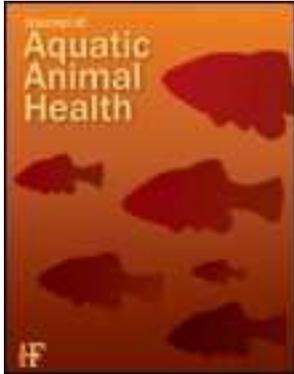


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Differential Gene Expression in Atlantic Salmon Infected with *Lepeophtheirus salmonis*

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Abstract.—*Lepeophtheirus salmonis* is an ectoparasitic copepod that can cause serious disease outbreaks in salmonids. Previous work has shown that Atlantic salmon *Salmo salar* show very little, if any, tissue response to infection, whereas less-susceptible host species exhibit strong inflammatory responses. The objective of this study was to examine whether a low-level *L. salmonis* infection in the absence of a stress response would have an effect on expression of Atlantic salmon immune-related genes over time. The effects of low-level infection (approximately 8–11 lice/fish) on kidney tissue and head kidney leukocyte immune-related gene expression were studied at two postinfection time points. At this level of infection, there was no evidence of a cortisol stress response. By use of real-time polymerase chain reaction, constitutive expression of the major histocompatibility (MH) class I gene was shown to be 2–10-fold lower in infected Atlantic salmon head kidneys by 21 d postinfection (dpi) than in head kidneys of uninfected fish held under the same conditions. Conversely, by 14 and 21 dpi, constitutive MH class II expression was significantly increased (>10-fold) in infected fish. Constitutive expression of interleukin (IL) 1 β also increased threefold in head kidneys of infected fish by 21 dpi; however, no differences were observed in cyclooxygenase (COX)-2 expression over the course of the infection. Adherent head kidney leukocytes from control Atlantic salmon showed an increase in MH class I and COX-2 expression after 3 h of lipopolysaccharide (LPS) stimulation for all three time points collected. The use of LPS stimulation on similar leukocytes from infected fish did not further increase levels of MH class I and COX-2 expression. This is the first report on host gene expression during an ectoparasitic copepod infection. The implications of these expression changes will be discussed with respect to the host–parasite relationship.

Lepeophtheirus salmonis is an ectoparasitic copepod with a direct life cycle consisting of 10 stages (Johnson and Albright 1991). The host species range of this parasite is considered limited to salmonid fish of the genera *Salmo*, *Oncorhynchus*, and *Salvelinus*; however, reports of infections on other hosts are becoming available (Jones et al., in press). Susceptibility to infection is variable among salmonid host species and has been linked to the host's inability to mount a significant inflammatory response (Johnson and Albright 1992; Fast et al. 2002). Host species (e.g., coho salmon *O. kisutch*) that exhibit a well-developed inflammatory response to the ectoparasite are able to easily resolve laboratory infections within approximately 140 degree-days (Johnson and Albright 1992; Fast et al. 2002). Although the course of *L. salmonis*

infections is well documented in the literature, studies have generally concentrated on host physiological responses, while the intricacies of the host–parasite relationship have largely been unexplored (Johnson and Albright 1992; Grimnes and Jakobsen 1996; Bjorn and Finstad 1997; Nolan et al. 1999; Bowers et al. 2000; Ross et al. 2000; Fast et al. 2002). In many of these studies, fish have been infected with relatively high levels of *L. salmonis*, similar to levels that reportedly cause disease (Johnson and Fast 2004). At these levels, infection is known to cause a generalized stress response that may ultimately result in reduced immune functions (Mustafa et al. 2000). It is unclear whether low levels of infection can affect immune functions in the absence of a generalized stress response.

Inflammation is a process by which tissues respond to injury, infection, or irritation. In the case of ectoparasitic infection, this involves closure of the epithelial breach and elimination of intruding foreign bodies. To initiate these processes, pro-inflammatory cytokines, such as interleukin (IL) 1 β and tumor necrosis factor (TNF) α , are produced by macrophages

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and monocytes at the site of injury and participate in leukocyte recruitment, lymphocyte activation, and the acute-phase response (Dinarelo 1994). After phagocytosis of foreign particles, antigen-presenting cells (APCs) express antigens on their cell surfaces in association with major histocompatibility (MH) class II molecules. Antigen presentation is a necessary step in the activation of T cells and the development of an acquired immune response. The mechanisms by which *L. salmonis* is able to avoid these responses on Atlantic salmon *Salmo salar* have not been determined. *Lepeophtheirus salmonis* infection has, however, been observed to decrease macrophage phagocytic capacity and respiratory burst in Atlantic salmon and rainbow trout *O. mykiss* in the absence of a cortisol response (Fast et al. 2002).

Lepeophtheirus salmonis secretions are known to contain prostaglandin E_2 (PGE_2) and trypsin (Firth et al. 2000; Fast et al. 2003, 2004). Furthermore, based on our knowledge of other arthropod parasites, it is likely that other, as-yet unidentified compounds with pro- and anti-inflammatory effects are also present (Fast et al. 2004). Prostaglandin E_2 has been shown to down-regulate IL-1 β and Th1 lymphokines (Betz and Fox 1991; Demeure et al. 1997; Fast et al. 2005). Prostaglandin E_2 is a 20-carbon fatty acid product biosynthesized from arachidonic acid with the assistance of cyclooxygenase (COX; enzyme number 1.14.99.1; IUBMB 1992). Up-regulation of the inducible form of COX, COX-2, has been linked with many inflammatory diseases such as adjuvant arthritis and can be further up-regulated by production of IL-1 β and PGE_2 in mammals (Raz et al. 1988; Hinz et al. 2000; Mancini et al. 2001).

The goal of this study was to determine whether a low-level *L. salmonis* infection would effect constitutive and inducible immune gene expression in Atlantic salmon. To achieve this, we infected Atlantic salmon with between 0.05 and 0.12 lice per gram of body weight, a level below which cortisol and glucose stress responses occur; we then monitored immune gene expression in the head kidney by real-time polymerase chain reaction (PCR). We also analyzed constitutive and lipopolysaccharide (LPS) inducible expression of immune genes in adherent head kidney leukocytes (AHKLs), which were isolated from infected and uninfected fish. Stimulation with LPS was used to investigate immunomodulatory effects of the infection.

Methods

Fish.—A single stock of postsmolt Atlantic salmon (Saint John River, New Brunswick, strain) was used for this study. Fish were acclimated to seawater for 2

months prior to the start of the experiment. Fish were maintained in 2,500-L tanks (1/3 volume) supplied with flow-through 10–13°C seawater and a 12 h light : 12 h dark photoperiod. Two weeks prior to infection, fish were separated into two tanks (one control, one test) containing 40 fish each. The weights (mean \pm SE) of the fish at 0, 12, and 21 d postinfection (dpi) were 68.5 \pm 8.83, 80.5 \pm 6.99, and 83.5 \pm 7.07 g, respectively.

Infection trial.—Copepodids were raised in the laboratory, and fish were infected by use of the methods described by Mustafa et al. (2000). Approximately 100 infective copepodids/fish were added to the designated test tank for 12 h under conditions of reduced water volume (15 kg/m³) and flow. Dissolved oxygen was maintained between 80% and 100% saturation (8.0–9.5 mg/L), and 100- μ m mesh was placed over outflows to prevent loss of copepodids. The control tank was subjected to the same period of reduced volume and flow as the test tank but without the addition of copepodids.

Sampling.—Control and test tanks were sampled ($n = 6$ fish) at each time point. Feed was withheld for 24 h prior to sampling. Fish were killed by an overdose of tricaine methanesulfonate and were bled within 3 min of immobilization; their head kidneys were then removed aseptically. Blood samples were centrifuged for 5 min at 4,000 \times gravity (g), after which the serum was decanted and stored at -80°C until use. Head kidneys were split into two samples: one was used for cell culture (see below), while the other was transferred into RNAlater storage reagent (Ambion, Austin, Texas) and stored at -80°C prior to RNA extraction for single-time-point gene expression studies. After tissue removal, all fish were individually examined under a microscope and *L. salmonis* were counted on all body surfaces.

Analysis of serum samples.—An enzyme-linked immunosorbent assay kit (Neogen, Lansing, Michigan) was used to determine serum cortisol levels for each fish (Afonso et al. 2003). The protocol followed the manufacturer's instructions and is briefly summarized here. Serum samples were thawed on ice and then diluted at a ratio of between 1:30 and 1:50 with the provided extraction buffer. Conjugate (120 μ L/plate) was mixed with enzyme immunoassay buffer (6.0 mL) and then was added at a ratio of 1:1 (50 μ L : 50 μ L) to standards (range = 0.4–10.0 ng/mL) and diluted samples in the provided 96-well plate. All standards and samples were run in duplicate. The plate was gently shaken, covered, and incubated at room temperature (20°C) for 1 h. Enzyme conjugate (horseradish peroxidase; 1.11.1.7) was then removed, and each well was washed three times with 250 μ L of

diluted wash buffer (20 mL of wash buffer and 180 mL of double distilled H₂O). After removal of wash buffer, 150 µL of K-Blue substrate (Neogen) was added to each well and the plate was shaken gently. The plate was incubated at room temperature (20°C) for 30 min and again shaken gently, and the absorbance at 650 nm was measured with a Thermomax microplate reader (Molecular Devices, Sunnyvale, California). Endpoint readings were determined in SoftMax Pro software (version 4).

Serum glucose was determined for each fish by means of a colorimetric assay kit (Sigma, Mississauga, Ontario; Afonso et al. 2003). Thawed serum samples and glucose standards (range = 10–90 mg/dL) were added (10 µL) to a 96-well plate in duplicate. Glucose reagent was also added to each well (190 µL), and the plate was incubated for 10 min at room temperature (20°C) (Trinder 1969). Absorbance at 505 nm was determined by use of the same microplate reader and software described above.

Adherent head kidney leukocyte isolation for cell culture.—A macrophage-enriched culture was obtained by isolating AHKLs from the anterior kidney with a modification of previously described methods (Ottinger et al. 1999). Anterior kidneys were removed aseptically from the fish and were placed immediately into 5 mL of L-15 media supplemented with 2% fetal bovine serum (FBS), 100 units of penicillin–streptomycin (PS)/mL, and 10 units of heparin/mL. Tissues were then stored on ice (1–2 h) until further processing. Tissues were dissociated by repeated passage through a 3-mL syringe, and fragments were allowed to settle for 10 min prior to removal of suspended cells. Cell suspensions were pelleted (500 × g for 10 min at 4°C) and were washed twice prior to layering on Percoll (Sigma) gradients (34–51%). Cells were centrifuged at 400 × g for 20 min at 4°C, and the macrophage-enriched fraction was collected at the 34–51% interface. Cells were then re-suspended in 10 mL of L-15 and 2% FBS, pelleted by centrifugation at 500 × g for 10 min at 4°C, washed with 10 mL of L-15 and 2% FBS, and then re-suspended in L-15 and 5% FBS with 100-units/mL PS. Viable cells were counted with the trypan blue exclusion method, and cell density was adjusted to 1 × 10⁷ cells/mL in L-15 and 0.1% FBS. Cells from each fish were then individually plated at 100 µL/well on 96-well plates and were incubated at 18°C for 2 h. After 2 h, media and nonadherent cells were removed and an equal volume of L-15 supplemented with 5% FBS was added. Cells were maintained for 1.5 d at 18°C prior to manipulation. After this period elapsed, media were removed and 100 µL of fresh L-15 and 5% FBS with *Escherichia coli* LPS (Sigma) at a concentration of 0.0, 0.5, 5.0, or 50.0

µg/mL was added. Stimulation of cells was carried out for 3 h at 18°C, at which time the media were removed and the cells were transferred into RNAlater and stored at –80°C until RNA extraction. Addition of LPS had no effects on cell viability, morphology, or adherence properties in this study (data not shown). After an initial incubation with three different levels of LPS, 5-µg/mL LPS was used throughout the rest of the study. All chemicals used for cell culture were obtained from Invitrogen (Burlington, Ontario) unless otherwise stated.

Extraction of RNA and synthesis of complementary DNA.—Total RNA was extracted by using 50 mg of head kidney tissue from each fish. For extracted AHKL incubations, cells were pooled for individual fish under a single incubation condition. Total RNA was extracted with the Nucleospin RNA II kit (Clontech, Mountain View, California), and the concentration was measured by a spectrophotometer. The RNA samples underwent PCR to verify the lack of DNA contamination. For reverse transcription, 1.0 µg of total RNA from each sample was dissolved in molecular biology–grade water. Reverse transcription was carried out in duplicate by use of the Enhanced Avian HS RT-PCR kit (Sigma) with random nonamers, as per supplier's instructions. The complementary DNA (cDNA) was stored at –20°C until use in real-time PCR assays.

Real-time PCR.—Sequences for real-time PCR primers were designed by use of Primer 3 software (Rozen and Skaletsky 2000) and Michael Zuker's mfold server (<http://www.bioinfo.rpi.edu/applications/mfold>). Primers were generated from available Atlantic salmon sequences (β-actin: National Center for Biotechnology Information number AF012125; MH class I: AF508864; MH class II: X70166; IL-1β: AY617117) and Expressed Sequence Tags databases (COX-2; Table 1; Fast et al. 2005). Although there is only one reported IL-1β sequence for Atlantic salmon, we designed the downstream primer in a region shown to be variable between IL-1β-1 and IL-1β-2 in rainbow trout to prevent amplification of a possible second IL-1β gene. For MH class I and II genes, well-conserved regions across different salmonid and nonsalmonid species were chosen for amplification. The TNFα-like gene primers were designed from comparisons of the highly conserved regions of those genes in rainbow trout and plaice *Pleuronectes platessa* (Laing et al. 2001; Fast et al. 2005). Using these primers, we obtained from Atlantic salmon a product with a 99% nucleotide identity to rainbow trout (TNFα-1). All primers used in this study are listed in Table 1. All primer sets were tested on head kidney cells isolated from Atlantic salmon to confirm single amplification products. The PCR products of β-actin, MH class I,

TABLE 1.—Sequences of oligonucleotide primers used in real-time PCR to determine gene expression in Atlantic salmon infected with parasitic copepods *Lepeophtheirus salmonis*. Abbreviations are as follows: COX = cyclooxygenase, MH = major histocompatibility, IL = interleukin, and TNF = tumor necrosis factor. The superscripted numbers in the third column indicate the locations of the PCR products in the corresponding Atlantic salmon gene sequence.

Genes	Primers	Sequences (5'-3')
β-actin	β-actin forward	²³⁰ CAACTGGGACGACATGGAGA ²⁴⁹
	β-actin reverse	³¹⁸ AGTGAGCAGACTGGGTGCT ²⁹⁹
COX-2	COX-2 forward	²³⁸ CAGTGCTCCAGATGCCAAG ²⁵⁷
	COX-2 reverse	³³⁷ GCGAAGAAGGCGAACATGAG ³¹⁸
MH class I	MH I forward	⁹⁷⁴ TGCTCGTTCGTGCTGTGTT ⁹⁹³
	MH I reverse	^{1,067} TCAGAGTCAGTGTCCGAAGTGC ^{1,048}
MH class II	MH II forward	⁷²² AAGGCTTGAAGACAGTTGC ⁷⁴¹
	MH II reverse	⁸²⁸ CAGTCCAGCAGTAACGTCCA ⁸⁰⁹
IL-1β	IL-1β forward	¹⁹⁷ ATGCGTCACATTGCCAAC ²¹⁴
	IL-1β reverse	²⁸⁷ GGTCTTGTCTTGAACCTCG ²⁶⁸
TNFα	TNFα forward	GGCGACATACCACCTCCTCT
	TNFα reverse	TCCGACTCAGCATCACCGTA

MH class II, COX-2, TNFα, and IL-1β were cloned into a *Taq*-polymerase-amplified (TA) cloning vector (pCR 4-TOPO; Invitrogen) and were sequenced to confirm the sequence of amplified products. Plasmid vectors were isolated and used as standards for real-time studies (Fast et al. 2005).

Real-time quantitative PCR was performed in an iCycler iQ Real-Time Detection System and SYBR Green kits (Invitrogen). The SYBR Green MasterMix kit was used according to the manufacturer's instructions with the following exceptions. SuperMix was added (25 μL) to template cDNA (2.5 μL), water (17.5 μL), and specific primers (125-nM forward and reverse final concentration), giving a total volume of 50 μL prior to allocation into separate wells for duplication of readings. Primer concentrations were optimized at 125 nM after testing a range of concentrations from 90 to 900 nM. To ensure that no genomic DNA contamination was added to the quantified cDNA, we ran non-reverse-transcription controls for each RNA isolation under the same PCR conditions and we observed them by use of 2.5% agarose gel electrophoresis.

The PCR profile was as follows: two initial 2-min denaturation steps at 50°C and then at 95°C, followed by 40–45 cycles of denaturation (15 s at 95°C), annealing (30 s at 56°C), extension (30 s at 72°C), and finishing with a final extension step of 72°C for 5 min. The sensitivity of reactions and amplification of contaminant products (e.g., primer dimers) that were indiscriminately detected by SYBR Green (i.e., SYBR Green binds to all double-stranded DNA) were evaluated by amplifying 10-fold dilutions of the clones (10–10⁻⁶ ng) and duplicate samples, and by performing a blank that lacked cDNA during each run. The relationship between the threshold cycle (Ct) and log(RNA) was linear (−3.5 < slope < −3.2) for all reactions.

Single-product amplification was further verified by melt curve analysis. Melting curves were obtained after 40–45 cycles of amplification on the Lightcycler (Bio-Rad, Mississauga, Ontario) by integrating the signal every 0.1 s during a linear temperature transition from 95°C to 70°C. Fluorescence data were converted by iCycler software (Bio-Rad) to remove background fluorescence and the temperature effect on fluorescence.

Statistical analysis.—Statistical analyses were performed in SigmaStat for Windows, version 3.0 (SPSS). All values shown are means (±SE) of individual fish. The statistical significance of gene expression differences was assessed on expression changes relative to β-actin expression by use of one-way analysis of variance (ANOVA; *P* < 0.05). A two-way ANOVA was used to determine whether there were any significant differences between the physiological parameters of infected and control fish over time. Multiple comparisons were carried out by use of Tukey's tests and paired *t*-tests. All nonnormal data were transformed with a square-root function. Pearson's product-moment correlations were used to determine whether there were correlations between lice count, cortisol level, and relative gene expression; however, no correlations were observed.

Results

Reference Gene Validation

The use of β-actin as a reference or housekeeping gene in this study was validated by real-time PCR. Normalizing the expression of β-actin to total RNA showed that there was a 1.5–2.0-fold difference in β-actin expression in head kidneys of Atlantic salmon over time. However, there were no significant differences between infected and uninfected fish on any day (Table 2). Furthermore, β-actin expression in AHKLs isolated from Atlantic salmon changed two- to three-

TABLE 2.—Mean (\pm SE) expression of β -actin relative to total RNA added in each real-time PCR analysis of Atlantic salmon infected with *Lepeophtheirus salmonis* ($n = 6$); dpi = days postinfection; AHKLs = adherent head kidney leukocytes.

Tissue and treatment	Prior to infection (day 0)	12 dpi	21 dpi
Head kidney			
Uninfected	$4.21 (\pm 1.83) \times 10^7$	$7.57 (\pm 1.88) \times 10^7$	$4.06 (\pm 2.33) \times 10^7$
Infected	$6.55 (\pm 1.67) \times 10^7$	$9.57 (\pm 3.1) \times 10^7$	$9.46 (\pm 2.77) \times 10^7$
AHKLS			
Uninfected	$8.10 (\pm 1.36) \times 10^7$	$2.65 (\pm 0.577) \times 10^7$	$5.08 (\pm 2.12) \times 10^7$
Infected	$4.86 (\pm 0.995) \times 10^7$	$3.35 (\pm 0.793) \times 10^7$	$5.71 (\pm 2.02) \times 10^7$

fold over time, but again the expression between groups was not significantly different (Table 2). There was no significant effect of LPS stimulation on β -actin expression at any of the concentrations tested. Expression of β -actin relative to total RNA ranged from 9.35×10^7 to 1.85×10^8 .

Infection Trial

Experimental infection of Atlantic salmon with *L. salmonis* resulted in mean lice numbers of 9.6 and 9.8 lice/fish at 12 and 21 dpi, respectively. The prevalence of *L. salmonis* on infected fish was 100%, and abundances ranged from 6 to 13 lice/fish. In uninfected fish, individual serum cortisol levels ranged from less than 0.04 to 129.1 ng/mL (sample means = 36.0–69.0 ng/mL) (Figure 1a). In infected fish, individual serum cortisol levels ranged from 2.3 to 116.5 ng/mL (sample means = 48.1–56.9 ng/mL). Although serum cortisol levels were generally higher in control fish than infected fish at 0 and 12 dpi, these differences were not significant (Figure 1a). Serum glucose levels, however, were significantly higher in uninfected fish than infected fish throughout this trial (Figure 1b).

There were no significant differences in constitutive MH class I gene expression in whole-kidney tissue between infected and uninfected groups prior to infection (0 dpi) or at 12 dpi (Figure 2). Expression of the MH class I gene was significantly lower in infected and uninfected fish at 12 dpi than in both groups at 0 dpi and the uninfected group at 21 dpi. At 21 dpi, MH class I gene expression was significantly (10-fold) lower in infected fish than in uninfected fish. At both 0 and 21 dpi, 67% of the uninfected fish had expression levels around 1.0 relative to β -actin expression, whereas at 12 dpi MH class I gene expression was less than 0.12 relative to β -actin for all fish. As was observed at 12 dpi, all infected fish at 21 dpi had an MH class I gene expression of less than 0.19 relative to β -actin.

Prior to infection, constitutive MH class I gene expression of AHKLs was similar between groups (Figure 3). The levels of constitutive MH class I gene expression in uninfected, unstimulated AHKLs re-

mained relatively constant over time, whereas MH class I gene expression in infected, unstimulated AHKLs at 12 dpi was significantly higher than that of uninfected, unstimulated AHKLs. By 21 dpi, MH class I gene expression in infected, unstimulated AHKLs had returned to a level similar to that seen in uninfected, unstimulated AHKLs.

The LPS stimulation of AHKLs collected from both groups prior to infection and at 12 dpi resulted in significant increases in MH class I gene expression (Figure 3).

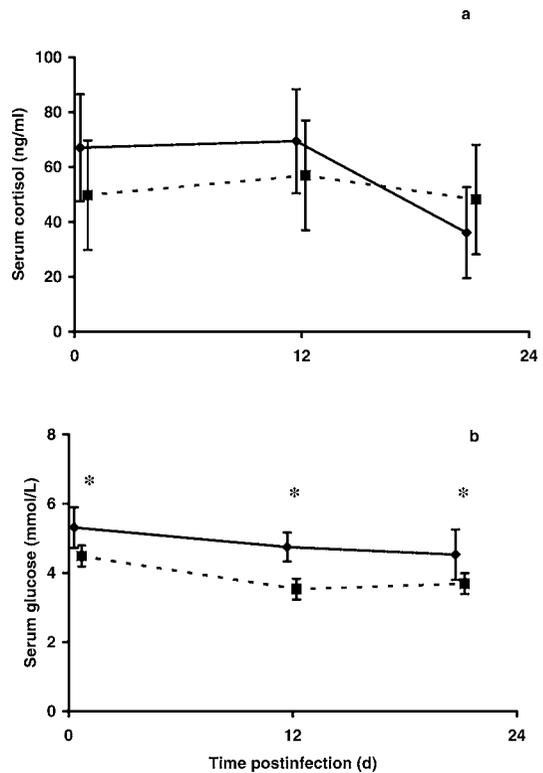


FIGURE 1.—Comparison of mean \pm SE (a) serum cortisol and (b) serum glucose levels between *Lepeophtheirus salmonis*-infected (squares) and uninfected (diamonds) Atlantic salmon. An asterisk indicates a significant difference between infected and control fish on a given day ($P < 0.05$, $n = 6$).

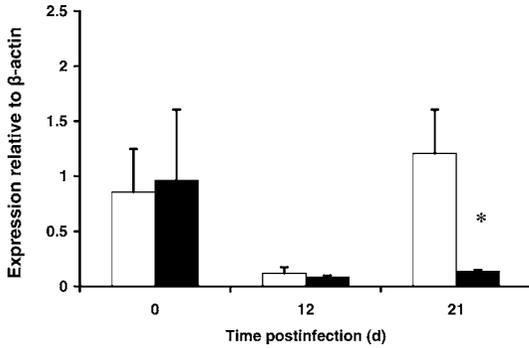


FIGURE 2.—Mean \pm SE expression of the major histocompatibility class I gene relative to β -actin expression in head kidneys of *Lepeophtheirus salmonis*-infected (black bars) and uninfected (white bars) Atlantic salmon 0, 12, and 21 d after the former were infected. The asterisk indicates a significant difference between infected and control fish on a given day ($P < 0.05$, $n = 6$).

Constitutive expression of the MH class II gene was lower than MH class I gene expression in whole-kidney tissue of uninfected fish (Figures 2, 4). In uninfected fish, mean expression levels relative to β -actin were 0.06, 0.05, and 0.20 at 0, 12, and 21 dpi, respectively (Figure 4). Expression of the MH class II gene was observed to increase throughout the experiment in infected fish (Figure 4). At 12 and 21 dpi, the mean levels of gene expression were significantly

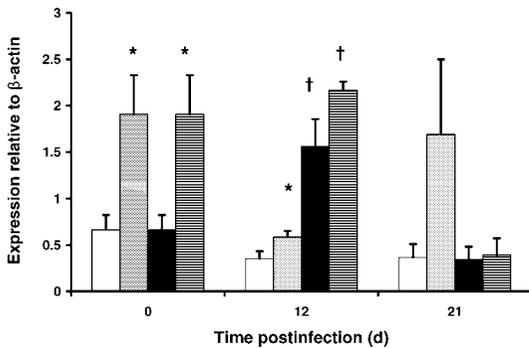


FIGURE 3.—Mean \pm SE expression of the major histocompatibility class I gene relative to β -actin expression in lipopolysaccharide (LPS)-stimulated and unstimulated adherent head kidney leukocytes (AHKLs) isolated from *Lepeophtheirus salmonis*-infected and uninfected Atlantic salmon. White bars denote uninfected, unstimulated AHKLs; light gray bars denote uninfected, LPS-stimulated AHKLs; black bars denote infected, unstimulated AHKLs; and dark gray bars denote infected, LPS-stimulated AHKLs. An asterisk denotes a significant difference from the unstimulated control group on a given day ($P < 0.05$); a dagger denotes a significant difference from uninfected fish under the same incubation conditions ($P < 0.05$, $n = 6$).

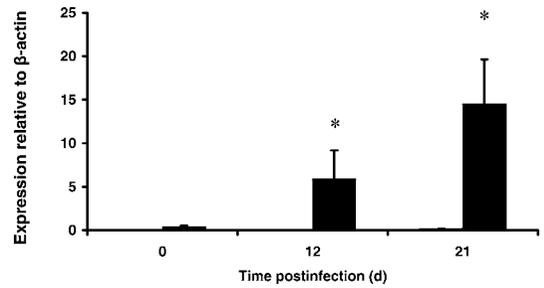


FIGURE 4.—Mean \pm SE expression of the major histocompatibility class II gene relative to β -actin expression in head kidneys of *Lepeophtheirus salmonis*-infected (black bars) and uninfected Atlantic salmon 0, 12, and 21 d after the former were infected. The values for uninfected fish were very small and therefore do not show up clearly. An asterisk indicates a significant difference between infected and control fish on a given day ($P < 0.05$, $n = 6$).

(>100-fold) higher in infected fish than in uninfected fish (Figure 4). However, the large increases in MH class II gene expression were observed in only three of six fish on both sampling days.

Adherent head kidney leukocytes from uninfected fish showed relatively low constitutive expression of the MH class II gene, similar to that seen in whole-head-kidney tissues from uninfected fish. Levels of expression were variable over time (0.01–0.034 relative to β -actin) and were not significantly affected by either LPS stimulation or *L. salmonis* infection.

Interleukin-1 β was constitutively expressed in whole-kidney tissues from uninfected fish but at much lower levels than MH class I gene expression (Figures 2, 5). At 21 dpi, IL-1 β expression in uninfected fish was similar to that seen at 0 dpi; however, infected fish showed a significantly (threefold) higher level of IL-1 β expression than did uninfected fish (Figure 5). Similar to MH class II gene expression, the increase in IL-1 β expression at 21 dpi was attributable to increases in only three out of six infected fish. However, this did not occur in the same fish that exhibited the MH class II increase.

Levels of constitutive IL-1 β expression in AHKLs from uninfected fish were lower than those observed in whole-head-kidney tissue (Figures 5, 6). Adherent head kidney leukocytes from uninfected fish showed variable responses to LPS stimulation, resulting in no significant difference in IL-1 β expression at 0 or 21 dpi but a significant (twofold) increase at 12 dpi (Figure 6). At 12 dpi, IL-1 β expression was significantly (sixfold) higher in unstimulated AHKLs from infected fish than in those from uninfected fish. At 12 dpi, LPS stimulation of AHKLs from infected fish resulted in

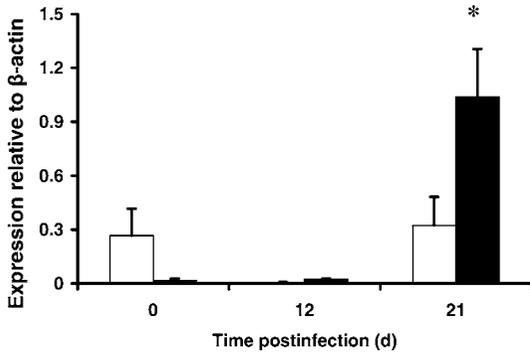


FIGURE 5.—Mean \pm SE expression of the interleukin-1 β gene relative to β -actin expression in head kidneys of *Lepeophtheirus salmonis*-infected (black bars) and uninfected (white bars) Atlantic salmon 0, 12, and 21 d after the former were infected. The value for uninfected fish on day 12 postinfection was very small. The asterisk indicates a significant difference between infected and control fish on a given day ($P < 0.05$, $n = 6$).

significantly lower levels of expression than were observed for unstimulated AHKLs. However, this level was still significantly (twofold) higher than levels recorded for unstimulated and LPS-stimulated AHKLs from uninfected fish (Figure 6). At 21 dpi, IL-1 β expression was relatively low in unstimulated AHKLs from infected fish, whereas LPS-stimulated AHKLs showed levels of expression that were similar to those seen at 12 dpi (Figure 6).

When compared with the other genes studied, COX-2 expression in whole-kidney tissues from uninfected and infected fish was low and highly variable throughout this trial (0.0002–0.004 relative to β -actin). There were no significant differences in the levels of COX-2 gene expression between uninfected and infected fish at any time.

Constitutive expression of COX-2 was higher in AHKLs than in whole-kidney samples (Figure 7). The LPS stimulation of AHKLs resulted in significantly increased expression of COX-2 in both groups at 0 dpi (threefold) and in the uninfected group at 21 dpi (twofold) (Figure 7). At 12 dpi, COX-2 expression was significantly lower in LPS-stimulated AHKLs from infected fish than in all other groups. At 21 dpi, COX-2 expression was significantly (up to fourfold) higher in unstimulated AHKLs from infected fish than in unstimulated and LPS-stimulated AHKLs from uninfected fish (Figure 7). At 12 and 21 dpi, LPS stimulation of AHKLs from infected fish did not further increase COX-2 expression (Figure 7).

We were unable to detect TNF α -like gene expres-

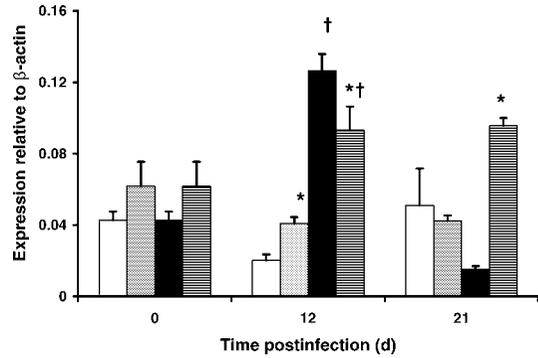


FIGURE 6.—Mean \pm SE expression of the interleukin-1 β gene relative to β -actin expression in lipopolysaccharide (LPS)-stimulated and unstimulated adherent head kidney leukocytes (AHKLs) isolated from *Lepeophtheirus salmonis*-infected and uninfected Atlantic salmon. White bars denote uninfected, unstimulated AHKLs; light gray bars denote uninfected, LPS-stimulated AHKLs; black bars denote infected, unstimulated AHKLs; and dark gray bars denote infected, LPS-stimulated AHKLs. An asterisk denotes a significant difference from the unstimulated control group on a given day ($P < 0.05$); a dagger indicates a significant difference from uninfected fish under the same incubation conditions ($P < 0.05$, $n = 6$).

sion in either whole-kidney tissue or AHKLs from both groups (data not shown).

Discussion

The use of β -actin as a reference or housekeeping gene in this study was validated by real-time PCR. The 1.5–3.0-fold changes in expression of β -actin in head kidneys and AHKLs suggest that β -actin may not be ideal as a reference for comparing gene expression changes over time. This is especially true for those genes that do not show expression changes of a log-unit higher than the variation observed in this control. Although some variation in the expression of β -actin was observed, expression levels were not significantly different between groups at any time. In some cases, control gene variability may have affected the magnitude of change between uninfected and infected fish without affecting the significance of the difference. Without comparing expression over time, we feel that our use of β -actin as a reference gene was warranted.

In this study, the effects of low levels of *L. salmonis* infection, between 6 and 13 lice/fish (0.04–0.12 lice/g), on constitutive and inducible immune-related gene expression in Atlantic salmon were examined. Because repression of nuclear factor κ B-dependent gene expression (IL-1 β , COX-2, etc.), for instance, is a major immunosuppressive activity of glucocorticoids (Brostjan et al. 1996; De Bosscher et al. 2000), low levels of

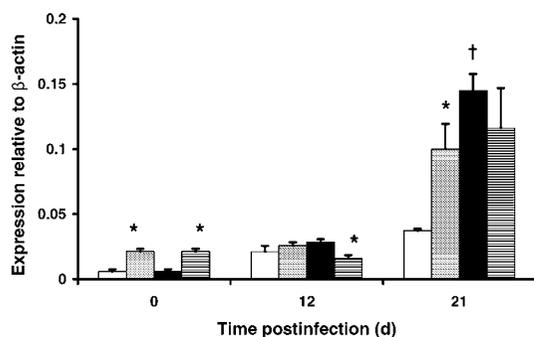


FIGURE 7.—Mean \pm SE expression of the cyclooxygenase-2 gene relative to β -actin expression in lipopolysaccharide (LPS)-stimulated and unstimulated adherent head kidney leukocytes (AHKLs) isolated from *Lepeophtheirus salmonis*-infected and uninfected Atlantic salmon. White bars denote uninfected, unstimulated AHKLs; light gray bars denote uninfected, LPS-stimulated AHKLs; black bars denote infected, unstimulated AHKLs; and dark gray bars denote infected, LPS-stimulated AHKLs. An asterisk denotes a significant difference from the unstimulated control group on a given day ($P < 0.05$); a dagger indicates a significant difference from uninfected fish under the same incubation conditions ($P < 0.05$, $n = 6$).

infection were chosen to reduce the possibility that a parasite-induced cortisol stress response would affect gene expression. Although there were no significant differences in serum cortisol levels between infected and uninfected fish, it is difficult to prove that none of the fish were chronically stressed to some degree. Pickering and Pottinger (1989), for instance, have suggested that normal plasma cortisol levels in salmonids are in the range of 0–5 ng/mL; similarly, Fast et al. (2002) found resting levels of plasma cortisol in the range of 3.5–7.2 ng/mL. In our study, serum cortisol levels were highly variable in both groups at the beginning of the experiment. Thus, while some fish may have undergone chronic stress during the period prior to the experiment, it appears to have occurred in both groups without affecting the relative differences between uninfected and infected fish.

Numerous immune-related genes have been identified in different teleost species, allowing studies to examine host gene expression under viral, bacterial, and parasitic infection (Hansen and La Patra 2002; Saeij et al. 2003a, 2003b; Singh et al. 2004a, 2004b; Ewart et al. 2005; Tafalla et al. 2005). In this study, the effects of *L. salmonis* infection on immune-related gene expression in whole-kidney tissue and AHKLs of Atlantic salmon were determined. We report marked differences in expression between whole-kidney tissues and AHKLs for several of the genes studied. As monocytes, macrophages, and other adherent cells

make up only a percentage of head kidney tissue, observed differences are probably due to influences from granulocytes and lymphocytes, which also play an important role over the course of infection. The MH class I and II genes were examined to determine the ability of host fish to elicit T-cytotoxic cell ($CD8^+$) and T-helper cell ($CD4^+$) responses, which are important in adaptive immunity. Generally, $CD4^+$ cells interact with MH class II molecules on the surfaces of APCs, whereas $CD8^+$ cells interact with MH class I molecules on APC surfaces. Major histocompatibility class I gene expression was significantly lower in whole-kidney tissue from infected fish at 21 dpi than in AHKLs. Down-regulation of the MH class I gene expression in whole-kidney samples of Atlantic salmon may be a result of active immunomodulation by *L. salmonis*. Prostaglandin E_2 has also been shown to down-regulate MH class I and II gene expression in APCs of mammals (Snyder et al. 1982; Harizi and Gualde 2002). As MH classes I and II are important in stimulating adaptive immune responses, their regulation by PGE_2 during parasitic challenge has been studied. Inhibition of MH class I and II gene expression by PGE_2 has been observed in *Leishmania donovani* infections of the murine macrophage cell line, P388D1, as well as in *Entamoeba histolytica* infections of bone-marrow-derived macrophages from Bagg albino (BALB/c) mice *Mus musculus domesticus* (Kwan et al. 1992; Wang and Chadee 1995).

Decreased MH class I gene expression may also be linked to the corresponding increase in MH class II gene expression over the course of the experiment. The importance of MH class II molecules in ectoparasitic infection has previously been observed in other arthropod parasite–host interactions. The sheep blowfly *Lucilia cuprina* and the European castor bean tick *Ixodes ricinus* have been shown to stimulate the influx of $CD4^+$ cells but not $CD8^+$ cells to sites of infection, suggesting that $CD4^+$ cells play a role in the host response to parasite invasion (Mbow et al. 1994; Nash et al. 1996). It is unknown whether an influx of $CD4^+$ cells is also occurring at the site of *L. salmonis* infection. However, if an increase in the trafficking of APCs to the head kidney is responsible for the increase in MH class II gene expression in this tissue, this response may trigger a positive feedback with $CD4^+$ lymphocytes. An influx of $CD4^+$ lymphocytes to the site of infection would then increase the magnitude and rate of antigen presentation, T-cell memory, and capacity to exert effector functions. A corresponding decrease in expression of the MH class I gene could result from a higher percentage of the head kidney being made up of cells that express relatively high

amounts of MH class II genes and relatively low amounts of MH class I genes.

At 12 and 21 dpi, MH class II gene expression was up-regulated in the whole-kidney tissue of infected fish relative to that of uninfected fish. This could be further broken down to show that half (3/6) of the infected fish on each day responded to infection by up-regulating MH class II expression. It is important to note that while changes in gene expression are important, more work needs to be done to show whether corresponding protein levels are also changed. For instance, the rainbow trout MH class II-associated invariant chain (INX) has shown posttranslational control wherein increases in protein levels occur after LPS stimulation without gene expression changes (Braunstein et al., paper read at the Canadian Society of Zoologists meeting, 2004).

Since MH class II molecules are important in presenting antigens from extracellular pathogens, it is not surprising that a host would increase expression during parasitic infection. In rainbow trout infected with the ectoparasitic ciliate *Ichthyophthirius multifiliis*, MH class II gene expression was also significantly elevated in the kidney at 4, 6, and 26 dpi (Sigh et al. 2004a). The authors of that study suggested that the up-regulation of the MH class II gene might be due to the presence of the pro-inflammatory cytokine, TNF α . Increased expression of TNF α -1 has been demonstrated over a 26-d period in rainbow trout head kidneys as the result of infection with *I. multifiliis* (Sigh et al. 2004b). In the current study, there was no evidence for TNF α -like expression in the tissues that were examined. Fast et al. (2005) reported that there was no constitutive or LPS-induced expression of this gene in a salmon head kidney cell line (SHK-1), and it was only after 24 h of LPS stimulation in the presence of PGE₂ that expression was evident. Whether the TNF α -like gene studied here functions in a manner similar to that of mammalian TNF α remains to be determined.

The role of IL-1 β as a central mediator in inflammation resulted in its inclusion in this study. Only one IL-1 β gene has been reported in Atlantic salmon; however, two of these genes have been reported for rainbow trout (Pleguezuelos et al. 2000). For this reason, we designed the reverse primer to amplify a region known to be variable between IL-1 β -1 and IL-1 β -2 in rainbow trout.

Interleukin-1 β was up-regulated at 21 dpi in infected fish. Interestingly, other parasitic infections in teleosts and mammals have also shown increased IL-1 β expression but often at much earlier time points. Common carp *Cyprinus carpio* that were infected with extracellular blood flagellates *Trypanoplasma borreli* exhibited increased IL-1 β expression in kidney tissues

at 1 and 2 dpi (Saeij et al. 2003a). Head kidney tissue from rainbow trout infected with *I. multifiliis* had significantly increased IL-1 β -1 expression at 1 and 6 dpi and increased IL-1 β -2 expression at 26 dpi (Sigh et al. 2004a, 2004b). Similarly, Nash et al. (1996) observed that IL-1 β gene expression at the site of *L. cuprina* infection at 6, 24, and 48 h and in the afferent lymph of infected sheep *Ovis aries* increased early on or prior to any substantial tissue damage or neutrophil influx. The late induction (21 dpi) of IL-1 β expression in Atlantic salmon head kidneys corresponds to the timing of an observation of mild inflammation in Atlantic salmon tissues in another *L. salmonis* infection (Johnson and Albright 1992). Unfortunately, no head kidney samples were taken from infected Atlantic salmon early on (<12 dpi) in our study. If a delayed IL-1 β response does exist, however, it may help to explain the lack of inflammation and subsequently the susceptibility of these hosts to *L. salmonis* infection.

Adherent head kidney leukocytes were isolated from infected and uninfected fish and were stimulated with LPS to investigate the effects of parasite infection on immune effector cell function. The expression of COX-2 and MH class I genes is rapidly induced by LPS, cytokines, and other inflammatory stimuli in mammals (rats *Rattus norvegicus*) and fish cell lines (Futaki et al. 1997; Brubacher et al. 2000; Fast et al. 2005). In this study, AHKLs from infected fish did not appear to have the ability to increase COX-2 and MH class I gene expression after stimulation with LPS at 12 and 21 dpi, respectively. As suggested previously, the monocyte subpopulations of the head kidneys of infected fish may have changed by these time points, and an inability to increase MH class I or COX-2 gene expression may alternatively reflect a change in the composition of leukocytes adhering to the plates. For instance, subpopulations of monocytes expressing low levels of CD14⁺ have been observed in human blood. As CD14⁺ is important in LPS binding and signaling, these cells have shown a reduced expression of pro-inflammatory genes, such as IL-10, upon LPS stimulation (Frankenburger et al. 1996). Dannevig et al. (1990) also reported that sinusoidal endothelial cells were present in significant numbers in suspensions of head-kidney-enriched macrophages from rainbow trout.

Down-regulation of MH class I gene expression with respect to levels in the head kidney at 21 dpi, coupled with a lack of LPS-stimulated expression in infected AHKLs, may help to explain the observed decreases in phagocytic capacity and respiratory burst seen in AHKL (macrophage-enriched) cultures from Atlantic salmon with infections at similar parasite densities (Fast et al. 2002).

The lack of LPS-stimulated effects on MH class II gene expression in AHKLs further supports previous findings. Fast et al. (2005) observed no effect of LPS stimulation on SHK-1 cell expression of the MH class II gene. Similarly, stimulation of a rainbow trout spleen cell line (RTS 11) with 10- $\mu\text{g}/\text{mL}$ LPS for 4 h had no apparent effects on MH class II gene expression. Knight et al. (1998) also found little or no effect of LPS stimulation on MH class II expression in primary isolates of rainbow trout head kidney macrophages.

Based on our knowledge of other arthropod parasites, it is reasonable to expect that *L. salmonis* modulates host immune responses at the site of infection and feeding. In this study, we have demonstrated that infection with low numbers of *L. salmonis* can also result in immune gene expression changes in tissues situated away from the site of attachment and feeding. When considering the implications for the host–parasite relationship, we must put these results into context. It would be most disadvantageous for *L. salmonis* to immunomodulate its entire host, because it is a long-lived parasite that exhibits an extremely long host attachment period. Therefore, any major changes resulting in decreased survival of the host would not benefit *L. salmonis*. The increases observed in MH class II gene expression over MH class I gene expression in the head kidney may be the result of some switching of cell populations within the lymph in response to parasitic infection. While there may be some increase in IL-1 β expression at the feeding site (skin) early on in the infection, perhaps similar to monogenean *Gyrodactylus derjavini* infections of rainbow trout (Lindenstrom et al. 2003), these observations may also be explained by the immunomodulatory capabilities of the parasite secretions. As the infection continues, however, neutrophils begin to migrate to the site of feeding and attachment, as was reported by Johnson and Albright (1992). The increased expression of IL-1 β in the head kidney at 21 dpi may therefore have resulted from the increase in tissue damage due to the more-detrimental mobile stages of *L. salmonis*, which develop by 21 dpi at these temperatures, and from the subsequent influx of neutrophils.

In this study, we noted individual variability in AHKL IL-1 β expression after LPS stimulation. Adherent head kidney leukocytes isolated from uninfected fish did not exhibit the consistent (due to variability between individuals) LPS-induced increase in IL-1 β expression seen in other studies (Brubacher et al. 2000; Fast et al. 2005). This may be explained by the use of cell lines in previous studies, which would greatly reduce variability. However, if some fish in both groups were chronically stressed prior to the trial as

suggested above, the immunosuppressive effects of cortisol may have also contributed to the observed inconsistent IL-1 β responses to LPS stimulation.

In the future, a larger study involving a greater number of individuals will be necessary to elucidate any correlations between cortisol and immune gene expression. The temporal changes in cytokine expression as well as the modulation of adherent leukocyte immune gene expression shown here give further insight into the mechanisms by which *L. salmonis* is able to maintain long-term infections on Atlantic salmon.

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