

Temperature affects settlement success of ectoparasitic salmon lice (*Lepeophtheirus salmonis*) and impacts the immune and stress response of Atlantic salmon (*Salmo salar*)

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Abstract

In this study, the effect of temperature on Atlantic salmon (*Salmo salar*) stress and immune response to the ectoparasitic salmon lice (*Lepeophtheirus salmonis*) was investigated. We found that infestation affected the expression of several immune and wound healing transcripts in the skin especially at the site of lice attachment compared to un-infested control fish. Moreover, expression patterns in the skin of infested fish suggest that host immune responses towards salmon lice are impaired at low temperatures. However, reduced lice infestation success and survival at the lowest investigated temperatures suggest that cold water temperatures are more detrimental to the lice than their fish hosts. Finally, temperature affected the stress response of the fish and infected fish had a higher increase in cortisol levels in response to handling (a stressor) than un-infested controls.

KEYWORDS

salmon, salmon lice, temperature, immune response, stress response

1 | INTRODUCTION

Temperature affects the survival, phenology, development and physiology of poikilotherms. However, the response to temperature is often non-linear and varies between species (Franke et al., 2019; Paull et al., 2012; Rohr et al., 2011). Hence, predicting the effect of temperature on interspecific interactions such as parasitism is challenging (Paull et al., 2012; Rohr et al., 2011). Understanding the net effect of temperature on host-parasite interactions (i.e., virulence and epidemiology) is necessary to predict how it influences the outcome of important pests in aquaculture.

Intensive farming of Atlantic salmon (*Salmo salar*) in Norway has increased rapidly during the last decades and in 2019 the production was more than 812,000 tons (Norwegian directorate of fisheries). High abundances of fish kept in open net pens along the coast have led to an increased abundance of parasitic salmon

louse (*Lepeophtheirus salmonis*) (Barrett et al., 2020; Costello, 2009; Krkosek et al., 2007; Krkosek et al., 2009; Serra-Llinares et al., 2014; Torrissen et al., 2013). Salmon lice feed on the blood, skin and mucus of their host and thereby cause problems with osmoregulation, increase susceptibility to secondary infections (Barker et al., 2019; Nylund et al., 1994) and enhance host mortality at high intensities (Fjellidal et al., 2020; Grimnes & Jakobsen, 1996). Salmon lice cause large economic losses to salmon farmers (Costello, 2009) and the cost of sea lice in Norway alone was estimated to be US\$436m in 2011 (Abolofia et al., 2017).

Salmon lice infestation of fish starts when the copepodid attaches to a suitable host, where the louse develops further through two sedentary stages (chalimi), two mobile preadult stages (preadults 1 and 2), before finally becoming adults (Hamre et al., 2013; Johnson & Albright, 1991). Atlantic salmon and consequently parasitic salmon lice are widely distributed in the northern Atlantic Ocean

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(Rikardsen et al., 2021), and thereby exposed to different water temperatures throughout their range. Additionally, temperatures vary seasonally, with the lowest temperatures in late winter. Salmon lice are ectothermic, and temperature influence their physiology such as development time, fecundity, infestation success, longevity, egg and body size (Boxaspen & Næss, 2000; Dalvin et al., 2020a; Hamre et al., 2019; Heuch et al., 2000, 2003; Nordhagen et al., 2000; Samsing et al., 2016; Skern-Mauritzen et al., 2020). Moreover, temperature could affect epidemiology by altering the generation time and the net reproductive rate of the louse (Groner et al., 2016). Hence, increased sea temperature due to global warming could exacerbate lice-infection pressure from Norwegian aquaculture (Sandvik et al., 2021).

The host fish are also ectotherms and hence ambient temperature influences their physiology like metabolism (Handeland et al., 2008), immune responses (Abram et al., 2017; Alcorn et al., 2002; Bowden, 2008; Magnadottir, 2006; Le Morvan et al., 1998) and wound healing capabilities (Jensen et al., 2015; Ream et al., 2003). The preferred temperature for Atlantic salmon is suggested to be around 16°C (Oppedal et al., 2011; Stehfest et al., 2017) and too high or low temperatures could negatively affect their physiology (Abram et al., 2017; Medcalf et al., 2021). Moreover, suboptimal temperatures could activate the hypothalamic–pituitary–interrenal axis resulting in the production of the stress hormone cortisol by interrenal cells in the head kidney, which is known to affect immune responses in fish (Alfonso et al., 2021; Rebl & Goldammer, 2018).

Comparable to other vertebrates the immune system of fish consists of an innate, complement and adaptive system (Lieschke & Trede, 2009; Secombes & Wang, 2012; Sunyer & Lambris, 1998; Watts et al., 2001). However, the function and organization of the immune system are different in fish than in mammals and most noticeable fish rely more on and have a more diverse innate immune system (Magnadottir, 2006; Secombes & Wang, 2012; Secombes et al., 2001). Furthermore, since teleost fish are unable to self-regulate their body temperature, the immune response is temperature dependent (Watts et al., 2001). Low temperatures generally impair immune functions in fish and especially the adaptive response is negatively affected (Abram et al., 2017; Alcorn et al., 2002; Bly & Clem, 1992). Suppression of immune responses at low temperatures has also been reported for salmonids. In rainbow trout (*Oncorhynchus mykiss*) major histocompatibility class II genes involved in adaptive immune responses (Nath et al., 2006) and respiratory burst activity are reduced at low temperatures (Nikoskelainen et al., 2004). Similarly, Atlantic salmon reared at low temperatures had a reduction in antibody response against the bacteria *Vibrio salmonicida* and *Aeromonas salmonicida* (Eggset et al., 1997) and rearing temperature also affects their innate antiviral responses (Ignatz et al., 2020).

Susceptibility to salmon lice varies between salmonids (Braden et al., 2020; Mackinnon, 1998; Nagasawa & Takami, 1993) and while some species in the Pacific ocean are partly resistant, species found in the Atlantic ocean (e.g., *Salmo salar*, *Salmo trutta* and *Salvelinus alpinus*) are susceptible. Differences in the Pacific and Atlantic sub-species of salmon lice may also affect the host–parasite interaction

(Skern-Mauritzen et al., 2020). Coho (*Oncorhynchus kisutch*) and pink salmon (*Oncorhynchus gorbuscha*) rapidly eliminate lice, but Atlantic and chum salmon (*Oncorhynchus keta*) are more prone to infestations (Braden et al., 2012, 2015; Fast et al., 2002). However, the mechanisms conveying lice resistance are not fully elucidated and vary between species. Lice rejection in pink salmon is linked to a rapid increase in proinflammatory cytokines (Jones et al., 2007), while resistance in Coho salmon is associated with both hyperplasia and inflammatory responses at the site of lice attachment (Johnson & Albright, 1992; Sutherland et al., 2014). Moreover, in addition to proinflammatory T helper like 1 (Th-1) responses (Braden et al., 2012, 2020; Fast et al., 2007; Jones et al., 2007; Skugor et al., 2008), resistance is also proposed to depend on regulatory Th-2 like pathways (Braden et al., 2015, 2020).

This study aimed to investigate the immune and stress response of Atlantic salmon to salmon lice at four different temperatures found in their native range and to explore how temperature affects parasite performance.

2 | MATERIALS AND METHODS

2.1 | Rationale

The occurrence of salmon lice epizootics in farmed fish is more frequent in farmed salmon at lower latitudes and during the summer and autumn along the Norwegian coast, suggesting that temperature could influence the host–parasite interaction. Hence, investigating the effect of different temperatures found along the Norwegian coast on this host–parasite interaction is eminent both to salmon farmers and regulatory authorities. To elucidate this, Atlantic salmon were kept at four different ambient temperatures (4, 8, 12 and 16°C, respectively) and samples for investigation of gene expression in the fish were taken at three timepoints after infestation with salmon lice. Sampling was conducted when the louse reached the chalimus 2 (sampling I), preadult 1 (sampling II) and adult stage (sampling III). However, since lice development is highly temperature dependent (Hamre et al., 2019), sampling at the different temperatures was not conducted on the same date.

2.2 | Handling of fish and infestation

Atlantic salmon ($n = 336$ (Aquagen strain reared in the laboratory); mean weight at termination 477 g (± 107 SE)) were raised in sea water with 35 ppt salinity at 8°C. To acclimatize to one of four experimental temperatures (4, 8, 12 and 16°C), fish were randomly distributed to 16 tanks (600 L, 21 fish/tank) 10 days prior to the infection. A laboratory louse strain was used in the experiment (Hamre et al., 2009) which were hatched and reared at 9.5°C and 35 ppt salinity prior to the infection. Infection was done by lowering the water level to one-third of its volume and adding infective salmon lice copepodids to the tanks. During the infection inflow

of water was maintained (12 L/min), but the outlet was blocked until the normal water level was restored. Infection success of salmon lice is temperature dependent (Dalvin et al., 2020a; Skern-Mauritzen et al., 2020), hence the number of copepodids per tank was adjusted to achieve similar lice intensities on the fish at different temperatures (i.e., 4°C, 1008 lice/tank; 8°C, 672 lice/tank; 12°C, 525 lice/tank and 16°C, 462 lice/tank). Fish in the control tanks were equally treated, but no copepodids were added to the tank.

2.3 | Sampling procedure

For each sampling, seven fish from each tank were individually carefully netted and sedated in 10 L of sea water mixed with 15 mg/L Finquel (Trikainmesilat) and subsequently humanely killed with a sharp blow to the head. Blood samples were immediately taken and kept on ice until they were centrifuged. 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 in infested 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 infestation success and survival).

Furthermore, two skin samples from infested and one skin sample from un-infested fish were taken immediately after the blood sample. In infested fish, one lice-positive skin sample was taken directly underneath a louse (i.e., local immune response in the skin), while the second lice-negative skin sample was taken at a similar location without lice (i.e., systemic immune response in the skin). At the chalimus stage, all samples were taken on the skin, despite that most individuals were found on fins. This was done so that we could compare them to subsequent samples. For the mobile stages (i.e., preadult and adult stage) lice-positive samples were preferably taken from the dorsal surface, where lice tend to be found on the fish. This was done to ensure that they were lice-positive since preadult and adult lice are mobile and can move on the host skin. Skin samples were frozen at -80°C in 1.4 ml PreMax™-plate tubes containing two stainless steel beads (Nerliens Meszansky) for later RNA extraction.

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 the manufacturer's recommendations in a total volume of 10 µl along with negative control (RTneg) and a no template control (NTC). The samples were diluted with nuclease-free, sterile water to get an RNA concentration of 400 ng/µl, 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. 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 first at 25°C for 10 min, thereafter at 42°C for 60 min and 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 of nuclease-free, sterile water and 5 µl of cDNA prior to the real-time qPCR assay.

2.6 | RT-qPCR

RT-qPCR was performed in the QuantStudio™5 system (Thermo Fisher 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, 0.10 µl (Table 1) 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 simplified $2^{-\Delta\Delta Ct}$ method as used by Dalvin et al. (2020b). Elongation factor 1-alpha (EF 1-Alpha) and receptor-like protein 1 (RLP 1) were used as reference genes. Results are presented as a change in fold in lice-infested fish (negative and positive) samples compared to un-infested control fish samples at the three sampling points (Tables S6–S8). Additionally, for lice-positive samples, fold change relative to lice-negative samples in infested fish was also calculated. Changes in threshold cycle (ΔCt) value were calculated for each temperature as differences between RNA levels of the gene of interest and the arithmetic mean of the reference genes. $\Delta\Delta Ct$ was quantified as the difference between ΔCt in infested fish (lice-positive and lice-negative samples) and the average ΔCt of un-infested control fish. For the comparison between lice-positive and lice-negative samples, the average ΔCt of lice-negative samples were compared to lice-positive samples. Only expressional differences between groups

TABLE 1 Forward (F) and reverse (R) primers used in RT-qPCR setup

Gene	Primers	Accession number	Amplicon length
Immunoglobulin T	F:GGTGGTCATGGACGTACTATTT R: CCTGTGCAGGCTCATATCTT	GQ 907004.1	98
Immunoglobulin M	F:TGAGGAGAAGTGTGGGCTACACT R:TTAATGACTACTGAATGTGCAT	S48652	67
Interleukin 8	F:GCATCAGAATGTCAGCCAGCC R:ACGCCTCTCAGACTCATCCC	NM_001140710.2	79
Interleukin 1-beta	F:GCTGGAGAGTGTGTGGAAGA R:TGCTTCCCTCTGCTCGTAG	NM_001123582.1	73
Interleukin 4/13a	F:CGTACCGGCAGCATAAAAATCACCATTCC R: CCTTGCATTTTGTGGTGGTCCCA	NM_001204895	147
Interleukin 10	F:GCTATGGACAGCATCCTGAAGTT R:GGTTGTTCTGCGTTCTGTTGTT	EF165028.1	76
Cluster of differentiation 4	F:GTGGAGGTGCTACAGGTGTTTTT R:GGGGAGGAGCCTAAAGCG	EU409794.1	158
Cluster of differentiation 8-alpha	F:TAGAGTGCAAGACAACGCTGGAATGGA R:TCTCGAGCCTTTTTGAAAGCCTTCAG	NM_001123583.1	150
Tumour necrosis factor-alpha	F: AGGTTGGCTATGGAGGCTGT R:TCTGCTTCAATGTATGGTGGG	NM_001123589	400
Elongation factor 1-alpha	F:CACCACCGCCATCTGATCTACAA R:TCAGCAGCCTCCTTCTCGAACTTC	NM_001123629.1	78
Complement factor 3	F:TCCCTGGTGGTACCAGTACAC R:ATGATGGTGGACTGTGTGGATC	BI468074	157
Serum amyloid A	F:AGCTGCTCGAGGTGCTAAAG R:ATGCCTCGACCCTGGAAC	NM_001146565.1	193
Collagen 10-alpha	F:TGGTGTCTTTGACTGCCTGTAA R:CATCCTGTGTGTGCAATATCACA	EG837148	180
Fibronectin precursor	F:GCATGTCTGAGACGGGCTTCAA R: AGTCACATCGGAAGTGTCCACTGC	XM_014123350.2	72
Matrix metalloproteinase 9	F:AGTCTACGGTAGCAGCAATGAAGGC R:CGTCAAAGGTCTGGTAGGAGCGTAT	NM_001140457.1	72
Transforming growth factor-beta	F:AATCGGAGAGTTGCTGTGTGCGA R:GGGTTGTGGTCTTATACAGAGCCA	EU082211	178
Receptor-like protein 1	F: ACTATGGCTGTCGAGAAGGTGCT R:TGTACTCGAACAGTCGTGGGTCA	CB516726	118

with a minimum of twofold differences in mRNA and $p < .05$ were considered significant.

2.7 | Statistical analyses

All data analyses were performed using the statistical program environment R 3.6.2 (<http://r-project.org>). For all generated 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.

2.7.1 | Lice infestation success and survival

A generalized linear model (glm) was fitted by combining two columns as response variables (i.e., lice that successfully infested and survived on the host (i.e., number of lice per fish when sampled) and those that did not) using the `cbind` function in R. The model was fitted with quasibinomial distribution. To test the effect of temperature a Chi-square test was performed.

To investigate the effect of the parasite development stage on the number of lice on the fish. A glm model for each of the investigated temperatures was fitted with quasi-Poisson distribution, a

number of lice as a response variable and development stage (chalinus, preadult and adult) as predictor variables.

2.7.2 | Plasma cortisol levels

To investigate factors influencing cortisol levels a lme model with interactions were fitted with cortisol as a response variable and sampling order (fish 1–7), temperature (4, 8, 12 and 16°C) and treatment (Control and infested) as predictor variables and tank as a random effect. Furthermore, to investigate the effect of lice numbers in infested fish a lme model was fitted with cortisol levels as response variable and number of lice, temperature and sampling order as predictor variable and tank as a random effect. This model was then compared with a model excluding lice numbers as predictor variable using the ANOVA function in R. Additionally, to compare cortisol levels at high (12 and 16°C) and low (4 and 8°C) temperatures, welch two-sample *t*-tests were done.

3 | RESULTS

3.1 | Parasite infestation and survival

Low temperatures impaired the ability of the lice to infest and survive on the host, resulting in lower lice intensities at these temperatures despite the higher number of copepodids they were initially infested with (Table 2). Mean infestation success and survival (%) for all stages ranged from 4.8% to 38.85% at 4 and 16°C, respectively (Table 2) and there was a significant effect of temperature on infestation and lice survival at all stages ($p < .001$). Moreover, the development time from chalinus to the adult stage increased from 18 days at 16°C to 49 days at 4°C (Table 3). There was no significant difference in the number of lice between any stage at 4 and 8°C, but at 12°C it was significantly lower at the adult than at the preadult stage (Table 4). Moreover, at 16°C the number of lice was higher at the preadult than at the chalinus stage (Table 4). This could suggest a slight underestimation of the lice number at the chalinus stage and lice loss between the preadult and adult stages at the two highest temperatures.

TABLE 2 Mean number of lice (\pm SE), minimum and maximum number of lice and infestation and survival (%) of lice depending on temperature and stage of the louse

Temperature (°C)	Chalinus			Preadult			Adult			Mean all stages (%)
	Mean (\pm SE)	Min-max	%	Mean (\pm SE)	Min-max	%	Mean (\pm SE)	Min-max	%	
4	2.0 (0.3)	0–4	4.2	2.9 (0.5)	1–6	6.1	2.0 (0.4)	1–4	4.2	2.3 (4.8)
8	5.8 (0.7)	2–11	18.1	6.9 (1.1)	1–15	21.0	4.7 (0.7)	0–9	14.1	5.8 (17.7)
12	8.2 (1.0)	3–15	32.9	8.6 (1.1)	1–14	34.6	5.7 (0.8)	2–11	22.9	7.5 (30.1)
16	7.4 (1.0)	1–14	33.4	10.5 (0.9)	6–17	47.7	7.8 (1.1)	1–17	35.4	8.6 (38.9)

3.2 | Host stress response

Cortisol levels in both control and infested fish were significantly higher at high (12 and 16°C) than at low (4 and 8°C) temperatures for all lice stages (Table S9). Sampling order, treatment (infested or not), and temperature all influenced plasma cortisol levels (Figure 1a). The response to a stressor (sampling) resulted in a different response in un-infested control fish than in those carrying lice. Sampling induced higher levels of cortisol in both groups, but whilst in control fish cortisol levels plateaued at 100 ng/ml, levels kept rising in infested fish with sampling order (Fish 1–7) (Figure 1a, Table S1). Further inspection of the data sorting samples according to temperatures revealed lower cortisol levels at 4°C and a leveling off in cortisol with sampling order in infested fish at 4°C, but not at higher temperatures (Figure 1b). The amount of cortisol at the chalinus stage was influenced by sampling order and treatment. Moreover, it was affected by interactions between treatment and temperature and between treatment, sampling order and temperature (Table S2). At the preadult stage, cortisol levels were influenced by sampling order, in addition to interactions between temperature and sampling order and between temperature, sampling order and treatment (Table S3). Lastly, at the adult stage cortisol was affected by sampling order, an interaction between sampling order and temperature and interaction between temperature, treatment and sampling order (Table S4). In infested fish cortisol levels were significantly affected by sampling order and interaction between temperature and sampling order. However, including numbers of lice per fish did not significantly improve the model at any stage (chalinus, $p = .07$; preadult, $p = .58$; adult, $p = .11$) and resulted in higher AIC values.

3.3 | Effect of temperature on transcription in control fish

The effect of temperature on the transcription of immune and wound healing genes in un-infested control fish was investigated. However, no consistent effect of temperature on CT values of the investigated genes was evident (Table S5).

3.4 | Significantly upregulated immune genes in lice-infested skin

Lice attachment sites had increased transcription of proinflammatory cytokines compared to both skin samples from control fish and lice-negative skin in infested fish. This included a significant upregulation of interleukin 1-beta for all temperatures at the chalimus stage in lice-positive samples compared to controls (Figure 2a). Except at 4°C this was also the case for sites of lice attachment compared to lice-negative sites in infested fish. A significant increase in transcription of interleukin 1-beta at the preadult and adult stage in lice-positive samples compared to both controls and lice-negative samples for most temperatures was evident (Figure 2b,c). Moreover, in infested fish there was an effect of temperature, with a lower transcriptional response at 4°C (chalimus stage) compared to higher temperatures (Figure 2a). However, contrary to that seen at the chalimus stage the highest upregulation of interleukin 1-beta at the preadult and adult stages was observed at 4°C. Tumour necrosis factor-alpha expression was significantly increased at all stages in infested fish (both in lice positive- and lice-negative samples) compared to controls at 16°C, but not at any of the lower temperatures (Figure 2d-f). Lastly, there was a significant upregulation of the cytokine interleukin 8 in lice-positive samples at all temperatures at

the chalimus stage, and for all temperatures at the preadult stage with exception of at 8°C relative to both lice-negative samples and controls (Figure 2g,h).

Temperature influenced the expression of the acute phase protein serum amyloid A, with a significant upregulation at 12 (adult stage) and 16°C (preadult stage) in lice-positive skin samples compared to controls (Figure 2i,j). SAA was also higher in lice-positive samples compared to lice-negative samples at 8°C (chalimus stage) (Table S8).

The expression of interleukin 4 increased in infested fish and was significantly higher at most temperatures and stages in lice-positive skin samples compared to controls (Figure 2k-m). Generally, the highest levels were observed at 16°C, and the lowest level of expression at the chalimus and preadult stage at 4°C (Lice-positive sample vs. controls). Additionally, a significant increase in expression in lice-negative samples compared to controls at 8, 12 (preadult stage) and 16°C (preadult and adult stage) was evident. Lastly, there was a significant difference in expression between lice-negative and lice-positive samples at chalimus (4, 8 and 16°C), preadult (16°C) and adult stage (4, 12 and 16°C).

3.5 | Significantly downregulated immune genes in lice-infested skin

Several gene transcripts in the skin were significantly decreased in infested fish. This included a significant reduction of a cluster of differentiation 8-alpha in lice-positive samples compared to controls at the adult stage for all temperatures except at 4°C (Figure 2n). The amount of downregulation of this gene at the site of lice attachment depended on temperature, being highest at 16 and lowest at 4°C. Relative to lice-negative samples a downregulation was seen at the sites of lice attachment at 16°C (preadult

TABLE 3 Sampling day post-infection (dpi) for the sampling of the different lice development stages at the four investigated temperatures

Temperature (°C)	Chalimus	Preadult	Adult
4	43	70	92
8	19	32	62
12	11	18	36
16	7	12	25

TABLE 4 Output from the glm model investigating differences in lice load between the stages at the investigated temperatures

Stage	Chalimus		Preadult		Adult		Temperature °C
	T-value	p-value	T-value	p-value	T-value	p-value	
Chalimus	NA	NA	1.8	0.07	0	1.0	4
Preadult	-1.8	0.07	NA	NA	-1.3	0.19	4
Adult	0	1.0	1.3	0.19	NA	NA	4
Chalimus	NA	NA	1.8	0.07	-1.1	0.265	8
Preadult	-1.8	0.07	NA	NA	-2.0	0.06	8
Adult	1.1	0.27	2.0	0.06	NA	NA	8
Chalimus	NA	NA	0.3	0.7638	-1.9	0.06	12
Preadult	-0.3	0.76	NA	NA	-2.2	0.03*	12
Adult	1.9	0.06	2.2	0.03*	NA	NA	12
Chalimus	NA	NA	2.1	0.047*	0.3	0.76	16
Preadult	-2.1	0.047*	NA	NA	-1.8	0.09	16
Adult	-0.3	0.76	1.8	0.09	NA	NA	16

*Denotes significant differences in lice load between the stages.

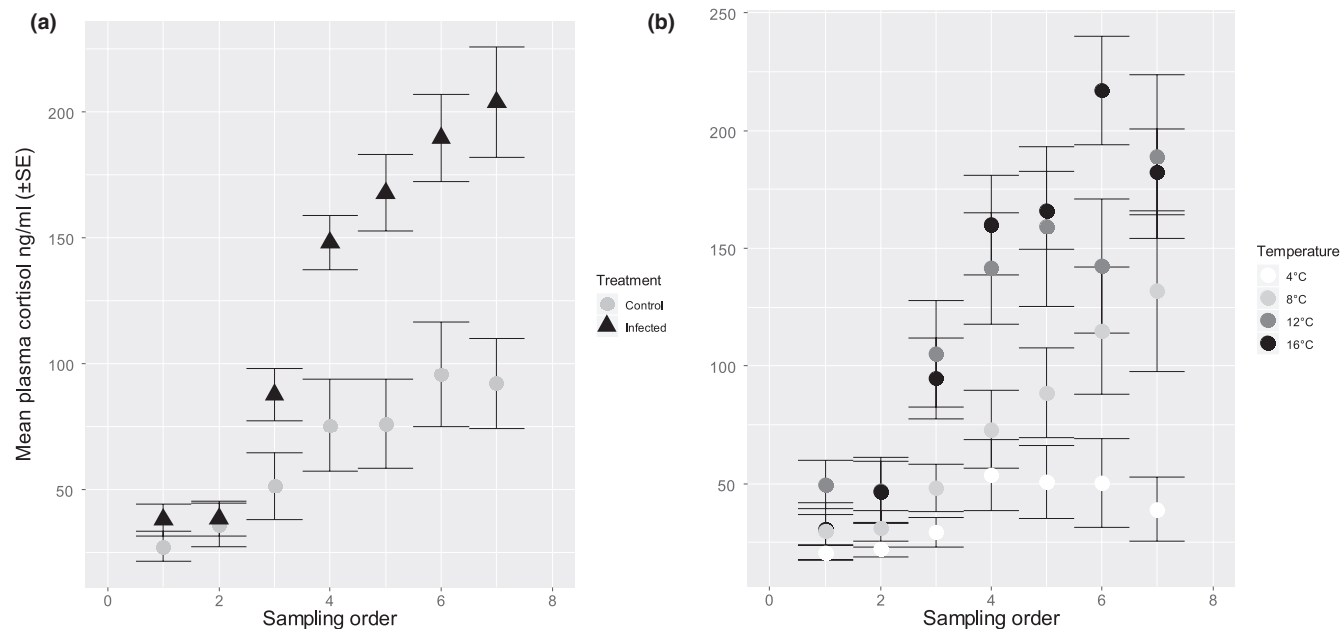


FIGURE 1 Mean plasma cortisol level (ng/ml) (\pm SE) in fish from all samplings depending on sampling order (fish 1–7), treatment (Infected vs. controls) (a) and as a function of temperature in infected fish (b)

and adult stage), in addition to at 12°C (adult stage). Contrarily, in lice-negative samples at 12°C at the preadult stage there was an upregulation compared to controls. In infested fish, the expression of immunoglobulin M deviated from that seen in controls, but the temperature did not affect transcription. A significant decrease in transcription in lice-positive samples compared to controls, at 4 and 12°C (chalimus stage), in addition to at 8 and 16°C (adult stage) (Figure 2o). On the contrary, it generally increased in lice-negative samples and significantly so at 8 and 16°C (chalimus stage) compared to controls. Hence, the transcription significantly differed in lice-positive compared to lice-negative samples at the chalimus stage (8, 12 and 16°C), preadult (12°C) and adult stage (4, 8 and 16°C). The expression of immunoglobulin T varied and was upregulated or downregulated depending on stage and temperature. However, the only significant difference in the site of lice attachment compared to the other treatment was at chalimus 12°C. Lastly, a significant reduction in transcription of interleukin 10 was found in lice-positive samples at 16°C (chalimus stage) and at 12°C (adult stage) compared to controls and at 12°C (adult stage) against lice-negative samples (Figure 2p,q). Moreover, at the site of lice attachment in infested fish the expression of this gene was enhanced at low temperatures.

3.6 | Immune genes with no significant change in expression in lice-infested skin

No difference in expression of cluster of differentiation 4, transforming growth factor-beta or in complement factor 3 between infested fish and un-infested controls was detected at any stage or temperature (Tables S6, S7).

3.7 | Significantly upregulated wound healing genes in lice-infested skin

Matrix metalloproteinase 9 was significantly upregulated in lice-positive samples compared to controls at 4, 8 and 16°C (chalimus stage), at 8 and 16°C (preadult stage) and at 12°C (adult stage) (Figure 2r,s). Compared to non-lice attachment sites in infested fish an upregulation in lice-positive samples at the chalimus stage occurred for all temperatures, at 4 and 16°C at the preadult and at the adult stage at 4°C (Table S8). The expression of fibronectin precursor significantly increased in lice-negative samples compared to controls at the chalimus stage at 8°C (Table S7).

3.8 | Significantly downregulated wound healing genes in lice-infested skin

Significant downregulation of collagen 10-alpha occurred in lice-positive samples at the adult stage for all temperatures compared to lice-negative samples and at 8°C compared to controls (Figure 3).

4 | DISCUSSION

Lice infestation affected the transcription of several immune and wound healing genes in the skin of Atlantic salmon and the response towards the parasite was also influenced by temperature for several of the investigated transcripts. Moreover, temperature affected lice performance and plasma cortisol levels. The latter was also influenced by sampling order and treatment.

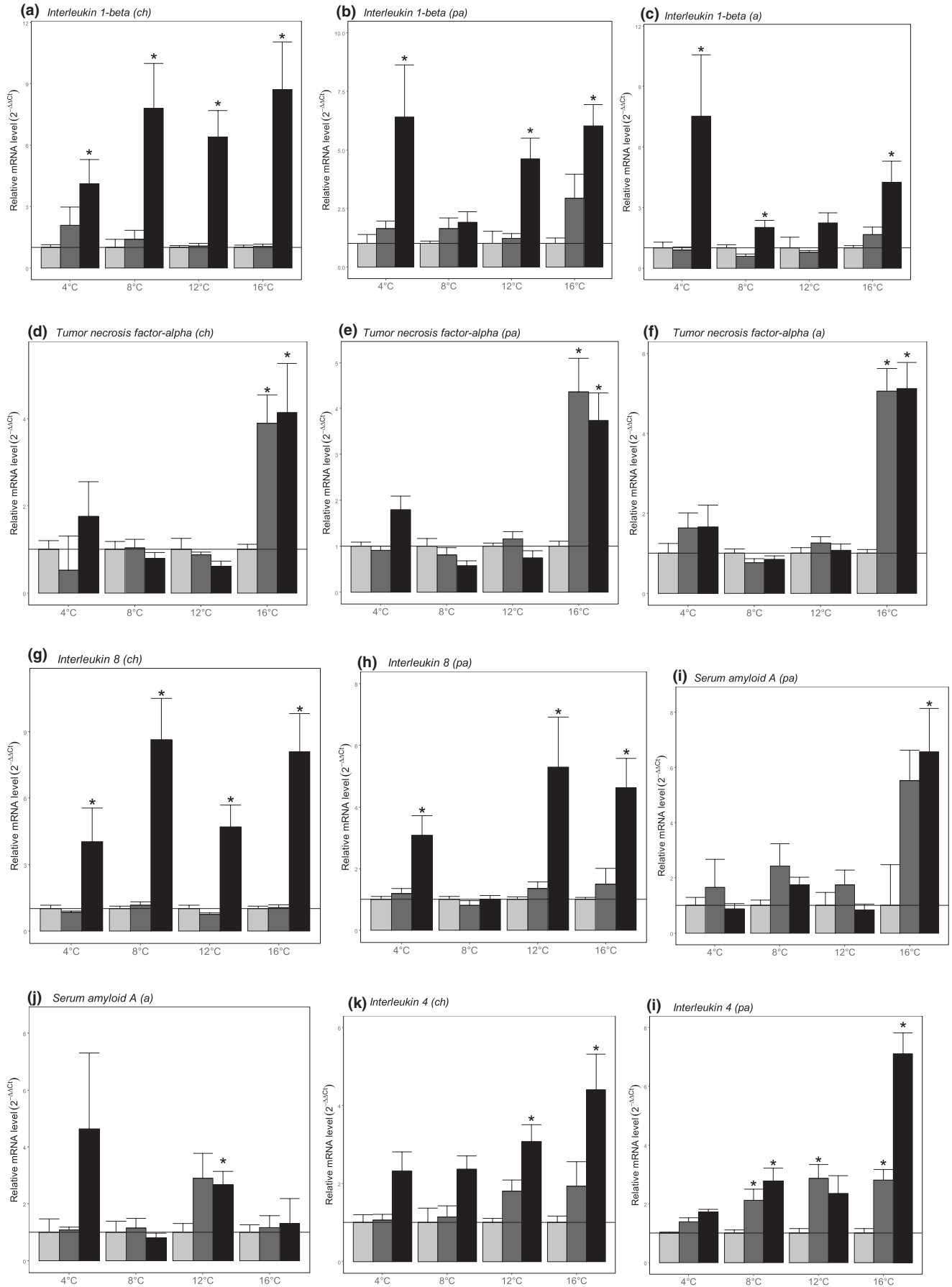


FIGURE 2 (Continued)

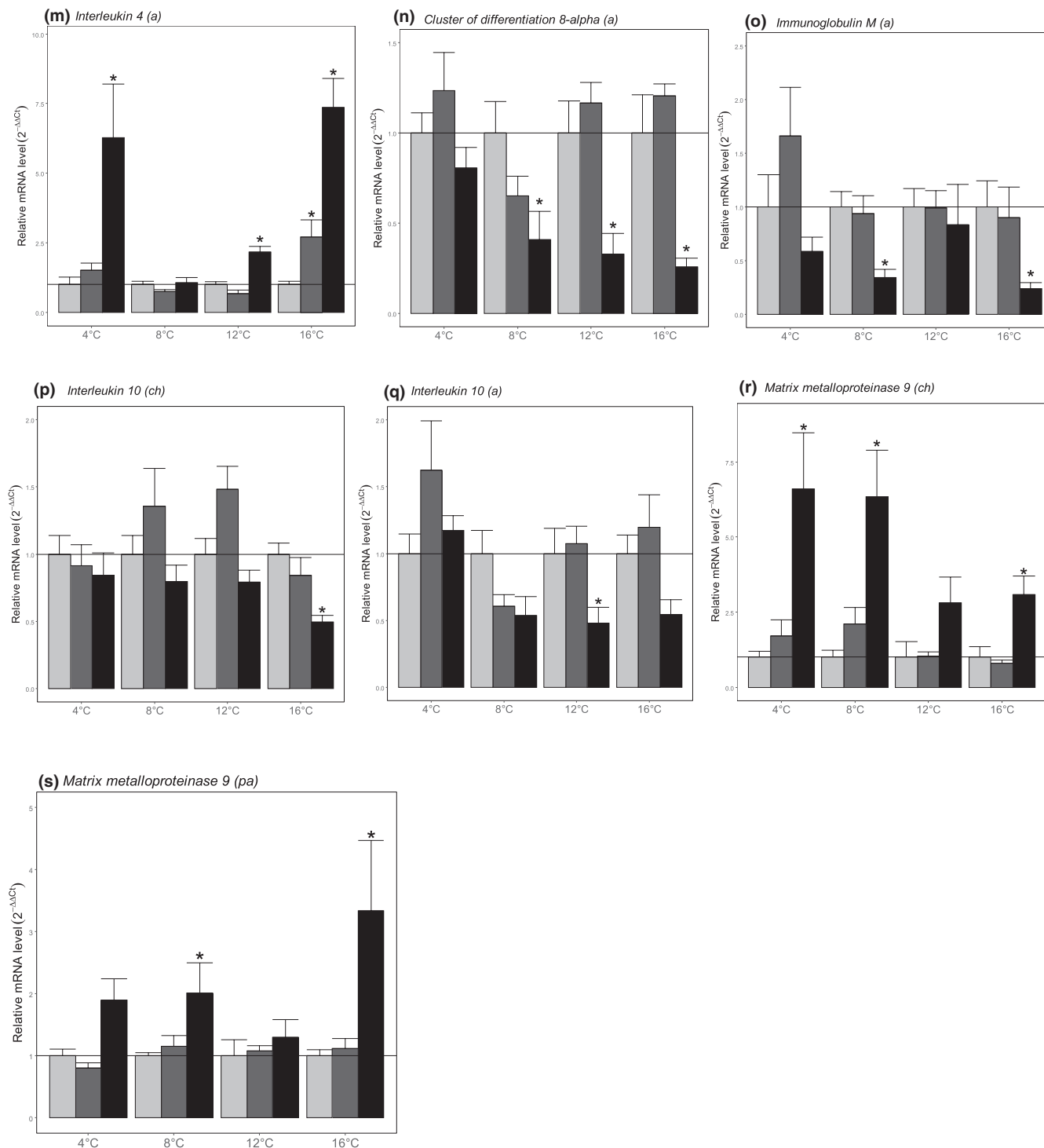


FIGURE 2 Relative mRNA levels (\pm SE) for selected immune and wound healing gene transcripts depending on temperature (4, 8, 12 and 16°C, respectively), sample (un-infested controls (light grey columns), lice-negative (dark grey columns) and lice-positive samples (black columns)) and lice development stage (ch = chalimus, pa = preadult and a = adult). The expression level is given as $2^{-\Delta\Delta CT}$ (\pm SE) in infested fish (lice-negative and lice-positive samples) compared to un-infested controls at the same temperature. The horizontal line is set at $y = 1$ in the figures. Statistically significant differences in expression relative to un-infested controls at the same temperature are denoted *

The reduction in lice survival and infestation success at low temperatures demonstrates that the overall effect of low temperatures is more detrimental to the louse than the host, despite reduced or delayed responses in several immune genes in the latter. This

therefore confirms findings in previous studies reporting decreased lice infestation success at low temperatures (Dalvin et al., 2020a; Hamre et al., 2019; Samsing et al., 2016), which could be caused by a reduction in energy reserves at the moult to the infective copepodid

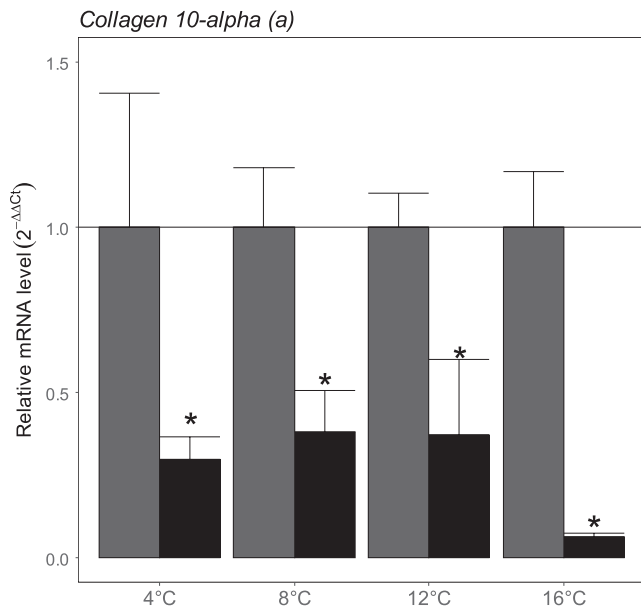


FIGURE 3 Relative mRNA levels (\pm SE) for collagen 10-alpha in lice-positive (black columns) and lice negative samples (grey columns) in infested fish depending on temperature (4, 8, 12 and 16°C, respectively) at the adult stage (a). The expression level is given as $2^{-\Delta\Delta CT}$ (\pm SE) in lice-positive samples compared to lice-negative samples in infested fish at the same temperature. The horizontal line is set at $y = 1$ in the figures. Statistically significant differences in expression in lice-positive compared to lice-negative samples at the same temperature are denoted*

stage (Skern-Mauritzen et al., 2020). The effect of temperature on lice performance reported herein may explain why lice abundances normally are lower at higher latitudes along the Norwegian coast (Jansen et al., 2012; Vollset et al., 2020). Hence, rising temperatures due to climate change are likely to result in higher infection pressure due to increased infectivity and shorter generation time of the parasite (Sandvik et al., 2021).

The higher increase in plasma cortisol levels in infested than in control fish in response to handling indicates that the lice infestation could affect their acute stress response even at relatively low intensities. Furthermore, the stress level was affected by temperature. The observed increase in cortisol levels in lice-infested fish in response to a stressor (handling), could decrease their ability to handle external stress and make them more susceptible to coinfections than control fish. Consequently, it is important to consider the effect of temperature when implementing delousing treatments that are likely to stress the fish (e.g., thermal and mechanical treatments). Enhanced cortisol levels have previously been reported in lice-infested fish, especially at high intensities and this could affect both osmoregulation and immune responses (Fjelldal et al., 2020; Mustafa et al., 2000; Ross et al., 2000). Suppressive effects of cortisol on host immune responses to salmon lice were demonstrated in Coho salmon supplied with cortisol implants (Johnson & Albright, 1992), however, increased cortisol levels mediated by cortisol implants in Atlantic salmon did not result in a higher number of salmon lice (Tadiso et al., 2011). Nevertheless, by downregulating immune genes

such as regulators of lymphocyte differentiation and reducing the upregulation of genes involved in wound healing (Tadiso et al., 2011), cortisol likely influences lice-induced pathology and immunological responses in the fish. In rainbow trout, suppressive effects of cortisol on the immune response against bacterial and fungal pathogens have been demonstrated (Pickering & Pottinger, 1989), but contrarily cortisol in feed decreased the number of *Argulus japonicus* on the same host species thereby suggesting enhanced immunity (Haond et al., 2003). Consequently, the effect of cortisol on host immune responses could be difficult to predict. However, these discrepancies could be caused by different kinds of stress, since acute stress is immune-stimulatory, while chronic stress is immune suppressive in fish (Tort, 2011).

Transcripts of several immune and wound healing genes in the skin were significantly differentially expressed locally at the site of lice attachment. However, there were fewer and less changes in lice-negative samples from infested fish compared to samples from non-infested control fish. These results therefore corroborate that immune response in salmonids against salmon lice results in more local than systemic responses (Dalvin et al., 2020a; Øvergård et al., 2018). The host's immune response to the parasite infestation was moreover dependent on temperature for several of the investigated genes. Thereby indicating that temperature could influence host immune responses to salmon lice and consequently susceptibility.

Levels of proinflammatory cytokines in infested fish were affected by temperature: This applied especially to tumour necrosis factor-alpha, where a significant upregulation in infested fish could only be detected at the highest tested temperature. Tumour necrosis factor-alpha is a proinflammatory cytokine associated with macrophages and enhances phagocytosis and respiratory burst activity against viruses, bacteria and parasites (Uribe et al., 2011). The increased expression has also been associated with reduced susceptibility to lice (Holm et al., 2015). Additionally, it is involved in the early phases of tissue repair (Braden et al., 2015), which could be important to heal wounds caused by the grazing activity of the louse. At lice-attachment sites, the expression of interleukin 1-beta increased relative to both controls and non-lice sites in infested fish. Furthermore, there was an effect of temperature on transcription of this gene, while it was lower at the chalimus stage, it was higher at the preadult and adult stage at 4°C relative to at higher temperatures. This could be caused by a delayed/weakened response early in the infestation at low temperatures and is congruent with findings in rainbow trout where low temperatures inhibited transcription of this gene (Zou et al., 2000). Interleukin 1-beta has several physiological functions in fish, including the regulation of immune responses (Secombes et al., 2001; Zou & Secombes, 2016). Enhanced transcription during salmon lice infestations proposes a role in the host immune response against the parasite (Braden et al., 2012, 2015; Øvergård et al., 2018). Lastly, an upregulation of interleukin 8 at lice attachment sites compared to the other treatments was evident, but the temperature did not affect the expression of this gene. Interleukin 8 is an important

proinflammatory mediator and attracts neutrophils, macrophages and lymphocytes to the site of infestation, and a rapid increase in interleukin 8 together with other proinflammatory cytokines in the skin beneath the louse is found in both Atlantic, Coho and sockeye salmon (Braden et al., 2015; Øvergård et al., 2018). Upregulation of tumour necrosis factor- α at the site of lice attachment only at the highest temperature, together with a diminished increase in expression of interleukin 1- β early in the infestation at the lowest temperature is indicative of impaired proinflammatory responses to salmon lice at low temperatures. Rapid proinflammatory responses are associated with lice rejection in several species (Braden et al., 2015; Jones et al., 2007), hence immune responses against salmon lice in Atlantic salmon could be impaired at low temperatures.

Increased transcription of the acute phase protein serum amyloid A at the site of lice attachment only at the two highest temperatures (12 and 16°C), suggests that the acute phase response to salmon lice is temperature dependent. Serum amyloid A is produced by macrophages in response to infections by a wide range of pathogens and is involved in opsonizing could interfere with pathogen functions and affects the production of both matrix metalloproteinases and cytokines (Chettri et al., 2014; Jensen et al., 1997). Acute-phase proteins including serum amyloid A have been linked to salmon lice resistance (Sutherland et al., 2014), which is corroborated by a higher increase in expression against lice in resistant Coho salmon compared to in more susceptible Atlantic and sockeye salmon (Braden et al., 2015). Enhanced transcription of serum amyloid A at the lice attachment site is also reported for rainbow trout (Dalvin et al., 2020b). Consequently, serum amyloid A is probably a part of the acute phase response to salmon lice in several species of salmonids and our findings show that this is temperature dependent, thereby potentially increasing susceptibility at low temperatures.

The complement system is important for the functioning of both the innate and adaptive parts of the immune system and consists of both plasma and cell-bound proteins (Chaplin, 2010). Amongst the central constituents is complement factor 3, which is expressed in the skin of Atlantic salmon and is involved in the activation of immune cells, recruitment of phagocytes and opsonization (Chaplin, 2010; Løvoll et al., 2007). Hence, it could be involved in host responses to salmon lice. However, we found no effect of treatment or temperature on the expression of this gene. This is congruent with previous findings in Atlantic salmon, where transcription of these genes was not significantly different in infested fish compared to uninfested controls (Holm et al., 2015). Contrary to our findings a reduction at the site of lice attachment is found in rainbow trout, indicating a suppression of the complement system in this species that could benefit the parasite (Dalvin et al., 2020b).

Enhanced expression of interleukin 4 in infested fish and most notably locally at the site of lice attachment suggests a role in the host's immune response to salmon lice. Transcription of interleukin 4 in infested fish is furthermore influenced by temperature and was highest at 16°C. Correspondingly the smallest upregulation was seen at the lowest temperature at both the chalimus and preadult stage

(no significant upregulation). However, at the adult stage transcription at the lowest temperature was elevated, suggesting a delayed response to infestation at low temperatures. The effect of temperature is supported by significant upregulation at 8°C but not at 4°C at the preadult stage at the site of lice attachment compared to uninfested controls. Interleukin 4 is associated with a specific subset of T-helper cells (Th-2) and enhances their proliferation and differentiation (Holm et al., 2015; Luckheeram et al., 2012). Interleukin 4 is highly expressed in the skin of salmonids (Takizawa et al., 2011) and a role in wound healing is also proposed (Braden et al., 2015), consequently it could be an important mediator of host responses to lice infestations. This is supported by an increase in expression of interleukin 4 transcripts at the site of lice attachment in rainbow trout (Dalvin et al., 2020b), Coho (Braden et al., 2015) and in Atlantic salmon at the copepodid stage (Øvergård et al., 2018). Furthermore, a significant upregulation of interleukin 4 mRNA in the most immune host, but not in the more susceptible Atlantic and sockeye salmon is indicative of a role in lice resistance (Braden et al., 2015). Thus, decreased expression of interleukin 4 in lice-infested fish early in the infestation at low temperatures could imply impaired Th-2 immunity.

No expressional change of the glycoprotein cluster of differentiation 4 was seen between the treatments at any temperatures or stage. A cluster of differentiation 4 is associated with T-helper cells, involved in regulating both cellular and humoral immune responses (Chaplin, 2010; Luckheeram et al., 2012). No difference in the transcriptome of the cluster of differentiation 4 in infested fish indicates a lack of influx of this subset of T-cells in the skin of lice-infested fish compared to controls and this is in agreement with findings in rainbow trout (Dalvin et al., 2020b). However, Skugor et al. (2008) and Tadiso et al. (2011) previously reported increased transcription of a cluster of differentiation 4 in the skin of infested Atlantic salmon, proposing involvement in host immune responses against lice. This discrepancy could be caused by variation in host physiology or other factors mediating the transcriptomic response in host skin (Tadiso et al., 2011) and/or differences in sampling regime. Contrarily, a reduction in the cluster of differentiation 8- α was evident, indicating a reduction of cytotoxic T cells at the site of lice attachment at the adult stage. The expression of this gene is also depending on temperature, with higher downregulation in infested fish at higher temperatures. Thus, fish infested with salmon lice at high temperatures could have a reduction in cytotoxic T-cells compared to those kept at lower temperatures. A similar reduction in the cluster of differentiation 8 transcripts at the site of lice attachment is reported for rainbow trout (Dalvin et al., 2020b) and in Coho, Atlantic and sockeye salmon in a study by Braden et al. (2015). Enhanced transcription of this gene increases antiviral responses at the site of attachment (Braden et al., 2015). Consequently, fish infested with adult salmon lice at high temperatures could have fewer cytotoxic T-cells in the skin beneath the louse and therefore be more susceptible to viral diseases than those kept at lower temperatures.

Immunoglobulins are involved in humoral immune responses and in fish three different isotypes are found either on the surface

of B-cells or secreted as antibodies (Hordvik, 2015). The most numerous, immunoglobulin M is involved in both innate and adaptive immune responses (Mashoof & Criscitiello, 2016). Expression of immunoglobulin M was significantly reduced in lice-positive samples at several stages and temperatures. Contrarily, an upregulation of immunoglobulin M transcripts in lice-negative samples compared to controls was evident at 8 and 16°C. A reduction at the site of lice attachment is previously reported for several susceptible salmonid species, including rainbow trout (Dalvin et al., 2020b), Atlantic and sockeye salmon (Braden et al., 2015). Interestingly, expression of immunoglobulin M is not reduced in resistant Coho salmon (Braden et al., 2015), indicating that B-cells or secreted immunoglobulins at the site of lice attachment could be involved in lice resistance. The immunoglobulin T isotype is specific for fish and is found especially in mucosal surfaces to protect against infections (Hordvik, 2015). The expression of immunoglobulin T was only reduced locally at lice attachment sites at 12°C (chalimus stage). Aberrant expression of the gene beneath the louse has been reported for both rainbow trout (downregulated) (Dalvin et al., 2020b) and early in the infestation in Atlantic salmon (upregulated) (Tadiso et al., 2011). This discrepancy could be caused by differences in the time of sampling and where the sample is taken on infested fish since only the former study took samples at the site of lice attachment and responses tend to be local. An important role of immunoglobulin T against pathogens is corroborated by findings in other host–parasite systems. Trout infected with intestinal parasites had a high number of immunoglobulin T positive B-cells and a role in protection against microbes is also proposed (Ángeles Esteban, 2012; Mashoof & Criscitiello, 2016). Reduction in immunoglobulin T transcripts and/or the lack of upregulation at the site of lice attachment, suggests little attraction or activation of these specific B-cells in the skin of lice-infested fish and could play a role in lice resistance.

Dysregulation of host immune responses could also be involved in susceptibility to salmon lice (Braden et al., 2020). Regulatory cytokines are important to mount appropriate immune responses against pathogens without inflicting excessive damage to the host. Interleukin 10 is a pleiotropic cytokine involved in regulating immune responses by inhibiting differentiation of monocytes, reduce phagocytosis, suppressing the expression of genes coding for MH class II molecules (Th-2 response) and proinflammatory cytokines (Chaudhry et al., 2011; Luckheeram et al., 2012; Rebl & Goldammer, 2018). Significant local downregulation of this cytokine at high temperatures (12 and 16°C) at the adult and chalimus stage suggests an effect of temperature on gene expression. Moreover, this is supported by the fact that the transcription is higher at low temperatures for all stages. Hence, decreasing levels of this cytokine with increasing temperatures could lead to excessive inflammatory responses at high temperatures in infested fish. Transforming growth factor-beta is another multifunctional regulatory cytokine, which both increases inflammation early in the infection and later suppresses inflammatory responses (Qi et al., 2016). We found no difference in the expression of this cytokine between treatments or temperatures. This is in agreement with findings by Braden

et al. (2015) who reported no change in transcription between treatments in Atlantic salmon. However, in the resistant Coho salmon transforming growth factor-beta was upregulated in infested fish together with interleukin10, suggesting that both these regulatory cytokines have a role in lice resistance in this species. Deviating transcription of transforming growth factor-beta has been associated with infections by other pathogens and studies in both trout and striped bass report a reduction in expression of this gene to combat pathogens (Rebl & Goldammer, 2018).

By feeding on the blood, skin and mucus, lice especially at the mobile stages inflict wounds on their host. Fish live in an environment rich in pathogens, so it is important for the host to rapidly repair these damages to avoid secondary infections (Uribe et al., 2011). Moreover, repairing the skin is also important to maintain the osmotic balance (Fjellidal et al., 2020). The skin health of Atlantic salmon is compromised at low temperatures and wound healing is delayed at four compared to at 12°C (Jensen et al., 2015). However, no effect of temperature on the transcription of genes involved in wound healing was evident in our study, but there was an effect of treatment. Most noticeable was a local increase in transcription of matrix metalloproteinase 9 at the site of lice attachment. Matrix metalloproteinases are involved in tissue remodeling of the extracellular matrix (Braden et al., 2020) and dysregulation of matrix metalloproteinases in lice-infested fish is associated with chronic wounds (Braden et al., 2012; Skugor et al., 2008). Activation of matrix metalloproteinase 9 at the site of lice attachment is earlier reported in Atlantic, Coho and sockeye salmon (Braden et al., 2015). The expression of Collagen 10-alpha was diminished at the adult stage for all temperatures in lice-positive samples compared to lice-negative samples in infested fish. Collagens are important parts of the extracellular matrix and important for wound healing and remodeling of tissue in fish (Castillo-Briceño et al., 2011). A reduction in the expression of this gene has also previously been found in lice-infested Atlantic salmon (Skugor et al., 2008). Together with our findings, this indicates that the presence of adult lice downregulates collagen, which could impair wound healing at the site of lice attachment. This could potentially be beneficial for the lice by prolonging the access to tissues beneath the epidermis. There was no difference in fibronectin expression between lice attachment sites in infested fish and controls. However, a significant increase in transcription was seen at 8°C in lice-negative samples compared to controls. This is incongruent with finding by (Skugor et al., 2008), who found a downregulation in this gene both early and late in the infestation at the site of lice attachment in Atlantic salmon, suggesting that expression could depend on the stage of the parasite or duration of the infestation.

We found that low ambient temperatures negatively affected the transcription of several immune genes in Atlantic salmon, but this did not result in more lice. On the contrary, the lice infestation success and survival in the host were reduced at low temperatures, suggesting that low temperatures are more detrimental to the lice than the host despite the effect on the host immune system. Consistent stage-specific transcriptional changes were evident at the adult stage, but only for two of the investigated genes.

This included a local downregulation at the site of lice attachment of cluster of differentiation 8 and collagen 10-alpha relative to un-infested controls in the former and compared to lice-negative samples in infested fish in the latter. The immune response of Atlantic salmon against salmon lice is weak and delayed compared to other salmonids resulting in low levels of resistance to infestations (Braden et al., 2020). The inefficient immune response against lice, could in parts be due to the immunomodulatory capabilities of the parasite (Braden et al., 2020; Fast et al., 2007; Øvergård et al., 2016, 2018), but the mechanism is currently not known (Dalvin et al., 2021; Eichner et al., 2015). However, the lack of an efficient immune response towards the ectoparasitic lice may also result from selection for tolerance rather than resistance in Atlantic salmon. To investigate this, more research on this economically important host-parasite interaction is needed to better understand how different ecological conditions affect the outcome of infestations.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

AUTHOR CONTRIBUTIONS

S.D. designed the study. S.D. and S.M. performed the experimental procedures in the fish facility and S.M. and M.S.U. performed the experimental procedures in the laboratory. MSU analysed the data and wrote the first draft. D.S. and S.M. provided critical revisions and comments to the manuscript.

DATA AVAILABILITY STATEMENT

Data are available as supplementary material.

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