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Comparison of Host Selection and Gene Expression of Adult Lepeophtheirus Salmonis and Salmo Salar During a Cohabitation of Initially Infected and Uninfected Fish

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Abstract

Lepeophtheirus salmonis is a common parasite of salmonid fish and has a significant economic impact on Atlantic salmon (Salmo salar) fish farms. Over time L. salmonis has developed resistance to a number of chemotherapeutants, making the discovery of new treatments important to maintain a profitable farming industry. Determining processes in both *L. salmonis* and Atlantic salmon important to host selection and colonization may provide new targets for treatment development. During a two week cohabitation of *L. salmonis* infected and uninfected Atlantic salmon, we were able to collect information on the ability of *L. salmonis* to switch hosts, and preference for infected or uninfected fish. Whole *L. salmonis* and Atlantic salmon tissues were collected at 2 and 14 days post cohabitation to determine if differential gene expression was occurring during this process. At 2 days post cohabitation there was no significant difference in the number of male lice on the initially infected and uninfected fish. Eight *L. salmonis* genes putatively associated with various facets of lice survival (CYP18 A1-like, cytochrome p450 Isoform 1-like protein, glycene receptor α-2-like, leukocyte receptor cluster member 9-like, nicotinic acetylcholine receptor subunit-like, tissue plasminogen activator precursor-like, peroxinectin-like, and Trypsin-1) were analysed in both adult male and female lice, as well as five genes indicating immune status in Atlantic salmon. Comparisons were made to look for differential gene regulation as well as correlation between expression of *L. salmonis* genes and Atlantic salmon genes. Only MMP9 expression in salmon spleen was differentially regulated during the study period, however, correlations between the expression of several louse and salmon genes were found. Notably, the expression of a peroxinectin-like gene in male and female *L. salmonis* was correlated with the expression of IL-1, IL-12, IgT and matrix metalloproteinase 9 intermittently in salmon. This paper provides new insight into the interactions between *L. salmonis* and *S. salar* during infection.

Keywords: Atlantic salmon; *Lepeophtheirus salmonis*; Host-parasite interactions; Peroxinectin

Introduction

Lepeophtheirus salmonis is a species of parasitic crustacean commonly referred to as sea lice. This species of lice has a circumpolar distribution, infecting fish in the marine waters of the Northern Hemisphere. *Lepeophtheirus salmonis* is primarily a parasite of salmonids, and is particularly pathogenic to Atlantic salmon (Salmo salar). The life cycle of *L. salmonis* has recently been revised and consists of eight stages; two nauplii and one copepodid free swimming stages, two chalimus stages which are completely host dependent and sessile on the surface of the host, and finally two preadult moults and an adult stage which freely move across the surface of the fish [1,2].

Shortly after commercial fish farming began in Europe, salmon farmers began to notice large numbers of *L. salmonis* colonizing their fish. By the early 1970s, salmon farmers were already beginning to use chemotherapeutants to remove lice [3]. Management of *L. salmonis* infection on farmed Atlantic salmon is imperative to the aquaculture industry, as sea lice can cause significant damage to salmon as they feed. As the lice feed on skin and mucus, lesions can occur and will result in difficulty maintaining osmoregulatory balance, decreased growth rates, secondary infections and decreased quality of life for the fish [4-6]. Total impact of sea lice infestation of Atlantic salmon on the aquaculture industry has a cost of nearly $500USD million annually, due to reduced value of final product and use of parasiticides [7].

In the past two decades salmon farmers have noted the development of resistance in *L. salmonis* populations to commonly used parasiticides [8]. This has increased the associated costs caused by the parasite by necessitating the use of higher doses of chemotherapeutants or requiring more frequent treatments to reduce infections to acceptable levels. Development of resistance has necessitated research into new control strategies. Ingvarsdóttir et al. [9] showed that *L. salmonis* host seeking behaviour can be activated by both Atlantic salmon conditioned water and *L. salmonis* conditioned water. The importance of chemical cues in *L. salmonis* host settlement initiated investigation into attractant chemicals as well as those that mask the cues used to identify an appropriate host. By understanding the mechanisms and pathways behind successful host recognition and settlement initiation, we may be able to interfere with this process and identify novel ways of controlling infection.

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The following study was designed to characterize gene regulation during the identification and colonization of Atlantic salmon by the sea louse species *L. salmonis* and to identify corresponding changes in immune responsiveness of Atlantic salmon. Cohabitation of Atlantic salmon initially infected and initially uninfected with adult *L. salmonis* allowed us to determine the rate at which the parasites switch hosts, observe any differentially regulated genes, and make correlations between the expression of these genes in both the host and the parasite during these events.

Materials and Methods

Cohabitation study design

Atlantic salmon were housed at the aquatics facilities of the Atlantic Veterinary College in 330L tanks with a closed recirculating artificial salt water system. Mean weight of all fish was 246.1 ± 6.8 g SEM and mean fish length was 27.6 ± 0.3 cm. Salinity was maintained at 34 ppt ± 2 ppt and temperature at 11°C ± 1°C; ammonia, dissolved oxygen, nitrate and nitrite levels were monitored weekly. Photoperiod was an 8 h:16 h light: dark cycle. During the study fish were fed 1% initial body weight of a 3.5 mm commercial salmon feed (Corey Nutrition Feed, Fredericton, NB, http://www.coreyaqua.ca/) split between two daily feedings.

Cohabitation entailed adding 14 *L. salmonis* infected Atlantic salmon into a tank containing 14 uninfected Atlantic salmon, repeated in a duplicate tank. Average lice load of initially infected fish was 4.0 ± 0.54 lice/fish. Infected Atlantic salmon had been infected with laboratory reared *L. salmonis* copepodids (exposed at 100 copepodids/fish) 72 days prior to the beginning of cohabitation, and individual lice numbers on each infected fish was recorded as it was transferred into the study tanks. The *L. salmonis* hatching and the initial infection procedure followed that described by Covello et al. [10]. Due to the duration of time between initial infection of fish and commencement of cohabitation, all lice in this study had moulted to the adult life stage. Uninfected fish were labelled with PIT tags one month prior to the beginning of the study to allow differentiation between initially infected and uninfected fish.

At 2 and 14 days post cohabitation (dpc) 7 initially uninfected and 7 initially infected fish were collected from each tank using a dip net, the first 7 from each group that were captured were euthanized with tricaine methanesulfonate (250 $mg_{TMS}/1L_{Tank\ water}$) (Syndel Laboratories, Qualicum Beach, BC). Weight and length was recorded for each fish, and PIT tag number was recorded for fish that were tagged. Louse gender, stage, and total number were also recorded for each fish, and lice from each individual fish were collected into a 1.5 mL microcentrifuge tube and stored on dry ice for the duration of sampling. Approximately 300 mg of spleen and a section of skin approximately 2 cm×4 cm were collected from a standardized site dorsal and posterior to the pectoral fin from each sampled salmon. Collected salmon tissues were stored on dry ice for the duration of sampling. On completion of sampling all samples were transferred and stored at -80°C. Lice found on initially uninfected fish were considered to have switched hosts, whereas those found on and initially infected host were considered to have not switched hosts.

RNA extraction

RNA was extracted from adult male and adult female lice whole body homogenates using a Tri-Reagent extraction method [11]. Lice were divided into four treatment groups defined by whether they had switched host during the cohabitation trial, consisting of: 1) lice that remained on an Initial Host at 2 dpc, 2) lice that remained on the Initial Host at 14 dpc, 3) lice that moved to a New Host at 2 dpc, and 4) lice that moved to a New Host at 14 dpc. No female lice were observed to have switched hosts, and therefore only the Initial Hosts at 2 and 14 dpc treatment groups exist for analysis of female lice. Ten lice were selected from each group to undergo RNA extraction, with an effort to select lice representing each duplicate tank, and minimizing use of multiple lice from the same fish. RNA was also extracted from the spleen and skin samples of the salmon from which the lice were collected. RNA from salmon tissues was extracted from 100-200 mg of each respective tissue. RNA samples were resuspended in molecular grade water and tested using a Nanodrop 2000 (Thermo Scientific, Wilmington, DE) to confirm purity and concentration.

Lice RNA generally had low 260/280 values, the lowest being 1.62 and an average ratio of ~1.80. Analysis of RNA quality using a BioRad Experion (Mississauga, ON), showed generally acceptable RNA Quality Indicator (RQI) values on a subset of samples, the lowest value being 4.7 and the highest being 9.8. Although some of these samples had an RQI <6, there did not appear to be any problems during downstream processing of the lower quality samples, and they were included in analysis. All salmon tissue RNA had 260/280 ratios greater than 1.85 and RQI values >7.0, with one exception of 4.2.

After RNA extraction, 1 µg of each RNA sample was treated with a DNase (TURBO DNA-*free* ™ kit, Ambion, Carlsbad, CA) to prevent genomic DNA from interfering with downstream processing. Treatment followed the manufacturer's protocol. Following DNase treatment samples were reverse transcribed (GoScriptTM Reverse Transcription System, Promega, Madison, WI) using the manufacturer's protocol.

Lepeophtheirus salmonis genes

A list of eight genes associated with different facets of *L. salmonis* survival, feeding, suppression of Atlantic salmon immune response, metabolism and neurotransmission were selected for gene expression analysis. Selected genes had been identified as being differentially regulated in a 38 K oligonucleotide array examining the effects of emamectin benzoate exposure on *L. salmonis* (data not shown). Putative gene functions were assigned by performing a BLASTx search in both the Swiss-Prot and Non-redundant protein sequence databases (blast.ncbi.nlm.nih.gov/Blast.cgi) with the sequences of interest, and using results with a Nr-Evalue and Swissprot-Evalue ≤ $1xE^{-10}$ (Table 1).

Primers were designed from *L. salmonis* sequences using Primer 3 software (simgene.com/primer3) and are shown in Table 1, except the reference genes which were taken from Igboeli et al. [21]. Amplified polymerase chain reaction (PCR) sequences were run on 2% agarose gels to confirm presence of a single amplicon; amplicons were confirmed via sequencing (Macrogen USA, Rockville, MD).

Atlantic salmon genes

Five salmon genes of interest associated with salmon immune responses to *L. salmonis* were selected for qPCR analysis; Interleukin (IL)-1β, IL-8, IL-12, Immunoglobulin T (IgT), and Matrix Metalloproteinase 9 (MMP9). These genes and primer sets were previously shown to be appropriate immunological markers for response to sea lice in Atlantic salmon [22,23].

Gene quantification

Relative gene expression was determined using quantitative real time PCR (qPCR). Quantitative PCR reactions were performed in duplicate with 96 well plates, using GoTaq® qPCR Master Mix

Gene	Function	Swissprot-ID	F/R	Primer Sequence
CYP18 A1-like	Ecdysteroid involved in moult and metamorphosis [12]	sp|Q95078|CP18A_DROME	F	TGGGAGGTGAAACCGTCGTAGT
			R	CCCCCAGAAGCTGGGATAACTCTGT
Trypsin-1	Serine protease involved in feeding, digests mucus and skin [13]	sp|P00765|TRYP_ASTFL	F	TGGTCGCAACTGCTCTTGCA
			R	GGCTCTGCCTCTTCACCACCG
Tissue plasminogen activator precursor-like	Degrades blood plasma proteins preventing clotting [14]	sp|P11214|TPA_MOUSE	F	AGGGAAATGCCATGGTGTGCAACT
			R	TGACACCATCATTACACGACCTCGT
Cytochrome p450 isoform 1-like Protein	Drug metabolism of aromatic hydrocarbons [15]	sp|Q9VEG6|PERC_DROME	F	TGGGCTTTGGCCGCTCCAAA
			R	GGCTGTGTCCGAATCGAAAGGCA
Peroxinectin-like	Louse immune system, encapsulation of invaders [16]	sp|Q96B70|LENG9_HUMAN	F	AGGTATACGGGAAGGCACAGACCT
			R	TGGCCAAAGGTACCCAGTCCT
Leukocyte receptor cluster member 9-like	Immune response [17,18]	sp|Q9VG82|CP9F2_DROME	F	TTGGGGTGGAAGCAGGCTGC
			R	ACGCAAAAACCAATGCTTGTCTCCA
Glycene receptor α-2 Like	Mediates inhibitory neurotransmission [19]	sp|P19019|GBRB3_CHICK	F	ACGACGCTTCACGTGTGGAGT
			R	AGAGGCCCGGAAAGTTGTTGAGT
Nicotinic acetylcholine receptor subunit-like	Excitatory neurotransmitter receptor [20]	Q9VG82	F	CTCTGCCGCACATCCACCCC
			R	TGGTGGAGGCGGAGGCTGAT

Table 1: Brief description of *Lepeophtheirus salmonis* putative gene function and primer sets. Putative functions were assigned by BLASTx searching full *L. salmonis* sequences in both the Swiss-Prot and nr databases and using a consensus to determine function.

Gene	Annealing Temperature (°C)	Standard Curve Efficiencies	Protocol
CYP18 A1-like	60	1.02	3 Step
Trypsin-1	65	0.99	3 Step
Tissue plasminogen activator precursor-like	60	1.07	3 Step
Cytochrome p450 isoform-1 like Protein	65	1.07	3 Step
Peroxinectin-like	60	0.99	3 Step
Leukocyte receptor cluster member 9-like	65	1	3 Step
Glycene receptor α-2 like	65	0.99	3 Step
Nicotinic acetylcholine receptor subunit-like	65	1.06	3 Step
Ribosomal Protein S20	61	0.92	2 Step
Glyceraldehyde 3-phosphate dehydrogenase	65.1	0.97	2 Step
Elongation Initiation Factor 1	65.1	0.91	2 Step

Table 2: Quantitative PCR protocols used to amplify reference genes and genes of interest in *Lepeophtheirus salmonis*. Three step protocols consist of: One cycle of 95°C for 10 minutes, forty cycles of 95°C for 15 seconds, $T_{Annealing}$°C for 15 seconds and 72°C for 15 seconds, and one melt curve. Two step protocol consisted of: One cycle of 95°C for 10 minutes, forty cycles of 95°C for 15 seconds, $T_{Annealing}$°C for 30 seconds and one melt curve.

Gene	Annealing Temperature (°C)	Standard Curve Efficiencies	Protocol
Interleukin 1β	55	0.91	3 Step
Interleukin 12	55	0.93-1.04	3 Step
Immunoglobulin T	55	0.93-0.95	3 Step
Matrix Metalloproteinase 9	55	0.90-0.91	3 Step
Interleukin 8	55	0.91-0.93	3 Step
Elongation Factor 1	55	0.91-0.93	3 Step
Elongation Initiation Factor	55	1-1.06	3 Step
Ribosomal Protein S20	55	0.93-0.95	3 Step

Table 3: Quantitative PCR protocols used to amplify reference genes and genes of interest in Atlantic salmon. Three step protocols consist of: One cycle of 95°C for 10 minutes, forty cycles of 95°C for 15 seconds, $T_{Annealing}$°C for 15 seconds and 72°C for 15 seconds, and one melt curve.

(Promega, Madison, WI), on a Mastercycler® ep Realplex model 2S thermocycler (Eppendorf, Mississauga, ON). Replicate samples with a difference in threshold cycle (cT) greater than 0.55 were re-run in duplicate to a maximum of three times, and if still outside the variance limit were excluded from analysis. Reaction conditions were optimized by generating a standard curve with appropriate efficiencies (0.9-1.1) and R2 values (>0.95). Genes of interest were amplified using a 3-step protocol, while reference genes were amplified using a 2-step protocol (Tables 2 and 3).

Three reference genes were used for normalization of *L. salmonis* gene expression; glyceraldehyde-3-phosphate dehydrogenase (GAPDH), eukaryotic translation elongation factor 1α (EF-1α) and structural ribosomal protein S20 (RPS20). Three reference genes were also used to normalize Atlantic salmon gene expression; EF-1α, elongation initiation factor (EIF), and RPS20. Reference gene stability was determined using GeNormPlus software (M value ≤ 1.0, CV ≤ 0.5) (Biogazelle, Zwijnaarde, Belgium); a fourth reference gene (18S rRNA gene) was excluded from *L. salmonis* analysis as it was identified as being unstable.

Samples were normalized using qBASE software (Biogazelle, Zwijnaarde, Belgium) by comparing expression of the genes of interest to the expression of the reference genes to generate a Mean Normalized Referenced Quantity (MNRQ) [24].

Statistical analysis

Statistical analysis of MNRQ values were completed using GraphPad Prism 6 (GraphPad Software Inc., La Jolla, CA). For each gene, within each treatment, samples with an MNRQ greater than two standard deviations from the group mean were removed from the respective data set as outliers. The MNRQ values for both *L. salmonis* and Atlantic salmon were checked to ensure that within each treatment group the data set was normally distributed using either the D'Agostino and Pearson test or the Kolmogorov-Smirnov test for groups with smaller sample numbers. A one-way ANOVA was performed using a Tukey's Test ($p<0.05$) to determine if there was a significant difference between normally distributed treatment groups. If within a gene, one of the treatment groups was not normally distributed or the treatment groups were found to have significantly different standard deviations using a Brown-Forsythe test, the non-parametric Mann-Whitney test was used to determine if differential gene regulation was occurring between treatment groups for that gene. Both parametric and non-

parametric tests were used to achieve the highest statistical power within each gene.

A second series of analyses were completed to determine if there was a correlation between the expression of salmon immune markers in the skin and spleen tissue of the salmon host, and also if there were correlations between *L. salmonis* gene expression and Atlantic salmon gene expression. Gene expression of each sampled *L. salmonis* was compared to the expression of the immune markers in the tissues of the Atlantic salmon from which the louse was collected using a Pearson correlation. Comparisons were made across the entire duration of the study, as well as a comparison of gene expression within each treatment group.

Finally, Pearson correlations were used to determine if salmon with a high louse infection had stronger correlations in expression between lice and salmon gene expression than fish with low numbers of lice. The initial categorization of fish as initially infected or uninfected hosts is not used for this analysis. Due to the limited number of lice in this study, fish with 0-3 lice were considered to have a low number of lice and fish with 4-14 lice were considered to have a high infection. Five fish and twenty lice were used in the high group, and seven fish and twelve lice were used in the low group. Comparisons were made in the same manner as the Pearson correlations used to elucidate correlation across the entire study.

Results

Host selection

This study utilized adult male and female lice, to compare and contrast stages that have differing requirements from their hosts (i.e. males searching for new mates; females remaining on host to produce offspring). Combined lice counts from both tanks indicated that male lice would transfer between hosts, while no female lice switched host (Table 4). After initiation of cohabitation lice were able to switch between hosts, at 2dpc there was no significant difference between the number of male lice on the Initially Infected and Initially Uninfected treatment groups. At 14dpc there was also no significant change in the number male lice on fish from both treatment groups (Table 4). Of the 111 lice that entered the study, 102 were recovered during sampling. All male lice were recovered (72/72) while nine female lice (30/39) were lost during the two week study period. This indicates that adult male lice are able to choose and move to a new host relatively successfully, but adult female lice lack either the desire or ability to transfer between hosts.

		Initially Infected Fish			Initially Uninfected Fish		
Tank	Days post cohabitation	Number of Fish	Number of Male Lice	Number of Female Lice	Number of Fish	Number of Male Lice	Number of Female Lice
1	0	14	40	19	14	0	0
4	0	14	32	20	14	0	0
1	2	7	9	4	7	8	0
4	2	7	16	16	7	3	0
1	14	7	11	9	7	9	0
4	14	7	8	1	7	8	0

Table 4: Movement of *L. salmonis* (lice) between initially infected and initially uninfected Atlantic salmon hosts. Day 0 post cohabitation (dpc) lice counts were done immediately before initiation of cohabitation. Number of fish indicates the number of fish lethally sampled to collect and count *L. salmonis*. There was no significant difference in the number of male lice/fish between groups ($p<0.05$). No female *L. salmonis* were observed to have switched hosts.

Lepeophtheirus salmonis gene expression

A one-way ANOVA with Tukey's test determined there were no significant changes in *L. salmonis* gene expression between treatment groups or over time during the two week cohabitation study in either male or female lice (Supplementary Figures 1 and 2). A Bartlett's test identified a statistically significant decrease in variation of Trypsin 1 expression in male lice that switched hosts.

Atlantic salmon gene expression

Two sets of analysis were used to determine if differential gene regulation was occurring in Atlantic salmon, the first being fish associated with infection by male lice, and the second being with fish associated with female lice. In the salmon there was only one instance of a significant change in gene expression in either salmon skin and spleen involving higher expression of MMP9 in the spleen of fish associated with infection by adult male lice in the Initial Host group at 2dpc as compared to those on New Hosts at 2dpc (Supplementary Figures 3A, 3B and 4).

Salmon skin-spleen interactions

Pearson correlations were completed to determine if any instances of significant correlations between the expression of immune markers in skin and spleen tissues had developed during the study (Table 5). This analysis determined that there was a positive correlation between IL-1 and IL-8 in spleen tissue with IL-12 expression in skin tissue. A full set of data is provided in Supplementary Table 1.

Host-parasite interactions

As there were no major trends within salmon or lice gene expression, analysis of individual interactions were performed. Due to tagging of fish and enumeration of lice on individual hosts, we were able to compare lice gene expression to the individual salmon from which it was collected. Using a Pearson correlation, relationships between the host and parasite began to be elucidated.

There were several instances where there was a single correlation between male louse gene expression and the gene expression in Atlantic salmon skin tissue within each of the four treatment groups. There were multiple instances of correlation of male louse expression of the peroxinectin-like gene to Atlantic salmon skin gene expression in the Initial Host groups (Table 6), as well as correlations between Atlantic salmon skin gene expression of IL-8 to louse expression of CYP450 I1-like (Pearson C:-0.844, P-value: 0.017) and nicotinic acetylcholine receptor subunit-like (Pearson C: 0.717, P-value: 0.045) in the Initial Host 2dpc group associated with colonization by female lice. Some fish were colonized by both male and female and were therefore included in both sets of analyses.

The correlation between peroxinectin-like gene expression in *L. salmonis* and expression of Atlantic salmon genes in skin tissue were present again in the female lice Initial Host treatment groups (Table 7A and 7B). Unlike the male lice, there were also correlations between peroxinectin-like expression and gene expression in Atlantic salmon spleen (Table 7A and B). Additionally, Atlantic salmon IL-12 gene expression was positively correlated with expression of several female *L. salmonis* genes, peroxinectin-like in the Initial Host at 2dpc treatment group (Pearson C: 0.676, P-value: 0.0455), and with both the glycene receptor α2-like (Pearson C: 0.760, P-value: 0.028) and Trypsin-1 (Pearson C: 0.828, P-value: 0.005) expression in the Initial host at 14 dpc treatment group. In male lice, correlations between the

	Initial Host 2 dcp					Initial Host 14 dpc					New Host 2 dcp					New Host 14 dpc				
Skin	IL-1	IL-8	IL-12	IgT	MMP9	IL-1	IL-8	IL-12	IgT	MMP9	IL-1	IL-8	IL-12	IgT	MMP9	IL-1	IL-8	IL-12	IgT	MMP9
Spleen																				
IL-1													+			+				
IL-8										+			+							
IL-12	-														-					
IgT																				
MMP9				-																

Table 5: Summation of the Pearson correlations between gene expression in Atlantic salmon skin and spleen tissues. Interleukin (IL)-1, IL-8, IL-12, immunoglobulin T (IgT) and matrix metalloproteinase 9 (MMP9) were analyzed. Positive (+) and negative (-) correlations with a p-value less than 0.05 are shown.

	IL-1β		IL-8		IL-12		IgT		MMP9	
	Pearson C.	**P-value**	**Pearson C.**	**P-value**	**Pearson C.**	**P-value**	**Pearson C.**	**P-value**	**Pearson C.**	**P-value**
Initial Host (2dpc)	-0.025	0.944	-0.498	0.173	0.049	0.894	0.641	0.046	-0.399	0.254
Initial Host (14dpc)	-0.759	0.011	-0.657	0.054	-0.533	0.140	0.726	0.042	-0.436	0.208
New Host (2dpc)	-0.358	0.34	-0.321	0.400	-0.084	0.844	-0.453	0.221	-0.219	0.572
New Host (14dpc)	0.603	0.086	-0.097	0.820	-0.582	0.078	-0.349	0.357	-0.284	0.427

Table 6: Pearson correlation (Pearson C.) of the mean normalized relative quantity (MNRQ) of adult male Lepeophtheirus salmonis Peroxinectin-like gene expression and the MNRQ of five markers of Atlantic salmon immune response in skin tissue. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with $p \leq 0.05$ are significantly different than 0.

A	IL-1		IL-8		IL-12		IgT		MMP9	
	Pearson C.	**P-value**	**Pearson C.**	**P-value**	**Pearson C.**	**P-value**	**Pearson C.**	**P-value**	**Pearson C.**	**P-value**
Spleen: Initial Host (2 dpc)	0.687	0.041	0.219	0.571	-0.621	0.07	0.668	0.049	0.657	0.054
Skin: Initial Host (14 dpc)	0.087	0.824	-0.093	0.813	0.476	0.195	0.569	0.141	0.398	0.289

Table 7A: Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult female Lepeophtheirus salmonis peroxinectin-like gene expression and the MNRQ of five markers of Atlantic salmon immune response in skin tissue. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with $p>0.05$ are significantly different than 0.

B	IL-1		IL-8		IL-12		IgT		MMP9	
	Pearson C.	**P-value**	**Pearson C.**	**P-value**	**Pearson C.**	**P-value**	**Pearson C.**	**P-value**	**Pearson C.**	**P-value**
Skin: Initial Host (2 dpc)	0.434	0.243	0.630	0.069	0.676	0.046	0.681	0.043	0.667	0.050
Spleen: Initial Host (14 dpc)	-0.006	0.988	-0.125	0.749	0.210	0.588	0.120	0.759	-0.430	0.248

Table 7B: Pearson correlation (Pearson C.) of the Mean Normalized Relative Quantity (MNRQ) of adult female Lepeophtheirus salmonis peroxinectin-like gene expression and the MNRQ of five markers of Atlantic salmon immune response in skin tissue. Pearson correlation approaching 1 and -1 have positive and negative correlation between genes, correlations with $p>0.05$ are significantly different than 0.

Lice Load	Correlations in Host Skin	Correlations in Host Spleen
Less or equal to 3	+13 and -1	+4 and -0
Greater or equal to 4	+3 and -4	+4 and -3

Table 8: Summation of the number of instances of significant positive and negative Pearson correlations between both male and female lice and the expression of several markers of Atlantic salmon immune status.

expression of peroxinectin-like gene in lice and markers of salmon immune status tended to increase in strength as time progressed (Table 6). The entirety of the time-based correlative data can be viewed in Supplementary Table 2.

There were changes in significant correlations between *L. salmonis* and salmon gene expression when differentiating between fish with low and high infection levels. In correlations examining male lice and salmon skin there were three instances of negative correlation between fish and louse in the high infection group and one instance of positive correlation. In the low infection group there was one instance of negative correlation and seven instances of positive correlation. In the comparisons between male lice and salmon spleen tissues there were three instances of positive correlation in the high infection group and one instance of positive correlation in the low infection group. In female lice and salmon skin comparisons there was one instance of negative correlation in the high infection group and two instances of positive correlation, in the low infection group there were six instances of positive correlation. Comparisons of female lice and salmon spleen revealed three instances of negative correlations and one instance of positive correlation in the high infection group, and three instances of positive correlation in the low infection group. This data is summarized in Table 8, and the entirety of the infection intensity correlative data can be viewed in Supplementary Table 3.

Discussion

Host selection

It was found that the male lice reached an equal distribution across all hosts, although female lice appear to have remained on the same fish for the duration of the study. Previous research has shown that adult male and preadult II female *L. salmonis* are able to switch hosts in both field and laboratory studies [25]. Another study using a related sea lice species, Caligus elongatus, found that male and female adult lice without a host could find and attach to a number of fish species over a period of several hours [26]. The lack of movement of adult female *L. salmonis* observed in this study compared to the movement of preadult II *L. salmonis* and adult *C. elongatus* in the two previous experiments may be attributed to morphological and energical demands associated with reproduction. The larger genital segment of adult female *L. salmonis* would be cumbersome and could potentially

reduce swimming efficiency. As these lice were already situated on an appropriate host, energy allocation to host seeking could potentially impair reproduction and be disadvantageous to the genetic success of the parasite.

Redistribution of male lice may be a survival strategy where pressure on each individual louse is minimized. The equal distribution of lice would result in reduced competition for food, space on the host and access to potential virgin female lice. It has been shown that *L. salmonis* exhibit positive taxis towards both salmon and lice conditioned water in Y-tube experiments [9]. It is possible that male *L. salmonis* are able to use semiochemicals excreted by Atlantic salmon to also make the best host selection, perhaps by choosing the least stressed host or a host secreting a compound indicating it is susceptible to infection.

Lepeophtheirus salmonis gene expression

No *L. salmonis* genes were found to be significantly differentially regulated during the 14 days cohabitation study between treatment groups. There was decreased variation in Trypsin-1, a digestive enzyme, in male lice that chose to move to a new host. This may be due to the increased energy required to seek and colonize a new host. Lice that remained on their initial host have had the opportunity to feed to satiation and may or may not need to be actively feeding which could lead to a large variation in trypsin secretion, while the lice on a new host may need to restore energy and expend energy reserves in order to suppress the activation of the immune system of the new host. Fast et al. [27] have shown a decrease in macrophage function during infection of naive Atlantic salmon infected with pre-adult *L. salmonis* at 21 days post infection which they hypothesize may be caused by the feeding of the parasite. A number of low molecular weight proteases, thought to be trypsin-like, were also found in Atlantic salmon mucus. This finding corresponded to the decrease in macrophage function [27,28], which indicates that trypsin or other *L. salmonis* secretory proteins may play a role in regulating host immune responses.

Atlantic salmon gene expression

There was one instance of significant differential gene regulation in Atlantic salmon in this study. The expression of MMP9 was significantly higher in the spleen of the Initial Host at 2dpc as compared to the New Host at 2 dpc. MMP9 is known to be involved in inflammation, having a role in regulating both the initiation and termination of this process [29]. It has been proposed to have a role in cell migration and tissue remodelling; Murakami and Mano [30] have shown an increase in MMP9 expression after skin wounding in Japanese flounder. Using carp head kidney phagocytes [29] were also able to demonstrate a significant increase in MMP9 expression 4 to 24 hours after lipopolysaccharide (LPS) challenge. Skugor et al. [22] previously demonstrated an increase in MMP9 expression in damaged skin tissue of salmon infected with *L. salmonis*. The low level of MMP9 expression observed in the spleen of New Hosts at 2dpc could indicate that while MMP9 is expressed to aid in tissue remodelling, it requires greater than 48 hours of stimulation by *L. salmonis* to be induced in the spleen. Although to mitigate damage caused by *L. salmonis* feeding MMP9 would need to be expressed in the skin, it is possible that it is only expressed very locally in skin tissues. *L. salmonis* adults move freely across the host as they feed, making it difficult to determine specific feeding sites. As the fish in this study had not developed any lesions typical of heavy *L. salmonis* infection, and a standardized section of skin was selected for gene expression analysis, changes in gene expression in the skin may have occurred and not been observed.

Salmon skin-spleen correlations

Due to the large number of correlations performed in this study, the opportunity for Type I errors to have occured was increased. To accomodate these potential errors, the authors have chosen to focus discussion on statistically significant correlations that have occured in multiple instances within a gene. In the New Host 2dpc treatment group both IL-1β and IL-8 expression in spleen tissue was positively correlated with IL-12 expression in the skin. This correlation is indicative of the progression of an initial inflammatory response, likely driven by IL-1β and IL-8 systemically, to a targeted TH1 type response driven by IL-12, occuring in the skin or closer to the site of parasite attachment and feeding.

Correlations in host-parasite gene expression

The peroxinectin-like gene appears to have an important role in the relationship between *L. salmonis* and Atlantic salmon, being significantly correlated with nearly all of the markers of salmon immunity in the Initially Infected hosts. Peroxinectin is widely known to be an important mediator of the innate immune response of arthropods, being involved with cell adhesion, degranulation, and promotion of encapsulation, opsonization, and peroxidase activities [16]. A review by Cerenius and Söderhäll [31] suggests that peroxinectin may also have a role in inducing the production of microbicidal compounds. The putative immune response function of the peroxinectin-like gene may indicate an *L. salmonis* response to an Atlantic salmon defensive or immune response, additionally, it could be a result of exposure to microbes consumed during feeding. The peroxinectin-like gene from males and females was positively correlated with IgT in host skin and spleen. While the function for IgT is unknown in terms of ectoparasitic copepod infection, it has been observed previously to increase in Atlantic salmon skin following *L. salmonis* infection [32]. Humoral responses and the lack of cell mediated responses in general have also been described in previous work on *L. salmonis* infections of Atlantic salmon [32]. This could be partially driven by parasitic modulation towards a T_H2 immune response in the host rather than activation of macrophages and cell mediated immunity associated with a T_H1 host response.

A second consideration, when trying to understand the effect the peroxinectin-like compound might have on salmon gene regulation, is that *L. salmonis* consume some level of blood meal and secrete serine proteases onto the skin of the fish, potentially to aid with feeding [33]. Peroxinectin is not known to be secreted by *L. salmonis*, but Arcà et al. [34] have shown that a salivary peroxidase is expressed in the salivary glands of the female mosquito, *Anopheles gambiae*, while it is minimally expressed in the carcass of the female and whole body of the male. Only female mosquitos consume blood , indicating that peroxidase activity of the peroxinectin-like gene may have an essential role in blood feeding [34]. Correlations between the expression of the peroxinectin-like gene by male lice and the host grew stronger over the course of the experiment in male lice, suggesting that length of time on the host was a significant factor regulationg the interactions between the host and parasite gene regulation. This was supported by a greater frequency of significant correlations occuring between the female louse peroxinectin-like gene and host responses at 2 dpc. This relationship was not maintained at 14 dpc, but could suggest that a threshold of infection by the parasite is required to affect host responses, since the female lice number was approximately 50% at 14 dpc of what it had been at 2 dpc.

The positive correlations between louse expression of the

peroxinectin-like, glycene receptor α-like, and Trypsin-1 genes and salmon skin expression of IL-12 in the Initial Host groups is likely due to IL-12's role in the innate immune sytem, and it's ability to initiate adaptive immune responses. Interleukin-12 is known to induce the production of the inflammatory cytokine Interferon λ, an inflammatory protein involved in innate immune responses. The ability of IL-12 to help induce the differentiation of T cells into T_H1 Cells can begin the initiation of an adaptive immune response, as T_H1 cells and IL-12 can induce proliferation of antibody producing B cells [35]. The long duration of infection of the initially infected fish, 70+ days, would be a chronic stimulation of the salmon's inflammatory and wound healing responses, and the developement of an adaptive immune response may be occuring. Trypsin-1's role as a secretatory molecule and the peroxinectin-like gene's potential role as a secretatory protein may be continuously stimulating the host immune system resulting in triggering of B and T-cell signalling and adaptive responses associated with IgT and IL-12.

The gene correlations examining the effects of high vs. low infection level revealed that fish with low infection levels tended to have positive correlations with *L. salmonis* gene expression, where the higher infection levels had more variation between the numbers of positive and negative interactions. The constant positive correlation at low lice density suggest a uni-directional and potentially stimulatory effect of lice infection, with stronger impacts on the skin. However, the results of the high infection group are suggestive of a more dynamic regulation at both the site of the infection and spleen which may be the result of parasite influence on the host response.

Conclusions

Interleukin-1, IL-8, IL-12, IgT and MMP9, expression in Atlantic salmon skin all appear to be important during lice infection, and re-infection [14]. Interleukin-1, IL-12, IgT and MMP9 were only significantly correlated with the peroxinectin-like gene in initially infected fish, suggesting Atlantic salmon may take some time to become responsive to *L. salmonis* infection. This would agree with previous research which demonstrated a significant increase in IL-12 in the skin of previously infected animals during CpG stimulation at 17 days post exposure and not at 7 days post exposure [14]. Interleukin-12's role as a T_H1 cytokine produced by antigen presenting cells and lymphocytes would suggest it may take longer to be stimulated and therefore measured. Furthermore, host response effects on louse gene expression may only become apparent after prolonged exposure to the louse and the 14 day exposure period to initially uninfected fish may not have been long enough to observe this effect. In most cases, *L. salmonis* gene expression had greater significant correlation with host expression over time, also suggesting that the host immune response itself took time to affect *L. salmonis* gene regulation.

This study highlights the importance of measuring both host and pathogen responses to each other at the individual level. In cases such as this, where out bred host and parasite populations are being used, individual variation may be quite high and make statistically significant observations across treatment groups difficult. By looking at host and parasite responses corresponding to each other we may be able to identify more subtle, yet still very important mechanisms at work in the host-parasite relationship.

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