

The effect of different intensities of the ectoparasitic salmon lice (*Lepeophtheirus salmonis*) on Atlantic salmon (*Salmo salar*)

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Abstract

The effect of different intensities of the ectoparasitic salmon lice (*Lepeophtheirus salmonis*) on stress, growth and the expression of immune and wound healing transcripts in the skin of Atlantic salmon (*Salmo salar*) was investigated. Lice infection success and survival were similar at the chalimus and preadult stage in the low and high dose group, but infection success and survival were significantly lower in the high than in the low dose group at the adult stage. The expression of investigated transcripts was not correlated to lice intensities, but several of them were significantly differently expressed locally in the skin at the site of lice attachment in infected fish compared to controls. This included an up-regulation of pro-inflammatory markers at the site of lice attachment (e.g., interleukin 1-beta, interleukin 8 and the acute phase protein serum amyloid A), a reduction of markers of adaptive immunity (cluster of differentiation 8-alpha and immunoglobulin M) and decreased expression of the anti-inflammatory cytokine interleukin 10.

KEYWORDS

Atlantic salmon, Immune response, parasite intensity, salmon lice, weight

1 | INTRODUCTION

Parasites rely on resources from a host to survive and reproduce. Thereby, they cause some degree of harm and reduce host fitness. The negative effects of macroparasites tend to increase with the number of parasites on a host (Wilson et al., 2002). Consequently, parasite intensity often influences both pathogenicity and the outcome of the host–parasite interaction.

To mount an immune response is energetically costly for the host and resources must be diverted away from other fitness-enhancing traits (Sheldon & Verhulst, 1996). Hence, hosts are under selection for optimization of the immune response, which could depend on both intrinsic and extrinsic factors (Schmid-Hempel & Ebert, 2003). Consequently, low-intensity infections could be tolerated if the cost of removal outweighs the benefits (Behnke, Barnard,

& Wakelin, 1992) and it has been suggested that host immune responses will be highest at intermediate parasite loads (Khokhlova, Spinu, Krasnov, & Degen, 2004). Parasite intensity is found to influence host immune responses both quantitatively and qualitatively. For instance, immune responses in rats (*Rattus norvegicus*) against the gastrointestinal nematode *Strongyloides ratti* increased with infective dose and shifted from a Th-1 response at a low dose to a Th-2 response at high dose (Bleay, Wilkes, Paterson, & Viney, 2007; Paterson & Viney, 2002). High infestation intensity of the freshwater ectoparasite *Argulus* sp. on goldfish (*Carassius auratus*) resulted in higher colonization by the opportunistic bacterium *Aeromonas hydrophila*, suggesting immunosuppression by the ectoparasite (Shameena et al., 2021). Hence, the effect of parasite intensity could depend on the specific host–parasite interaction and is difficult to predict.

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Most Atlantic salmon (*Salmo salar*) caught in the northern Atlantic Ocean are infected with ectoparasitic salmon lice (*Lepeophtheirus salmonis*), but parasite intensity is usually low (Jacobsen & Gaard, 1997; Todd et al., 2000; Torrissen et al., 2013). However, as reported for other parasites (Anderson & May, 1978; Poulin, 2007; Shaw & Dobson, 1995; Shaw, Grenfell, & Dobson, 1998; Wilson et al., 2002), salmon lice tend to show an aggregated distribution (Costello, 2006; Murray, 2002). Hence, parasite intensity is highly variable, and it also increases with host age in the wild and with body size in the laboratory (Costello, 2006; Hamre & Nilsen, 2011). Heterogeneity in parasite intensities could be caused by differences in infection pressure or be due to variation amongst hosts in either behaviour, physiology and/or immunological responses influencing susceptibility (Anderson & Gordon, 1982; Murray, 2002; Shaw et al., 1998; Wilson et al., 2002). Additionally, parasite intensity could be affected by interactions amongst lice on the host (Costello, 2006; Ugelvik, Mo, Mennerat, & Skorping, 2017; Ugelvik, Skorping, & Mennerat, 2017). This could have important implications for both the host–parasite and parasite–parasite interaction in the fish.

Salmon louse has a direct life cycle with six parasitic stages (Hamre et al., 2013), feeding on the blood, skin and mucus of its host; Atlantic salmon, sea trout (*Salmo trutta*) and arctic char (*Salvelinus alpinus*) (Costello, 2006; Pike & Wadsworth, 1999). Negative effects include disturbance of the osmotic balance (Fjellidal, Hansen, & Karlsen, 2020; Grimnes & Jakobsen, 1996), behaviour (Bui, Oppedal, Samsing, & Dempster, 2018; Øverli et al., 2014), stress and increased susceptibility to secondary viral infections (Barker et al., 2019; Nolan, Reilly, & Bonga, 1999). The physiological effects of infestation depend on lice intensity, for instance, is the osmotic balance of Atlantic salmon post-smolts negatively affected at 0.18 lice g⁻¹, while higher lice intensities induce host mortality (Fjellidal et al., 2020). Moreover, pathogenicity depends on the developmental stage of the parasite, and mortality is generally not observed before the moult to the preadult stage (Grimnes & Jakobsen, 1996). Variation in the host–parasite interaction could partly be caused by differences in louse gene expression between and within stages (Eichner, Dondrup, & Nilsen, 2018) or changes in louse exocrine glands possibly involved in the host–parasite interaction (Øvergård et al., 2016).

Salmonids exhibit large variations in susceptibility to salmon lice (Braden, Monaghan, & Fast, 2020; Mackinnon, 1998) and while young pink salmon (*Oncorhynchus gorbuscha*) and coho salmon (*Oncorhynchus kisutch*) rapidly eliminate lice, Atlantic salmon are highly susceptible to infestations (Braden, Barker, Koop, & Jones, 2012; Braden, Koop, & Jones, 2015; Fast et al., 2002). Interestingly, mechanisms conveying lice resistance vary between species. In pink salmon, parasite rejection is linked to a rapid increase in pro-inflammatory cytokines (Jones, Fast, Johnson, & Groman, 2007), while resistance in coho salmon is associated with both hyperplasia and inflammatory responses at the site of lice attachment (Johnson & Albright, 1992). Additionally, resistance is proposed to depend on regulatory Th-2 like pathways (Braden et al., 2015; Braden et al., 2020). Lastly, it has been suggested that salmon lice could down-modulate host immune responses (Braden et al., 2020; Fast, Johnson, Eddy, Pinto, &

Ross, 2007). Thereby making infected hosts more susceptible to new infections (Ugelvik, Mo, et al., 2017), which could increase aggregation in this host–parasite system.

The expansion of farming of Atlantic salmon during the last decades has increased the availability of susceptible hosts for the salmon louse (Torrissen et al., 2013). Epidemiological theory (Anderson & May, 1978) and findings from other host–parasite systems suggest that such an increase in host density is likely to enhance parasite numbers, by increasing the probability of infective stages coming into contact with a susceptible host (Arneberg, Skorping, Grenfell, & Read, 1998). Accordingly, the growth of salmon farming has resulted in enhanced salmon lice abundances (Krkosek, Lewis, Morton, Frazer, & Volpe, 2006; Middlemas, Fryer, Tulett, & Armstrong, 2013; Morton, Routledge, Peet, & Ladwig, 2004; Serra-Llinares et al., 2014; Taranger et al., 2015; Tully & Whelan, 1993) and has been associated with declining stocks of wild salmonids (Costello, 2009; Ford & Myers, 2008; Krkosek et al., 2013; Taranger et al., 2015; Thorstad et al., 2015).

Hence, although the effect of salmon lice infestation on fish is well documented, knowledge of how different lice intensities affects hosts is to our knowledge not been documented previously. Here, the effect of parasite intensity was systematically studied by infecting Atlantic salmon with salmon lice using two different doses and thereafter investigating if cortisol levels, weight and the transcription of selected immune and wound healing genes were affected by parasite intensity.

2 | MATERIALS AND METHODS

2.1 | Handling of fish and lice infestation

One month prior to the infection, 300 Atlantic salmon (Aquagen strain) was sedated in 10 L of sea water mixed with 15 mg L⁻¹ Finquel (Trikaimesilat), weighed (mean weight 319 g [\pm 3 SE]), tagged with passive integrated transponder tag (PIT tag) and randomly divided into 15 (5 tanks per treatment), 600L tanks (20 fish tank⁻¹) filled with sea water (35 ppt salinity and 12°C). Fish were fed with 3-mm commercial salmon feed pellets (Skretting) based on the number of fish and the biomass in the tank according to standard husbandry practices. Water flow was maintained at 12 L min⁻¹ and oxygen levels were monitored and kept between 70% and 110% throughout the experiment. Fish were later exposed to three different doses of infective salmon lice copepodids (control, 0 lice fish⁻¹; low dose, 35 lice fish⁻¹; high dose, 100 lice fish⁻¹).

The lice used in the experiment were originally acquired from a commercial salmon farm in Sognefjord (Norway) and maintained in the laboratory for one generation at 12°C at 35 ppt salinity, prior to the infection. Infection was performed by lowering the water level in the tank to one-third and adding salmon lice copepodids to the tanks. During the infection inflow of water was maintained (12 L min⁻¹), but the outlet was blocked until the normal water level was restored. Fish in control tanks were exposed to identical procedures except that no copepodids were added to the tank.

2.2 | Sampling procedure

Sampling was conducted at three sampling points; when the louse had developed into chalimus 2 (sampling I, 12 days post-infection (dpi), preadult 1 (sampling II, 17 dpi) and the adult stage (sampling III, 35 dpi). For each sampling, 5 fish from each tank were individually carefully netted and sedated, the PIT tag was read, and the fish was subsequently humanely killed with a sharp blow to the head. Weight was recorded and blood samples were taken with a 0.7 × 25 mm needle from the caudal vein and kept on ice in Eppendorf tubes® until the samples were centrifuged. The blood samples were centrifuged at 11,000 *g* for 2.5 min at 4°C. The plasma was stored at -80°C until analysis. Plasma cortisol concentration was determined using an ELISA assay kit (IBL International GmbH) with a Sunrise microplate reader (Tecan). The number of lice on infected fish was enumerated and this was used as a proxy for the number of lice that successfully attached to and survived on the host until sampling (hereafter referred to as lice infection success and survival). Additionally, two skin samples from infected fish (lice-positive and lice-negative samples) and one skin sample from uninfected controls were taken. In infected fish, the lice-positive sample was taken directly underneath a louse as a proxy for local immune responses in the skin and the lice negative sample from a site without lice as a proxy for systemic responses in the skin of infected fish. To be able to compare samples taken from different lice development stages, samples at the chalimus stage were taken in the skin although most were found on fins. At the preadult and adult stages, the louse is mobile and to ensure that lice-positive samples were positive they were preferably taken on the dorsal surfaces, where lice are often found. All fish were handled individually and swiftly to avoid degradation of RNA samples. Skin samples were frozen at -80°C in 1.4 ml PreMax™-plate tubes containing 2 stainless steel beads (Nerliens Meszansky) for later RNA extraction. Samples from 15 fish per treatment from each sampling were used to investigate transcriptional changes in the skin.

2.3 | Bacterial infection prior to the 2nd sampling

Seven days prior to the second sampling, wounds were observed on the skin of some fish in the experiment. This was most noticeable in control fish, even though fish belonging to all treatments received water from the same header tanks. Fish with visible lesions were removed from the experiment (control, *n* = 9; low dose, *n* = 6; high dose and *n* = 5), humanely killed and bacterial samples were taken and cultivated. However, no unusual bacteria were identified in these samples. The bacterial infection was not evident at the first or last sampling.

2.4 | RNA purification

A 500 µl Tri reagent (Sigma Aldrich) was added to the 1.4 ml PreMax™-plate tubes containing skin samples and homogenized for 2 min at 1400 rpm (FastPrep 96, MP Biomedicals). Thereafter samples were

kept at room temperature for 5 min before adding 100 µl chloroform (Sigma Aldrich), then vortexed for 1 min at 1400 rpm (FastPrep 96, MP Biomedicals) and centrifuged at 16,000 rcf at 4°C for 15 min. A 200 µl supernatant was withdrawn and 400 µl RLT (Qiagen) and 600 µl 70% ethanol were added. RNA was further extracted following the RNeasy-Micro protocol (Qiagen). The quality and quantity of RNA were assessed with a NanoDrop™-1000 spectrophotometer (NanoDrop Technologies, ThermoFisher Scientific) and purified RNA was stored at -80°C until further use.

2.5 | cDNA

Reverse transcription was carried out using SuperScript® VILO™ cDNA synthesis kit (ThermoFisher Scientific) according to manufacturer recommendations in a total volume of 10 µl along with the negative control (RTneg) and no template control (NTC). The samples were diluted with nuclease-free, sterile water to get an RNA concentration of 400 ng µl⁻¹, and 3 µl RNA was transferred and mixed with 7 µl Vilo™ cDNA synthesis mix (containing 4 µl nuclease-free, sterile water, 2 µl 5XVilo™ reaction mix and 1 µl 10X superscript® enzyme mix) in a total of 1200 ng µl⁻¹. An RTneg was prepared by replacing the 10X superscript® enzyme mix with nuclease-free, sterile water, while only nuclease-free, sterile water was pipetted into the NTC wells.

Samples were incubated following the manufacturer's instruction for 10 min at 25°C, followed by 60 min at 42°C before the reaction was terminated at 85°C for 5 min. Samples were frozen at -20°C and cDNA was later diluted (1:20) by mixing 95 µl nuclease-free, sterile water and 5 µl of cDNA prior to the RT-qPCR assay.

2.6 | RT-qPCR

RT-qPCR was performed in the QuantStudio™ 5 system (ThermoFisher Scientific). Assays were run in 7 µl reactions, including 3.5 µl master mix (BrilliantIII ultra-fast SYBR® green qPCR master mix, Agilent), 0.28 µl of forward primer, 0.28 µl of reverse primer (Table 1), 0.10 µl reference dye (1:500), 0.84 µl nuclease-free, sterile water and 2 µl template. qPCR cycling conditions were 95°C for 3 min, then 40 cycles of 95°C for 5 s and 60°C for 20 s.

Analysis of mRNA levels was conducted using the 2^{-ΔΔCt} method as used by Dalvin et al. (2020). Elongation factor 1-alpha (EF 1-alpha) and receptor-like protein 1 (RLP 1) were used as reference genes (Table 1). C_t-values for the reference genes at the different treatments are found in the (Table S3). Results are presented as a change in the fold in lice-infested fish (negative and positive samples) compared to un-infested controls at three sampling points. Furthermore, in infested fish fold change in lice-positive samples relative to lice-negative samples was calculated. Changes in threshold cycle (ΔC_t) value were calculated as differences between RNA levels of the gene of interest and the arithmetic mean of the reference genes. ΔΔC_t was quantified as the difference between ΔC_t in infested fish (lice-positive or

TABLE 1 Forward (F) and reverse (R) primers for the investigated immune and wound healing genes used in RT-qPCR setup

Gene	Primers	Accession number	Amplicon length
Immunoglobulin F ^f	F:GGTGGTCATGGACGTACTATTT R: CCTGTGCAGGCTCATATCTT	GQ 907004.1	98
Immunoglobulin M ^f	F:TGAGGAGAACTGTGGCTACACT R:TTAATGACTACTGAATGTGCAT	S48652	67
Interleukin 8 ^a	F:GCATCAGAATGTCAGCCAGCC R:ACGCCTCTCAGACTCATCCC	NM_001140710.2	79
Interleukin 1-beta ^a	F:GCTGGAGAGTGCTGTGGAAGA R:TGCTCCCTCTGCTCGTAG	NM_001123582.1	73
Interleukin 4/13a ^a	F:CGTACCGGCAGCATAAAAATCACCATTCC R: CCTTGCATTTTGTGGTGGTCCCA	NM_001204895	147
Interleukin 10 ^e	F:GCTATGGACAGCATCCTGAAGTT R:GGTTGTTCTGCGTTCTGTTGTT	EF165028.1	76
Cathelicidin 2 ^b	F: ACACCCCTAACACTGACC R: CCTCTTCTGTCCGAATCTTCT	AY542961.1	382
Cluster of differentiation 8-alpha ^a	F:TAGAGTGCAAGACAACGCTGGAATGGA R:TCTCGAGCCTTTTGAAGCCTTCAG	NM_001123583.1	150
Tumour necrosis factor-alpha ^b	F: AGGTTGGCTATGGAGGCTGT R:TCTGCTCAATGTATGGTGGG	NM_001123589	173
Elongation factor 1-alpha ^a	F:CACCACCGCCATCTGATCTACAA R:TCAGCAGCCTCCTTCTCGAACTTC	NM_001123629.1	78
Complement factor 3 ^d	F:TCCCTGGTGGTACCAGTACAC R:ATGATGGTGGACTGTGTGGATC	BI468074	157
Serum amyloid A ^c	F:AGCTGCTCGAGGTGCTAAAG R:ATGCCTCGACCACTGGAAC	NM_001146565.1	193
Collagen 10-alpha ^{e,h}	F:TGGTGCTCTTTGACTGCCTGTAA R:CATCCTGTGTGTTGCAATATCACA	EG837148	180
Fibronectin precursor ^{e,h}	F:GCATGTCTGAGACGGGCTTCAA R: AGTCACATCGGAAGTGCCACTGC	XM_014123350.2	72
Matrix metalloproteinase 9 ^{e,h}	F:AGTCTACGGTAGCAGCAATGAAGGC R:CGTCAAAGTCTGGTAGGAGCGTAT	NM_001140457.1	72
Transforming growth factor-beta ^b	F:AATCGGAGAGTTGCTGTGTGCGA R:GGGTTGTGGTCTTATACAGAGCCA	EU082211	178
Receptor-like protein 1 ^g	F: ACTATGGCTGTCGAGAAGGTGCT R:TGTACTCGAACAGTCGTGGGTCA	CB516726	118
Interleukin 2 ^h	F:GCGGATGTAGAGAAAAGCATTG R:CATTCTGACGAGTCCGTTCTGAT	HE805272.1	155
Inducible nitric oxide synthase ^b	F: GGAGAGCCTTCTGGTTG R: ACCTTAACTTGTCTCTGAGATAC	AF 088999.1	116

^aØvergård et al. (2018).^bHolm et al. (2015).^cBridle, Morrison, Cunningham, & Nowak (1996).^dLøvoll et al. (2007).^eSkugor et al. (2008).^fHolm et al. (2017).^gJorgensen, Kleveland, Grimholt, & Gjoen (2006).^hDenotes genes involved in wound healing in Atlantic salmon. Source of the used primers is denoted with a letter after the gene name in the table.

lice-negative samples) and the average ΔC_t of un-infested control fish. For the comparison between lice-positive and lice-negative samples in infested fish, the average ΔC_t of lice-negative samples were compared to lice-positive samples. Additionally, t-tests investigating differences in ΔC_t values in lice-positive and lice-negative samples between the high and low dose group were performed (Table S4). Only expressional differences between groups with a minimum of twofold differences in mRNA and $p < .05$ were considered significant as previously used in fish immunology studies (Dalvin et al., 2020; Zuo et al., 2020).

2.7 | Statistical analyses

All data analyses were performed using the statistical program environment R (R core team, 2022). For all models, normality and heteroscedasticity of residuals were performed by visual inspection. Tank as a random effect resulted in lower AIC values for linear mixed effect models (lme) and was kept in the final models.

2.8 | Transcriptional changes in the skin

To investigate transcriptional changes between the different treatments t-tests were used. This included between controls and infected fish (a separate test was performed for the site of lice attachment and at non-lice sites for both infective doses). Moreover, we compared expression at non-lice sites in the low dose group to the site of lice attachment in both the low and high dose group and compared to non-lice sites in the high dose group. Lastly tests investigating differences in the same samples (lice-positive or lice-negative) from the low and high dose group were performed.

2.9 | Host weight gain

To investigate the effect of treatment (infected vs. control) a lme model was fitted with weight as a response variable, treatment as a predictor variable and tank as a random effect for each sampling.

2.10 | Plasma cortisol levels

To investigate factors influencing cortisol levels a lme model was fitted with cortisol as response variable and treatment (control versus infected) as predictor variable and tank as a random effect for each sampling.

2.11 | Lice infection success and survival

A generalized linear model (glm) was fitted by combining two columns as response variables (i.e., lice that successfully infected and

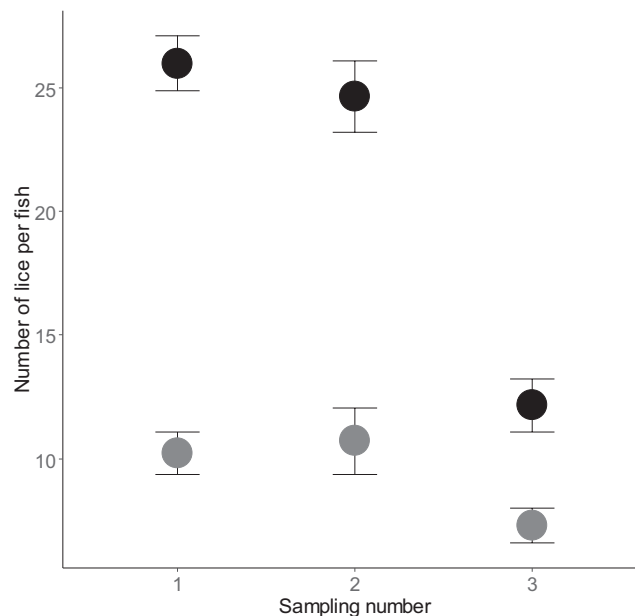


FIGURE 1 Infection levels. Number of lice in infected fish (\pm SE) depending on sampling number (1 = chalimus 2 stage ($n = 50$), 2 = preadult stage 1 ($n = 50$) and 3 = adult stage ($n = 50$) depending on infective dose (high dose = black and low dose = grey)

survived on the host and those that did not) using the cbind function in R and dose (low or high) as a predictor variable.

3 | RESULTS

3.1 | Lice infection success and survival

The mean number of lice on the sampled fish at the chalimus stage was 10 at the low 26 at the high dose, while at the preadult stage it was 24 at the high and 11 at the low dose group (Figure 1). Lastly, in the adult stage, the mean number of lice was 12 in the high and 7 in the low dose group. Lice infection success and survival in the host did not differ between the infective doses for the chalimus ($p = .221$, $t = 1.2$) or the preadult stage ($p = .141$, $t = 1.5$) but was significantly lower in the high than in the low dose group when lice had developed to the adult stage ($p < .001$, $t = 3.9$; Table 2, Figure 1). This could indicate similar infection success but decreased survival of lice in more heavily infected fish later in the infection.

3.2 | Host weight gain

Start weight was measured as the fish were distributed to experimental tanks and did not differ between infected and control fish ($p = .72$, t -value = -0.36). Moreover, there was no difference in weight at the time of sampling between infected and control fish (sampling 1, $p = .59$, t -value = -0.55 ; sampling 2, $p = .53$, t -value = 0.64 and sampling 3, $p = .42$, t -value = -0.84).

TABLE 2 Mean infection success and host survival (\pm SE) depending on the stage and infective dose (low or high)

Dose	Stage	N	Mean infection success and survival	\pm SE
High	Chalimus	25	0.26	0.01
High	Preadult	25	0.25	0.01
High	Adult	25	0.12	0.01
Low	Chalimus	25	0.29	0.02
Low	Preadult	25	0.31	0.04
Low	Adult	25	0.21	0.02

3.3 | Plasma cortisol levels

Plasma cortisol levels did not differ between infected and control fish at the chalimus and adult stage ($p = .58$ and $p = .79$), however, it was higher in control than in infected fish at the preadult stage ($p = .01$, t -value = 2.86).

3.4 | Differentially expressed transcripts

Several of the investigated transcripts were up or downregulated in infected fish compared to controls. The highest upregulation was

Gene	Treatment	Mean fold change	Stage
Interleukin 4	High-positive	11	Chalimus
	Low-positive	10	
	Low-negative	4	
Interleukin 8	High-positive	5	
	Low-positive	4	
Interleukin 1-beta	Low-positive	3	
	High-positive	3	
Serum amyloid A	Low-positive	3	
	Low-negative	3	
Matrix metalloproteinase 9	Low-positive	2	
Interleukin 4	High-positive	9	Preadult
	High-negative	8	
	Low-positive	4	
	Low-negative	3	
Interleukin 1-beta	High-positive	4	
	Low-positive	3	
Interleukin 8	High-positive	3	
Matrix metalloproteinase 9	Low-positive	3	
Inducible nitric oxide synthase	High-positive	0.4	
Interleukin 1-beta	High-positive	7	Adult
	Low-positive	6	
Serum amyloid A	Low-positive	5	
Interleukin 4	High-positive	5	
	Low-positive	5	
Interleukin 2	Low-positive	4	
	High-positive	3	
Complement factor 3	Low-positive	3	
	High-positive	3	
Cathelicidin 2	Low-negative	<0.5	
Interleukin 10	Low-positive	<0.5	
Collagen 10-alpha	Low-positive	<0.5	
	High-positive	<0.5	
Cluster of differentiation 8	Low-positive	0.4	
Inducible nitric oxide synthase	High-positive	0.3	
	Low-positive	0.1	

TABLE 3 Genes that were significantly different (sorted from high to low transcriptional changes) in infected fish than in controls at the different lice development stages. High-positive are samples taken at the site of lice attachment in fish infected with the high lice dose, while low negative is from non-lice sites in fish infected with the low dose

observed in interleukin 4 at the chalimus stage at the site of lice attachment, while the highest observed downregulation was observed in inducible nitric oxide synthase at the site of lice attachment in fish infected with the low dose (Table 3).

3.5 | Relative mRNA expression depending on parasite development stage

3.5.1 | Chalimus stage

Several immune genes had higher transcription in the skin at the site of lice attachment compared to samples from un-infected controls, this included interleukin 1-beta (Figure 2a), interleukin 8 (Figure 2d) and interleukin 4 (Figure 2i). Additionally, the expression of interleukin 4 was higher at lice-negative sites in infected fish than in controls in the high dose group (Figure 2i). Transcription of the acute phase protein serum amyloid A was upregulated in both samples in infested fish at the low dose group (Figure 2n). Matrix metalloproteinase 9, a gene involved in wound healing showed increased transcription in lice-positive samples compared to controls at a low dose (Figure 2p).

In infected fish, there was an upregulation of transcripts of interleukin 1-beta (Figure 3a), interleukin 8 (Figure 3c), interleukin 4 (Figure 3j) and matrix metalloproteinase 9 (Figure 3g) in lice-positive compared to lice-negative samples at both doses. Transcription of the antimicrobial peptide cathelicidin 2 was higher in lice-positive than at no-lice sites in infected fish in the high dose group (Figure 3e). Lastly, the expression of interleukin 10 (Figure 3n) and immunoglobulin M (Figure 3m) was reduced in lice-positive compared to lice-negative sites in infected fish in the high dose group.

3.6 | Preadult stage

In fish carrying lice at the preadult stage, there was an upregulation of interleukin 1-beta (Figure 2b) and interleukin 4 (Figure 2j) in lice-positive samples at both doses compared to samples from un-infected controls. Moreover, for interleukin 4 there also was a higher expression in lice-negative samples compared to controls (Figure 2j). Interleukin 8 was upregulated locally at the site of lice attachment only in the high dose group (Figure 2e), while transcription of the enzyme inducible nitric oxide synthase was reduced in the high dose group compared to un-infected controls (Figure 2f). The only gene involved in wound healing included in the study that showed higher expression at this stage was matrix metalloproteinase 9 in lice-positive samples at a low dose compared to controls (Figure 2q).

In the infected fish matrix, metalloproteinase 9 was significantly higher expressed in lice-positive than in lice-negative samples in the high dose group (Figure 3h).

3.7 | Adult stage

In fish carrying lice at the adult stage transcription of interleukin 1-beta (Figure 2c), interleukin 2 (Figure 2h), interleukin 4 (Figure 2k) and complement factor 3 (Figure 2r) was higher at both infective doses in lice-positive than in samples from un-infected controls. Additionally, there was downregulation of the enzyme inducible nitric oxide synthase (Figure 2g) and collagen 10-alpha (Figure 2s) in lice-positive samples compared to controls. The acute phase response protein serum amyloid A had higher transcription (Figure 2o), while interleukin 10 (Figure 2l) and the cluster of differentiation 8 (Figure 2m) were reduced in lice-positive samples at the low dose compared to controls. The only difference in transcription between lice-negative samples in infected fish and un-infested controls was a reduction in the antimicrobial peptide cathelicidin 2 at the low dose.

In infected fish, there was a significantly higher transcription of interleukin 1-beta (Figure 3b), interleukin 8 (Figure 3d), interleukin 4 (Figure 3k), cathelicidin 2 (Figure 3f) and matrix metalloproteinase 9 (Figure 3i) in lice-positive than in lice-negative samples at both doses. Moreover, at the low dose there was an upregulation of the acute phase protein serum amyloid A and complement factor 3 in lice-positive compared to lice-negative samples in infected fish (Table S2). Lastly, the expression of inducible nitric oxide synthase, collagen 10-alpha (Figure 3l) and the cluster of differentiation 8-alpha (Figure 3o) was significantly decreased at the site of lice attachment at both infective doses compared to non-lice sites.

3.8 | Genes with no transcriptional changes

No difference in transcription between infected and un-infected fish or between lice-positive and lice negative samples in infected fish at any stage was evident for immunoglobulin T, tumour necrosis factor-alpha, fibronectin precursor or transforming growth factor-beta (Tables S1 and S2).

3.9 | Effect of parasite intensity on relative gene expression

No consistent effect of infection density (lice g^{-1}) on the relative mRNA level of the investigated wound healing and immune genes was found in lice-positive samples. The only significant correlation between fold change and infection density was a negative correlation at the chalimus stage for interleukin 10 (p -value = .038, t -value = -2.164 and $R^2 = 0.1312$) and a positive correlation between the relative expression of interleukin 4 and infection density at the preadult stage (p -value = .02861, t -value = 2.323 and $R^2 = 0.1775$). Accordingly, interleukin 4 had higher expression at the preadult stage in both lice-negative and lice-positive samples in the high than in the low dose group (Table S4). At the chalimus stage, the level of interleukin 10 was

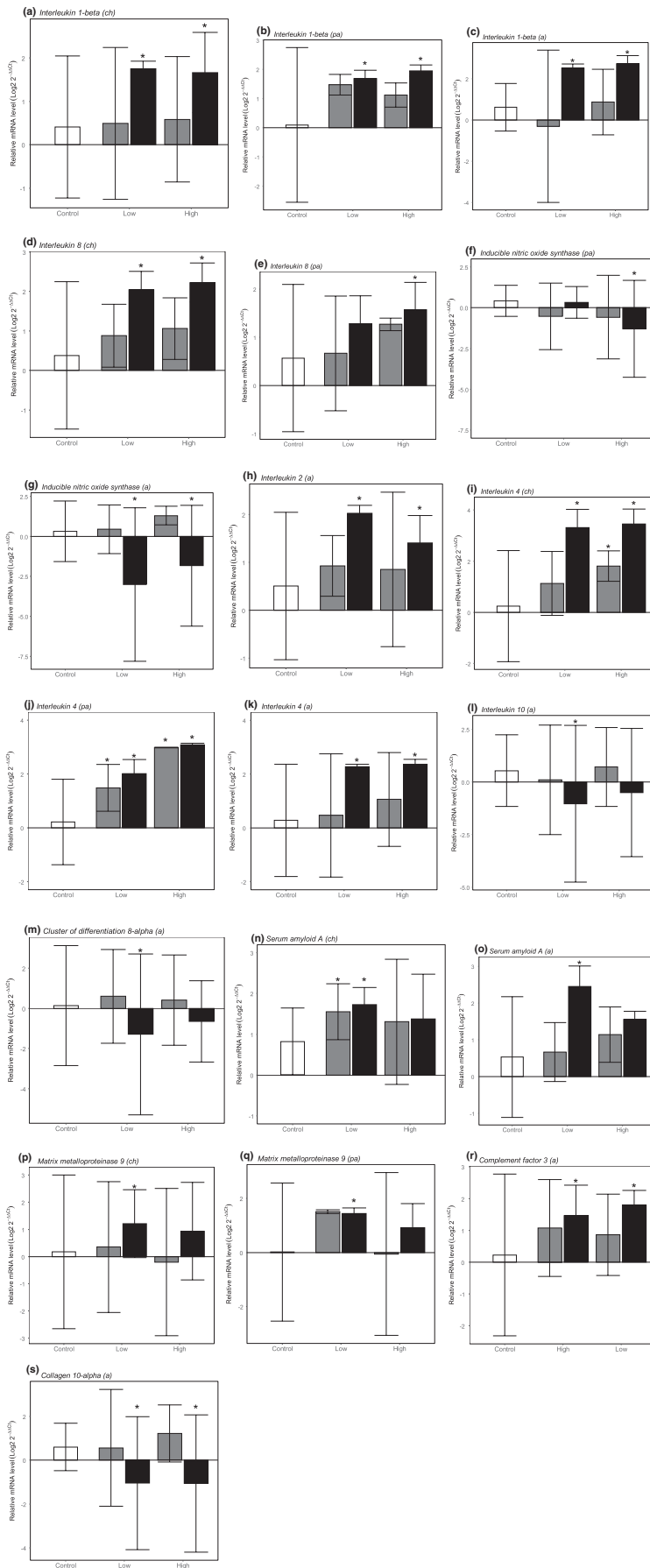


FIGURE 2 Relative log₂ transformed mRNA levels (\pm SE) in skin samples for selected immune and wound healing gene transcripts depending on infective dose (un-infested control, low or high dose, respectively), sample (un-infested controls (light grey columns), samples taken away from the site of infection (dark grey columns) and samples from under the lice (black columns)) and lice development stage (ch = chalimus 2, pa = preadult 1 and a = adult). The expression level is given as $2^{-\Delta\Delta CT}$ (\pm SE) in infected fish (lice-negative or lice-positive samples) compared to un-infested controls. The horizontal line is set at $y = 0$ in the figures. Statistically significant differences in expression relative to un-infested controls are denoted*

lower, while complement factor 3 had higher expression at the site of lice attachment in the low compared to the high dose group (Table S4).

4 | DISCUSSION

Infection status (infected vs. control) did not consistently affect cortisol levels or weight. Additionally, transcription of the wound and immune genes in infected fish was not affected by parasite intensity. However, transcription of several of the investigated genes in lice-positive skin samples differed significantly from lice-negative samples from infected fish and controls. This suggests that at the investigated doses, infective status but not the number of lice determined host responses. Lice infection success was unaffected by infective dose, but survival was reduced at the adult stage in the high dose group, which could indicate increased lice loss at high lice intensities.

There was no effect of treatment (infected vs. controls) on fish weight in our study. This could be due to the moderate lice loads and/or caused the time gap between the recording of start weight and lice infection. Which could have masked the effects of lice. However, if lice had a significant effect on fish weight gain this should become evident as the lice infection proceeded and should therefore be most noticeable at the adult stage. We did not find higher cortisol levels in infected fish, contrarily at the pre-adult stage, it was higher in control than in infected fish. This is probably due to the concomitant bacterial infection, which was more pronounced in control than in infected fish at this sampling point. Contrarily, previous studies have found higher cortisol levels in infected fish and cortisol levels were positively correlated to lice load and plasma ion levels, suggesting that at high intensities salmon lice could initiate a dose-dependent stress response (Fjelldal et al., 2020). Higher plasma cortisol levels in infected fish at high intensities could subsequently increase susceptibility to pathogens, due to the immunosuppressive effects of cortisol (Johnson & Albright, 1992; Pickering & Pottinger, 1989).

No consistent effect of parasite load on the transcription of the investigated wound healing and immune genes in infected fish skin was observed. Only two transcripts investigated had a significant correlation to lice intensities. Although these correlations were significant, little of the variation was explained by parasite intensity. The severity of host immune responses is suggested to increase with antigenic stimulation (Anderson & May, 1978). However, empirical studies have found no effect, enhanced or reduced immune responses at high parasite intensities, suggesting that the effect of parasite intensities on host responses varies for different host-parasite interactions (Bleay et al., 2007; Buchmann, 2020; Khokhlova et al., 2004; Misumi, Leong, Takemura, & Lewis, 2012; Paterson & Viney, 2002; Shameena et al., 2021).

Atlantic salmon are highly susceptible to salmon lice infections (Braden et al., 2012), and this could partly be due to parasite modulation of host immune responses (Braden et al., 2020; Fast et al., 2007). It has also been suggested that the immunosuppressive effect of

the parasite increase with parasite intensity (Holm et al., 2015). However, the mechanism behind salmon lice immunosuppression is not elucidated (Dalvin, Eichner, Dondrup, & Øvergård, 2021; Eichner, Øvergård, Nilsen, & Dalvin, 2015; Øvergård et al., 2016). A parasite intensity-dependent host immune response in the skin is not supported by our findings here. Furthermore, salmon lice infections result in more local than systemic immune responses in the skin (Dalvin et al., 2020; Øvergård, Hamre, Grotmol, & Nilsen, 2018), it seems unlikely that parasite intensities within the range tested here should significantly influence host immune responses. Local immune responses agree with findings in other host-ectoparasite systems, where pro-inflammatory responses are stronger at the site of infection (Covello et al., 2009; Gonzalez, Buchmann, & Nielsen, 2007). Salmon lice prefer some locations on the host over others (Bui, Oppedal, Nola, & Barrett, 2020), so even at the lice intensities investigated here competition for lice-favoured locations with reduced drag or better excess to nutrients could occur. Hence, the reduction in infection success and survival at the high dose at the adult stage found here, could result from intensified competition amongst lice at high intensities. A similar mechanism may also be the cause of the observed maximum intensity of lice found on salmon in the Pacific ocean (Costello, 2006). However, we cannot rule out that immune genes not included in this study are differently expressed depending on lice intensities or that a dose-dependent immune response could occur at higher lice intensities than in the current study.

At the second sampling (lice at the preadult stage) fish health in some tanks belonging to all treatments but most noticeably control fish were negatively affected by a bacterial infection in the skin. Samples were not taken from fish with visible lesions, but we cannot rule out that this co-infection affected our data at this sampling. Transcription of the investigated immune and wound healing genes at this stage, could be up- or downregulated not only in response towards the louse in infected fish but also in the response towards the bacteria in the skin of both infected and control fish. This could therefore potentially mask the response towards the louse by affecting transcription in control fish. However, despite the potential confounding immune and wound healing response towards the bacteria, aberrant expression at the site of lice attachment suggests that there is a lice-specific response in the skin for several of the investigated transcripts also in this sampling. Fortunately, this bacterial infection was not evident at the first and was cleared at the third sampling. Hence, results from these samplings are less likely to be affected.

4.1 | Immune responses in the skin

Th-1 like immunity is important for the clearing of intracellular pathogens and we observed an upregulation of the several pro-inflammatory cytokines at the site of lice attachment. This included a local increase in transcription of interleukin 1-beta at the chalimus and adult stage. Interleukin 1-beta is produced by various immune cells upon stimulation and is a chemoattractant for macrophages and leukocytes in fish (Zou & Secombes, 2016). Moreover, it affects

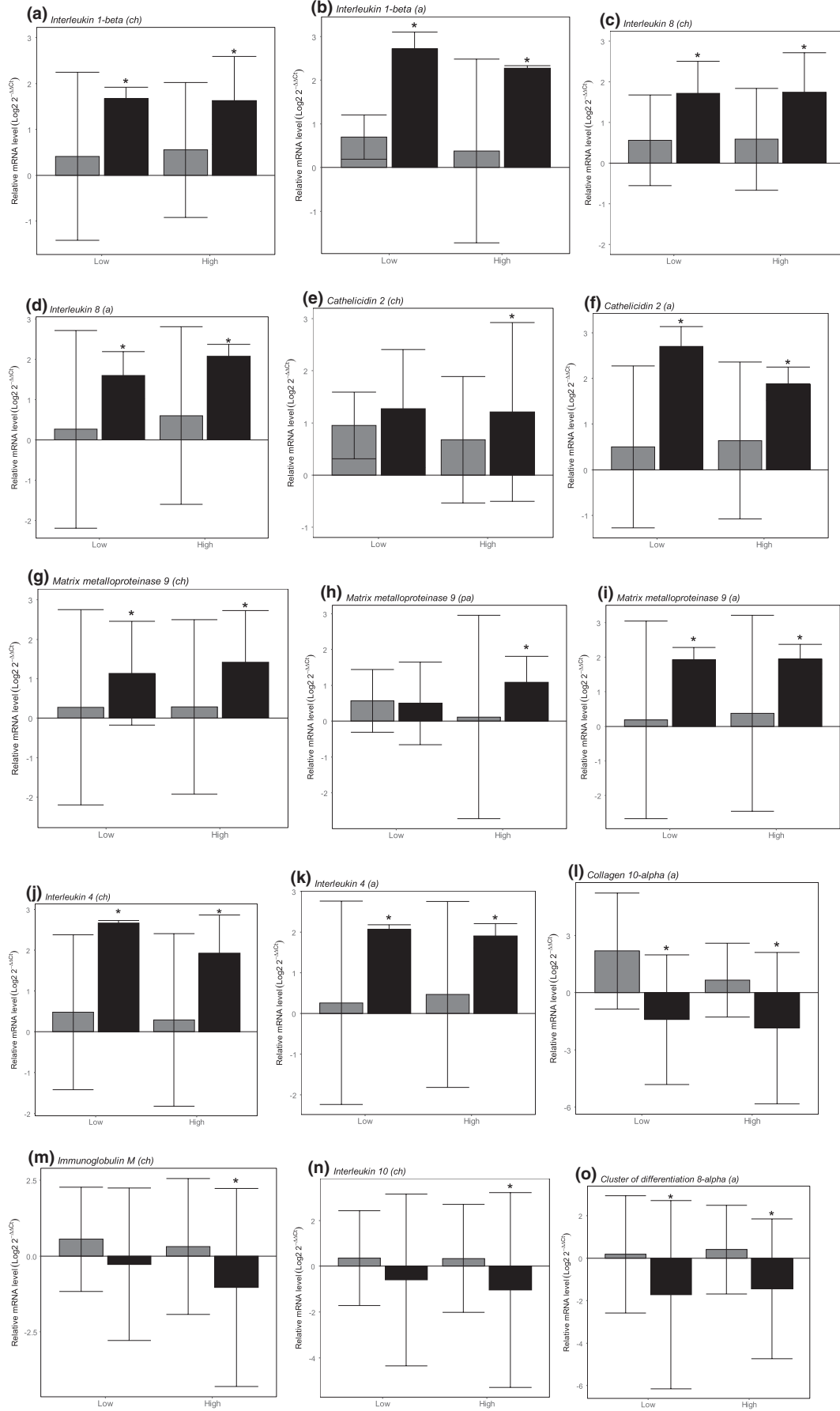


FIGURE 3 Relative log₂ transformed mRNA levels (\pm SE) in skin samples for selected immune and wound healing gene transcripts depending on infective dose (low or high), sample (sample from under the louse = black and samples taken away from the site of infection = grey) and stage of the louse (ch = chalimus 2, pa = preadult 1 and a = adult stage) in infected fish. The expression level is given as $2^{-\Delta\Delta CT}$ (\pm SE) in lice-positive samples compared to lice-negative samples in infected fish. The horizontal line is set at $y = 0$ in the figures. Statistically significant differences in expression in lice-positive compared to lice-negative samples is denoted*

the lysozyme activity in trout and could induce the immunosuppressive cytokines interleukin 10 and transform growth factor-beta in macrophages (Zou & Secombes, 2016). Higher expression of this cytokine is also previously reported in fish infected with salmon lice (Braden et al., 2012; Braden et al., 2015; Dalvin et al., 2020; Øvergård et al., 2018), suggesting a role in host responses against the ectoparasite in salmonids. Moreover, pro-inflammatory responses in infected fish are supported by enhanced expression of interleukin 8 both at the site of lice attachment and in lice-negative samples compared to controls. Interleukin 8 is associated with leukocytes and macrophages and mediates the migration of immune cells (Brunner, Varga, & Dixon, 2020; Covello et al., 2009), and findings here and from previous studies on Atlantic, coho and sockeye salmon (Braden et al., 2015; Øvergård et al., 2018) suggest an influx of these cells or a higher expression of this chemokine in the skin of infected fish. Higher expression of the antimicrobial peptide cathelicidin 2 was detected at the site of lice attachment compared to non-lice sites. Cathelicidin 2 is known to upregulate interleukin 8 in peripheral blood leucocytes (Brunner et al., 2020) and is also involved in wound healing, increased phagocytosis and innate immune responses (Brunner et al., 2020). However, cathelicidin 2 transcription was reduced in lice-negative samples in infected fish compared to controls at the adult stage (low dose), suggesting aberrant expression between lice-positive and lice-negative samples in infected fish at this stage. Higher mRNA levels of cathelicidin 2 at the site of lice attachment are also reported previously (Braden et al., 2015; Dalvin et al., 2020) confirming a putative role in host responses in several salmonids. Transcription of the acute phase protein serum amyloid A was higher locally at the site of lice attachment. Serum amyloid A originates from hepatic cells stimulated by cytokines from activated macrophages and opsonize pathogens, neutralize enzymes, and activate the complement system (Grayfer et al., 2018). Serum amyloid A and other acute-phase proteins have been associated with lice resistance (Sutherland et al., 2014) and the transcriptional changes are more pronounced in resistant coho salmon than in the more susceptible sockeye and Atlantic salmon (Braden et al., 2015).

The enzyme inducible nitric oxide synthase was locally downregulated at the site of lice attachment at the adult and preadult stage. This is similar to findings in rainbow trout where a downregulation was evident at the site of lice attachment (Dalvin et al., 2020), but an upregulation has previously been reported in Atlantic salmon infected directly with adult lice (Braden et al., 2012; Braden et al., 2015). This discrepancy could be caused by different infection procedures. Inducible nitric oxide synthase is a marker of activated macrophages (M1 subset) and is involved in the production of nitric oxide (NO) (Grayfer et al., 2018). No significant increase in transcription of the pro-inflammatory cytokine tumour necrosis factor-alpha,

together with reduced transcription or the enzyme inducible nitric oxide synthase suggests no influx or a reduction in M1 macrophages at the site of lice attachment, which could increase susceptibility to new infections.

Th-2-like responses are important for the defence against parasites and could protect fish skin from damage by inflammatory Th-1 and Th-17 responses (Takizawa et al., 2011). Dysregulation of host immune response is associated with increased susceptibility to parasites and pathogens, hence regulatory cytokines could be important for the outcome of a lice infection. One of these regulatory cytokines, interleukin 2 is produced by T cells and is known to regulate both Th-1 and Th-2 cytokines and enhance the proliferation of immune cells and increase phagocytosis (Wang et al., 2018). In infected fish, there was higher expression of interleukin 2 at the site of attachment relative to controls at the adult stage. Hence, interleukin 2 could have a role in regulating Th-1- and Th-2-like responses in the skin towards adult lice.

Transcription of interleukin 4 was enhanced at the site of lice attachment as previously reported for salmonids (Braden et al., 2020; Dalvin et al., 2020; Øvergård et al., 2018). Suggesting activation of a subset of anti-inflammatory macrophages (M2a) by Th-2, eosinophils, basophils or natural killer T cells (Grayfer et al., 2018; Luckheeram, Zhou, Verma, & Xia, 2012). Additionally, increased expression was evident in lice-negative samples in infected fish compared to controls at the highest dose, which could be indicative of systemic response in the whole skin. The role of interleukin 4 in host defence against salmon lice is supported by a higher expression in resistance than in susceptible species, and a role in wound healing is also suggested (Braden et al., 2015).

Lastly, we found a reduction in transcription of the anti-inflammatory cytokine interleukin 10 at the site of lice attachment. This is congruent with the concurrent upregulation of pro-inflammatory cytokines. Interleukin 10 regulates immune responses by inhibiting differentiation of monocytes, reducing phagocytosis, suppressing genes coding for MH class II molecules and pro-inflammatory cytokines (Chaudhry et al., 2011; Luckheeram et al., 2012; Rebl & Goldammer, 2018). Previously changes in mRNA transcripts of interleukin 10 have only been reported in resistant coho salmon (Braden et al., 2015).

The protein complement factor 3 is plentiful in fish serum and is involved in the lysis of foreign cells and opsonizing pathogens for phagocytosis (Grayfer et al., 2018; Holland & Lambris, 2002). The transcription of complement factor 3 was higher locally at the site of lice attachment than in controls at both doses and compared to non-lice sites from infected fish at the lowest dose at the adult stage. This suggests activation of the complement system at the site of lice attachment.

Transcription of the cluster of differentiation 8, a marker of cytotoxic T cells and immunoglobulin M was reduced locally at the site of lice attachment, while immunoglobulin T was not affected by treatment. Cytotoxic cells are important for antiviral immunity in fish by eliminating virus-infected cells (Somamoto, Koppang, & Fischer, 2014), hence susceptibility to viral infection could be enhanced in lice-infected fish. Immunoglobulin M is important for both adaptive and innate immune responses (Mashoof & Criscitiello, 2016) and it has been linked to lice resistance (Braden et al., 2015). Reduced expression of cluster of differentiation 8 and immunoglobulin M at the site of lice attachment is also previously reported in salmonids (Braden et al., 2015; Dalvin et al., 2020) and could suggest impaired adaptive immune responses locally at the site of lice attachment.

4.2 | Wound healing

Matrix metalloproteinases are important for remodelling of the extracellular matrix and aberrant expression in lice-infected fish is associated with chronic wounds (Braden et al., 2012; Braden et al., 2020; Skugor, Glover, Nilsen, & Krasnov, 2008) and this was confirmed in the present study. Increased transcription at the site of lice attachment is reported for several salmonid species including Atlantic salmon (Braden et al., 2015). Contrastingly, the expression of collagen 10- α was reduced at the site of lice attachment, which corresponds to previous findings in Atlantic salmon (Skugor et al., 2008). Collagen is crucial for the extracellular matrix, and it has a role in both wound healing and tissue remodelling in fish (Castillo-Briceño et al., 2011). Aberrant expression of matrix metalloproteinase and collagen 10 could negatively affect wound healing capabilities in lice-infected fish. No aberrant expression of these transcripts at the preadult stage, which could be expected if the sampled fish were affected by a bacterial infection in the skin.

In conclusion, we found little evidence for an effect of dose in our investigated transcripts. The intensities in this study are similar to that found in wild (Torrissen et al., 2013) and in Atlantic salmon kept in sea cages (Bui et al., 2020). Hence, results presented here are relevant to understanding how lice intensities affect host responses towards the parasite and interactions between lice on the fish in both farmed and wild salmon. Our investigation included key indicators of fish welfare including growth, stress and immune status, hence given the tested conditions, the dose-dependent response appears to be limited. Lice infection success and survival did not differ between the high and low dose group at the chalimus and preadult stage, but it was significantly lower in the high than in the low dose group at the adult stage. Increased lice loss late in the infection could be due to enhanced competition amongst lice at high lice intensities as lice prefer some locations on the host skin over others (Bui et al., 2020).

AUTHORS CONTRIBUTION

S.D. designed the study. S.D. and M.S.U. performed the experimental procedures in the fish facility and M.S.U. performed the

experimental procedures in the laboratory. MSU analysed the data and wrote the first draft. S.D. provided critical revisions and comments to the manuscript.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article

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