm specificity.

Genes of interest for this study were associated with acute phase response (serum amyloid a, saa; tumour necrosis factor, tnf) cytokines
Kaplan–Meier survival curves (n significant differences in survival between groups SL− line. Circles denote sampling events. Letters denote statistically tank after sampling at 7 dpl). The V−/SL+ line is hidden by the V−/SL− line. (R Core Team, 2018; Spiess, 2018). Target gene expression was normalized to that of the three most stable reference genes and log2 transformed for further analysis. mRNA turnover protein 4 homolog (mrto4). The three most stable candidates were elongation factor-1alpha (ef-1α), beta-actin (β-actin), dynein (dyn), eukaryotic translation initiation factor 3 subunit 6 (etif3s6) and mRNA turnover protein 4 homolog (mrto4). The three most stable reference genes were determined for each tissue type using geNorm (Vandesompele et al., 2002). The most stable genes in both skin and reference genes were determined for each tissue type using geNorm and data log10 transformed if non-normality was indicated. Kaplan–Meier survival curves and log-rank analysis of differences in mortalitry were generated using the survminer package in R (Kassambara & Kosinski, 2018) which generated adjusted p values (Bonferroni) of the pairwise comparisons. As skin damage data were non-continuous, differences in values between treatments at a time point were analysed by a Kruskal–Wallis test followed by Dunn’s multiple comparison (Holm-adjusted p values).

To evaluate the effect of treatment, time and their interaction on physiological parameters, gene expression, lice abundance and virus copy number, a linear mixed-effect model was employed using the nlme package (Pinheiro, Bates, DebRoy, Sarkar, & Team, 2018). The random effect term in the model was tank, and a term for unequal variance between treatments was included. The number of virus copies/µg RNA was log10 transformed prior to analysis. For the post hoc analysis, least-square means were generated in R using lsmeans from the package emmeans (Lenth, 2019) with the lme model. The adjusted p values (Tukey) of the pairwise comparisons of the means were then used for the analysis and are reported. A Spearman’s rank-order correlation matrices between log2 CNRQ gene expression values and either virus copy/µg RNA or total number of lice for kidney or skin, respectively, were performed in R version 3.4.3.

Results for all analyses were considered significant if p ≤ 0.05. Graphs were prepared in R using the ggplot2 package (Wickham, 2009). R code examples are given in the Supporting Information Material (Data S1).

3 | RESULTS

3.1 | Survival and skin damage

The earliest and greatest number of mortalities was observed in the V+/SL+ group, where average cumulative mortality reached 60% and occurred from 12 to 21 dpl. This represented 6 (1 and 5 per tank) mortalities out of the 10 fish remaining after sampling at 7 dpl. In the V+/SL− group, average cumulative mortality was 10% (1 of 10 remaining fish) with the lone mortality occurring at 22 dpl. No mortalities occurred in either the V−/SL− or the V−/SL+ group. All mortalities were positive for IHNV by quantitative RT-PCR. Survival in the V+/SL+ group was significantly lower than that in the V−/SL− and V−/SL+ groups but was not significantly different from the V+/SL− group (p = 0.024; Figure 1).

Median skin damage scores for the V−/SL+ group were significantly greater relative to the V−/SL− group at 3, 5 and 7 dpl (p < 0.05; Figure 2). At these times, median scores between the V−/SL+ and the V+/SL+ groups were not different, but at 7 dpl, the median score in the latter was also significantly greater than that of the V−/SL− group (Figure 2).

3.2 | Pathogen load and prevalence

The prevalence of lice infections in the V−/SL+ and V+/SL+ groups declined over time (Table 1). In the V−/SL+ group, parasite abundance at 32 dpl was significantly lower than 3, 5 and 7 dpl (p < 0.05). In the V+/SL+ group, abundance at 32 dpl was significantly lower than 3 and 7 dpl but not 5 dpl (p < 0.05). The proportion of samples positive for IHNV infection peaked at 7 dpl (5 dpl) in the V+/SL− (9 of 10) and the V+/SL+ (8 of 10) groups (Figure 3). Furthermore, viral
load was not statistically different between the V+/SL− and V+/SL+ groups at any of the sample times.

3.3 | Physiological response

Treatment, but not time, had a significant effect on plasma osmolality, K⁺ and haematocrit (Figure 4; Supporting Information Table S2). Collectively, plasma osmolality values in the V+/SL+ group were higher than those in the V−/SL− group ($p = 0.036$). Similarly, plasma K⁺ values in the V+/SL+ group were significantly higher relative to all other groups ($p < 0.05$). Lastly, haematocrit values were significantly lower in the V+/SL+ group as compared to the V−/SL− group ($p = 0.028$).

Changes in the mean values of plasma Na⁺ and Cl⁻ in each group were dependent on time ($p < 0.05$). At 3 dpl, mean plasma Na⁺ was significantly higher in the V−/SL+ group relative to the V−/SL− and V+/SL− group (Figure 4b). However, at 5 dpl, mean plasma Na⁺ in the V−/SL+ group was significantly higher than the uninfected control but not the V+/SL− group. Differences in mean plasma Na⁺ among groups were not detected at 7 and 32 dpl. A similar trend was observed for mean plasma Cl⁻ values. At 3 and 7 dpl, mean plasma Cl⁻ in the V+/SL+ group was significantly higher than the V−/SL− group (Figure 4c). However, at 5 dpl, differences between the V+/SL+ and V−/SL− groups were not significant although mean plasma Cl⁻ values in the V+/SL+ group were significantly higher than the V−/SL− group. There were no significant differences in mean plasma Cl⁻ values between the uninfected control and V+/SL− group at any time point.

3.4 | Gene expression in anterior kidney

Treatment but not time had an effect on the expression of rsad2 which was significantly elevated in the V+/SL− group as compared to the V−/SL− group ($p = 0.01$). The effect of treatment on the relative expression of several innate immune response genes (saa, il-1β, il-10, mx-1, hep-1 and mmp-9) changed over time.
Compared to the uninfected control, expression of saa was significantly elevated in the V-/SL+ group at 3 dpl but not at 7 dpl. At 7 dpl, expression of saa in the V+/SL− and V+/SL+ groups was significantly elevated relative to the uninfected control (Figure 5a). Expression of il-1β, il-10 and mx-1 did not differ between groups at 3 dpl, but significant differences were observed at 7 dpl. The expression of il-1β in the V+/SL− and V+/SL+ groups was elevated as compared to the V-/SL− group (Figure 5b).

Similarly, il-10 expression at 7 dpl was significantly greater in the V+/SL− group compared with the V-/SL− and V-/SL+ groups (Figure 5d). Lastly, mx-1 expression was significantly greater in the V+/SL− group compared with the V-/SL+ group (Figure 5e). Conversely, significant differences in hep-1 and mmp-9 expression were observed only at 3 dpl. Expression of hep-1 in the V+/SL− group was significantly downregulated relative to the V-/SL− and V+/SL+ groups (Figure 5h). The expression of mmp-9 was
FIGURE 5 Gene expression for sockeye salmon kidney sampled at 3 and 7 days post-lice infection (1 and 5 days post-virus infection). Each dot represents an individual fish. The black bar denotes the mean expression value for that group. Letters denote statistically significant differences in values between groups within a sampling time if there was a significant interaction of treatment and dpl for that parameter ($p \leq 0.05$) [Colour figure can be viewed at wileyonelibrary.com]
significantly elevated in the V+/SL+ group compared with the V+/SL− group (Figure 5).

### 3.5 | Gene expression in skin

Treatment but not time had a significant effect on il-1β, mmp-9, mx-1 and tf expression (p < 0.05). The expression values of il-1β and mmp-9 were significantly higher in the V−/SL+ and V+/SL+ groups relative to the V−/SL− and V+/SL− groups. Expression of tf was significantly higher in the V−/SL+ group than in the V−/SL− and V+/SL− groups but not the V+/SL+ group. Although expression of mx-1 in the V+/SL+ group was significantly higher compared to the V+/SL− group, there were no significant differences among the V+/SL−, the V+/SL+ and the V+/SL− groups.

The effect of treatment on the expression of il-10, rsad2, mhc I and hep-1 changed over time (p < 0.05; Figure 6). With the exception of hep-1, significant differences in the expression of these genes were only observed at 7 dpl. Thus, expression of rsad2 and il-10 was significantly lower in the V−/SL+ group compared with the V−/SL− and V+/SL+ groups (Figure 6d). In the V+/SL+ group, rsad2 expression was significantly greater compared to the V−/SL− group (Figure 6f). Expression of mhc I was significantly greater in the V+/SL− group than in all other groups (Figure 6g). At 3 dpl, expression of hep-1 was significantly greater in the V+/SL+ and V+/SL− groups than in the V−/SL− and V+/SL− groups. At 7 dpl, expression of hep-1 in the V+/SL+ group was significantly greater than the V−/SL− group.

### 3.6 | Correlation analysis

Correlation analysis showed that in the V+/SL− group at 7 dpl, expression values of genes associated with the host response to IHNV (il-1β, il-10, mx-1, rsad2 and sao) were significantly correlated to virus load in the anterior kidney. In the presence of sea lice (V+/SL+), correlations between viral load and expression of sao and il-10 were no longer evident (Table 2). In skin, the expression values of most genes associated with the host response to L. salmonis infection (hep-1, tf, mmp-9, il-1β) were not correlated to the total number of lice present. However, tf expression was significantly correlated to total number of lice in the V+/SL+ group but not the V+/SL− group (Table 2). Finally, mx-1 expression was correlated with number of sea lice in the V+/SL+ group but not the V+/SL− group (Table 2).

### 4 | DISCUSSION

Previous gene expression analyses of Pacific salmon infected with L. salmonis postulated that parasitized sockeye salmon would be more susceptible to viral infections (Braden et al., 2015; Sutherland et al., 2014) due to the suppression of antiviral responses. In the study herein, although survival was reduced in the co-infected group as compared to the other groups, there was no difference in the prevalence of virus infections or mean viral load between virus-only and co-infected salmon indicating that reduced survival of the co-infected salmon was not a consequence of increased infection with IHNV. The lack of statistical significance between the co-infection and virus-only groups was likely due to the low number of biological replicates in each group (n = 10). We hypothesize that co-infection of sea lice and IHNV in sockeye salmon altered the host capacity to modulate the effects of sea lice infection indicating a synergistic interaction between L. salmonis and IHN virus. Decreased survival in Atlantic salmon co-infected with L. salmonis and infectious salmon anaemia virus (ISAV) has also been reported (Barker et al., 2019). Barker et al. (2019) concluded that sea lice-infected fish modulated the host immune system resulting in increased susceptibility to ISAV. Given the differences between the two studies (host species, virus, study design and sample numbers, parameters examined), further investigation is necessary to determine whether co-infection increases host susceptibility and modulates host response to the physiological effects of sea lice infection, or both.

Sockeye salmon are highly susceptible to L. salmonis and infection results in increased plasma osmolality and Na+ and Cl− concentrations along with severe cutaneous lesions and subcutaneous oedema (Braden et al., 2015; Jakob, Sweeten, Bennett, & Jones, 2013; Johnson et al., 1996; Long et al., 2019). Host osmoregulation is affected by both the direct (attachment and feeding) and indirect (passive loss of water across the gills due to stress) effects of L. salmonis infection (Wendelaar Bonga, 1997). In contrast, the effects of IHNV on host osmoregulation are not well documented although Amend and Smith (1975) reported reduced plasma osmolality in moribund rainbow trout Oncorhynchus mykiss. In the current study, co-infected salmon had higher skin disruption scores, elevated osmoregulatory indicators and lowered haematocrit values as compared to the uninfected control. There was no disruption in osmoregulatory indicators in salmon infected only with IHNV, whereas elevated osmoregulatory indicator values were transient in salmon infected only with sea lice. Therefore, we conclude that the reduced survival in co-infected sockeye salmon resulted from the osmoregulatory consequences of the sea lice infections which were amplified in the presence of infection with IHN virus.

Upon infection with a virus, the host immune system initiates differentiation of Th lymphocytes into Th1 cells resulting in the production of cytotoxic T cells and interferon-γ as well as promoting macrophage activation (Bradley & Jackson, 2008; Cox, 2001). In contrast, Th2 cells are recruited when extracellular pathogens such as parasites are present, and this response is associated with up-regulation of il-4/13A, il-10 and transforming growth factor beta. Activation of B cells and proliferation of eosinophils due to cytokine production is a hallmark of the Th2 response to parasite infection (Cox, 2001). Braden et al. (2015) reported activation of a Th2-type regulatory pathway in the skin of L. salmonis-resistant coho salmon Oncorhynchus kisutch and hypothesized this as a mechanism of resistance as activation of this pathway was not detected in the susceptible sockeye and Atlantic salmon. In the current study, sockeye salmon displayed no evidence of a Th2-type pathway as il-4/13A expression was unchanged during L. salmonis infection and up-regulation of il-10 only occurred in response to IHNV exposure. This
FIGURE 6  Gene expression for sockeye salmon skin sampled at 3 and 7 days post-lice infection (1 and 5 days post-virus infection). See caption for Figure 5 [Colour figure can be viewed at wileyonlinelibrary.com]
TABLE 2  Results of Spearman's rank-order correlation between gene expression values and virus copy number/μg RNA (kidney) or total number of lice (skin) for individual fish. $r_p$ values are given for individual genes. If the correlation was statistically significant ($p < 0.05$), $r_p$ values are bolded and the $p$-value is given in parentheses. N.D. denotes not done

| Gene | Kidney V+/SL− | Kidney V+/SL+ | Skin V−/SL− | Skin V+/SL+ |
|------|--------------|--------------|-------------|-------------|
| saa  | 0.72 (0.030) | 0.5          | 0.62        | 0.11        |
| hep-1| 0.78 (0.013) | 0.36         | 0.52        | 0.20        |
| igM  | −0.42         | 0.02         | N.D.        | N.D.        |
| igT  | −0.067        | −0.40        | −0.55       | 0.62        |
| il-1β| 0.77 (0.016)  | 0.86 (<0.01) | 0.59        | 0.25        |
| il-10| 0.8 (<0.01)   | 0.60         | −0.018      | 0.34        |
| il-4/13A| −0.65      | −0.45        | −0.40       | 0.30        |
| mhc I| −0.1          | 0.52         | −0.40       | 0.68 (0.032) |
| mmp-9| 0.067         | −0.71 (0.047)| 0.5         | −0.03       |
| mx-1 | 0.88 (<0.01)  | 0.76 (0.028) | −0.26       | 0.74 (0.013) |
| tf   | 0.5           | 0.62         | 0.65 (0.043)| 0.13        |
| rsad2| 0.88 (<0.01)  | 0.74 (0.037) | −0.49       | 0.39        |
| trf  | N.D.          | N.D.         | 0.18        | −0.06       |

Matrix metalloproteinases are primarily responsible for extracellular matrix degradation and tissue remodelling which occurs during the inflammatory response (Chadzinska, Baginski, Kolaczkowska, Savelkoul, & Verburg-van Kemenade, 2008). In the skin and kidney of sockeye salmon, mmp-9 was up-regulated in response to *L. salmonis* infection which is in agreement with previous studies (Braden et al., 2015; Skugor et al., 2008; Sutherland, Jantzen, Sanderson, Koop, & Jones, 2011; Tadiso et al., 2011). In the present study however, up-regulation of mmp-9 in response to IHNV infection was observed in neither tissue although mmp-9 was induced in the kidney of IHNV-infected rainbow trout (MacKenzie et al., 2008). Inflammation due to infection typically results in up-regulation of il-1β leading to an influx of leucocytes which preferentially express mmp-9 (Hong, Peddie, Campos-Pérez, Zou, & Secombes, 2003; Krasnov, Timmerhaus, Afanasyev, & Jørgensen, 2011). Significant up-regulation of il-1β was not observed in the kidney of salmon infected with virus alone until 7 dpl (5 dpv), suggesting the possibility that mmp-9 expression was up-regulated after 7 dpl.

Serum amyloid A (SAA) is an acute phase protein whose levels increase in response to inflammation (Jensen et al., 1997; Rebl, Goldammer, Fischer, Köllner, & Seyfert, 2009). Several cytokines, including interleukin-1β (IL-1β), can induce transcription of this gene (Jørgensen, Lunde, Jensen, Whitehead, & Robertsen, 2000). In fish, saa has been induced in immune organs in response to viral, bacterial and parasitic infections (Braden et al., 2015; Chettri et al., 2014; Sutherland et al., 2014; Villarroel et al., 2008). Villarroel et al. (2008) have proposed that SAA is involved in local defence against pathogens as they were unable to detect SAA in plasma of fish infected with *Flavobacterium psychrophilum*, but the gene was expressed in kidney, liver and spleen cells. In the current study, there was no evidence of up-regulation of saa in skin in response to either pathogen. Expression of saa in kidney was up-regulated in a pathogen-dependent pattern. At 3 dpl (1 dpv), expression was only associated with salmon lice infection, whereas at 7 dpl (5 dpv), expression of saa occurred in the virus-infected groups, perhaps indicative of time-dependent patterns of inflammation.

IL-1β is a pro-inflammatory cytokine that enhances migration of leucocytes, modulates expression of IL-17 by Th17 cells and induces anti-inflammatory cytokines including IL-10 (Hong et al., 2003; Skugor et al., 2008; Zou & Secombes, 2016). Increased expression of il-1β in response to either *L. salmonis* or IHNV has been documented (Braden et al., 2015; Fast, Ross, Muise, & Johnson, 2006; Peñaranda, Purcell, & Kurath, 2009; Purcell, Kurath, Garver, Herwig, & Winton, 2004; Purcell, Marjara, Batts, Kurath, & Hansen, 2011; Sutherland et al., 2014). In the current study, il-1β expression varied depending on tissue and target organ of the individual pathogen, regardless of co-infection status. In kidney, il-1β expression was up-regulated during virus infections while in skin, expression was up-regulated during sea lice infections. These results agree with previous single infection studies, and therefore, we can conclude that expression of il-1β does not appear to be negatively impacted by co-infection in sockeye salmon (Braden et al., 2015; Chettri et al., 2014; Purcell et al., 2004).
IL-10 is a pleiotropic, anti-inflammatory cytokine that down-regulates inflammatory Th responses (Zou & Secombes, 2016). As such, increased expression of il-10 in kidney during virus-only infections was likely in response to increased il-1β expression in this tissue. Concurrently, the absence of statistical differences in the expression of il-10 between co-infected salmon and any of the other groups may indicate modulation of gene expression due to L. salmonis infection in the co-infected fish. Down-regulation of il-10 in skin from L. salmonis-infected sockeye salmon has been previously reported (Braden et al., 2015) and was again observed in the current study, demonstrating that regulation of the inflammatory response is impaired in skin of susceptible salmon species during the infection.

Expression of this gene in rainbow trout infected with IHNV varies by tissue, days post-exposure and virus strain with the highest fold change reported in liver and spleen at 7 days post-exposure to highly virulent IHNV isolates (ATCC #VR-1392, 220-90 and BLK94; Landis, Purcell, Thorgaard, Wheeler, & Hansen, 2008; Purcell et al., 2011). In contrast, sea lice are anticipated to down-regulate mhc I in kidney, as L. salmonis infection of Atlantic salmon had decreased mhc I levels (Fast et al., 2006). In our study, no significant differences in mhc I were observed in sockeye salmon infected with sea lice alone. Overall, there were no significant changes of mhc I expression in kidney, despite sampling during the time of peak virus load. However, increased expression of mhc I is often reported 3 days after virus infection (Hansen & LaPatra, 2002; Landis et al., 2008) and may have been missed in this study due to the timing of sampling.

In skin tissue, the lack of change of mhc I expression in sockeye salmon exposed to sea lice versus those uninfected agrees with previous studies that failed to demonstrate a difference in expression regardless of L. salmonis infection status (Braden et al., 2015; Fast et al., 2006). Conversely, in sockeye salmon infected only with IHNV, mhc I expression was elevated indicating virus-induced expression. As IHNV replicates in skin cells both in vivo and in vitro, up-regulation of mhc I in this tissue is not unexpected (Harmache, LeBerre, Droineau, Giovannini, & Brémont, 2006; Yamamoto, Batt, & Winton, 1992). However, expression of mhc1 is reduced in co-infected fish, it is probable that the virus-induced expression of mhc I was negatively impacted by L. salmonis exposure.

Both mx-1 and rsad2 are strongly induced by type I interferon, and their products are key components of the host antiviral response (Robertson, 2008). Down-regulation of mx-1 has been reported in both anterior kidney and skin of Pacific salmon infected with L. salmonis (Braden et al., 2015; Sutherland et al., 2014). Conversely, in salmon infected with IHNV, mx-1 and rsad-2 levels typically peak between 2 and 3 days after infection (Peñaranda et al., 2009; Purcell et al., 2011). At 7 dpi (5 dpv), mean expression of these genes in both tissues was lower in salmon infected with sea lice only compared with those infected with IHNV only. We had hypothesized that L. salmonis infections would result in down-regulation of interferon-induced genes in co-infected fish; however, expression of these genes did not differ between the virus-infected and the co-infected salmon. It should be noted that expression levels of both genes in co-infected salmon did not differ from those of the negative control in either tissue. Mapping protein expression will be necessary to determine whether the observed differences in the transcriptomic response result in measurable differences in the amount of protein produced.

Analysis of cytokine gene expression in skin highlighted an interesting pattern of expression in salmon infected with the virus alone. The genes rsad2, mx-1 and mhc I were all up-regulated indicative of an antiviral response. Furthermore, although il-10 expression was greatest in this group at 7 dpi (5 dpv), the expected decrease in il-1β expression was not detected. A similar observation was made in Atlantic salmon infected with infectious pancreatic necrosis virus (IPNV); up-regulation of il-10 in conjunction with a lack of induction of il-1β and il-8 (Reyes-Cerpa et al., 2012). The authors hypothesize that IPNV triggered an anti-inflammatory response which the virus then used to aid in establishment of persistence, a strategy which has been reported for other animal viruses (Wilson & Brooks, 2010). Replication of IHNV in epidermal tissue and persistence of the virus in brain tissue of sockeye salmon have been reported (Müller, Sutherland, Koop, Johnson, & Garver, 2015; Yamamoto, Batt, Arakawa, & Winton, 1990). Therefore, we hypothesize that IHNV also regulates il-10 expression which would allow for virus replication in epidermal tissue and potentially enable persistence in infected hosts. To determine whether IHNV employs such a strategy, further testing is needed in which expression of other pro-inflammatory cytokine genes such as il-8 is measured to determine whether il-10 is up-regulated in response to these genes. In addition, it is necessary to measure gene expression at additional time points to see how cytokine gene expression in skin changes through the course of an IHN infection.

In our study, co-infection did not appear to alter IgT expression in either skin or kidney tissue while expression of IgM was not detected in skin from sockeye salmon. This is in contrast to previous studies indicating increased transcript levels of both genes during ectoparasite infection. Chettri et al. (2014) observed increased expression of IgM in skin from rainbow trout infected with Ichthyobodo necator at 9 dpi. Similarly, Tadiso et al. (2011) reported highest IgM and IgT levels in skin of Atlantic salmon infected with L. salmonis at 15 days post-copepodid infection. In addition to the differences in host species, samples were collected when parasite load was high, greater than 50 parasites/fish, in contrast to the current study in which average parasite load was less than 7 lice/fish at both sampling times. Given these conflicting results, further work is needed to develop a better understanding of the effects of co-infection on the kinetics of antibody-mediated immunity.

Correlation analysis of gene expression of individual fish to pathogen load revealed that viral load strongly influenced the magnitude of the antiviral response while the level of host response to sea lice was not necessarily dictated by the parasite load. Expression values for genes associated with the host response to L. salmonis such as hep-1, tf, mmp-9 and il-1β were not significantly correlated
to the total number of lice per fish. In contrast, expression values of genes associated with the host response to IHNV (saα, il-1β, il-10, mx-1 and rsad2) in salmon infected with virus alone were correlated to viral load. A similar trend has been reported for both IHNV and viral haemorrhagic septicaemia (Avunje, Kim, Park, Oh, & Jung, 2011; Purcell, LaPatra, Woodson, Kurath, & Winton, 2010; Zou et al., 2014). In co-infected salmon, expression values of only il-1β, mx-1 and rsad2 were correlated to viral load.

5 | CONCLUSION

This study showed that the outcome of L. salmonis and IHNV co-infections differed from those of single infections in sockeye salmon. Survival in co-infected fish was reduced compared to both single infection groups, indicating that the two pathogens interacted synergistically with one another during co-infections. There was a significant physiological disruption in co-infected fish, suggesting the presence of IHNV partially impaired the host recovery from L. salmonis. With regard to gene expression, the only evidence of L. salmonis-induced modulation of the host antiviral response was down-regulation of mhc I although the possibility of modulation cannot be ruled out for interferon-induced genes. There was no effect of co-infection on the expression of genes associated with the host response to L. salmonis. This research highlights the need for whole organism analysis in conjunction with transcriptomic analysis to fully understand the impacts of co-infection on the susceptible host.

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CONFLICT OF INTEREST

The authors have no conflict of interest.

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