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# Rainbow trout *Oncorhynchus mykiss* skin responses to salmon louse *Lepeophtheirus salmonis*: From copepodid to adult stage

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ABSTRACT

The marine crustacean *Lepeophtheirus salmonis* (salmon louse) is a common ectoparasite of wild and farmed salmonids. The parasite has a complex ontogeny comprising eight instars. The planktonic copepodid stage settles on host skin and pass through five instars to reach the adult stage. The present study comprises an experimental infestation of *Oncorhynchus mykiss* (rainbow trout) with salmon lice and describes histopathology and host immune responses in skin beneath the louse at multiple time points encompassing all louse developmental stages. Each fish was exposed to 80 infective copepodids, a mean no. of 32 parasites reached the preadult I stage whereas a mean no. of 11 parasites reached the adult stage. A progression in the severity of cutaneous lesions was observed, and levels of immune gene transcripts at the attachment site revealed a dynamic response, initially related to innate immunity. Later, immune cells accumulated in the dermis concomitant with a moderate decrease in levels of transcripts characteristic of both innate and adaptive immune responses. The present study also demonstrates that the cutaneous immune response was mainly induced at lice affected sites, while non-affected skin resembled the skin of untreated control. This indicates that the skin cannot be regarded as a uniform organ and requires careful sampling at all salmon louse stages.

## 1. Introduction

Infestations with the salmon louse (*Lepeophtheirus salmonis*), a marine copepod ectoparasite of salmonid fishes, represent a major challenge to aquaculture in the Northern Hemisphere [1,2]. Both Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) are susceptible to the salmon louse [2–4], where the louse damages the skin through attachment and feeding, in which can cause stress, secondary infections and osmotic imbalance [5–7]. Hence, if left unchecked, the parasite poses a threat to both farmed and wild salmonids [8–10]. Due to resistance towards the majority of chemotherapeutants in use [11], there is a need for novel control measures such as vaccines, functional feeds or fish breeding for louse resistance. Especially for vaccine development, an understanding of the host-parasite interaction is a prerequisite.

The lifecycle of the salmon louse comprises eight instars. After two planktonic nauplius stages, the salmon louse copepodid attaches to fish skin, where the louse passes through two chalimi and two preadult stages before the final moult to a sexually mature adult [12–14]. The copepodid attaches to the fish by appendages [15]. During the first moult it extrudes a frontal filament, which fixates the chalimus to a restricted part of the skin. Preadults and adults, on the other hand, adhere to the fish by the aid of a suction cup shaped cephalothorax, allowing free movement over the host surface [13,16]. While the small immobile juveniles cause limited skin damage [17], the mobile preadults and adults appear to be more virulent [5,18,19].

Ectoparasites may modulate the host immune system with the aid of exocrine gland products, where, for the salmon louse, prostaglandins and proteases have been suggested to be among important effector molecules [20–26]. All parasitic salmon louse stages possess salivary glands [26]. In addition, preadults and adults have a set of exocrine glands that empty along the margins of the cephalothorax where the louse adheres to host skin. Hence, with a complex system of exocrine glands, mobile lice may exert a more sohisticated host immunomodulation compared to chalimi and copepodids.

The immunological responses associated with salmon lice

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infestations have been studied for several salmonid species, some fully susceptible, others moderately or highly resistant [4,27,28]. However, it is challenging to extract coherent knowledge from the published literature due to a high variation in the applied methodologies, including sampling schemes, infection intensities, parasite stage and the transcripts analyzed. In some studies fish were infested with copepodids, while in others, adult lice were placed on individual fish [29–33]. The first method reflects the natural infestation, whereas the latter provides better control of lice numbers on each fish. Additionally, the sampling methods, hereunder type and amount of tissue sampled, sampling location, and whether skin samples were retrieved from infested areas or not, vary among studies. Many studies, in particular those performed with juvenile lice, have focused on the general skin immune response away from the site of infestation, and on effects on primary and secondary immune organs [28,30,34–39]. Some results, however, imply that the cutaneous immune response is restricted to the attachment site of the salmon louse, while not prominent in unaffected skin away from the parasite [29,32,33,40]. Despite the well known ontogenetic changes in mode of attachment, louse feeding activity, and gland development, it remains unclear in what way these factors affect local immune responses during the course of an infestation. The aim of this study was thus to investigate the temporal changes in histopathology and immune gene transcription induced by the salmon louse in skin of rainbow trout by comparing responses precisely at the parasite attachment site with that at unaffected skin. Fish infested with a salmon louse cohort were examined throughout parasite development from the copepodid stage, via chalimi, to preadult and adult lice including egg bearing adult females to monitor the progression of the immune response in the fish.

## 2. Materials and methods

### 2.1. Animals and experimental set-up

All procedures involving animals were performed according to the Norwegian Animal Welfare Legislation. These experiments were approved by the Animal Ethics Committee by the Norwegian Food Safety Authority (approval number 4538) at University of Bergen. Rainbow trout were hand fed on a commercial diet and reared in seawater with a salinity of 34.5 ppt with a temperature of 12 °C. A total of 112 fish, on average 330 g ( ± 80 g) were distributed between four tanks. After two weeks of acclimatization, infestation with salmon lice was conducted by lowering the water level in two tanks and thereafter slowly adding copepodids (80 copepodids/fish). The other two tanks that contained control fish were exposed to the same procedure but without addition of copepodids. Salmon lice were obtained from a laboratory stock (LsOslofjord) [41]. Fish were sampled from all tanks eight times on the following days post infestation (dpi): 2, 5, 7, 10, 16, 24, and 41. The number and stage of salmon lice on each fish were recorded. At each time point, four fish from each tank were sampled, giving eight fish in each group (a total of 16 fish per sampling).

### 2.2. Sampling procedure

Immediately before sampling, fish were sedated in a bucket containing seawater with 100 mg/l benzocaine and 10 mg/l metomidate and subsequently euthanized with a sharp blow to the head. Blood was drawn prior to tissue sampling to avoid blood contamination of skin samples.

By dissection using a scalpel, skin samples were excised, and subcutaneous tissue removed. Samples were obtained only from skin with scales, excluding the lateral line system. The preferred site of louse attachment changes during lice development; sampling sites were adjusted accordingly. When lice were at the copepodid or chalimus stages, samples were taken ventral to the lateral line, between the pectoral and pelvic fins. At the preadult and adult stages, samples were taken from areas where many lice were feeding, caudal to the dorsal or adipose fin. From infested fish, two different sample types were collected: one of skin directly under a louse (attachment site), and one immediately adjacent (non-attachment site). From control fish, skin samples were always taken from the equivalent anatomical site as those from infested fish. Samples taken when lice were at the copepodid or chalimus stages were small. Thus, sample pools were needed to obtain sufficient amounts of tissue for RNA purification. Each pool consisted of three anatomically close samples from a single fish.

For quantitative PCR (qPCR), individual or pooled samples were placed in RNAlater (Sigma-Aldrich) or in 4% paraformaldehyde in phosphate buffered saline (PBS) for immunohistochemical and histological analysis. Finally, to confirm similar infestation levels on individual fish, counting of lice was performed by visual inspection. Early stages of lice are inherently difficult to count due to the small size of the parasite and the coloring of the fish. Accordingly, early lice counts were mainly performed to confirm similar infestation levels on individual fish.

### 2.3. Histological analysis

Skin samples from 2 to 3 individuals per treatment group and at each time point were processed. These were fixed in neutral formalin for 24 h, transferred to EtOH (70%) until dehydration in graded EtOH series (70–99.9%) with a final step in xylene substitute (Thermo scientific) before embedding in paraffin and sectioning (4 μm) using a Leica RM2135 microtome. Following mounting on slides, sections were deparaffinized with xylene substitute (Thermo scientific) and rehydrated to water through a graded series of ethanol. Sections were stained with Mayer's hematoxylin (Dako), with Alcian blue (1%, in 3% Acetic acid, Sigma Aldrich) to detect acid mucopolysaccharides, or used for immunohistochemistry. Slides were qualitatively assessed by visual examination.

### 2.4. Immunohistochemistry

To quench endogenous peroxidase activity, slides were incubated with 1.5% $H_2O_2$ in Tris-buffered saline (TBS) (Dako, Denmark) for 10 min. Antigen retrieval was performed by boiling in 10 mM Tris, 1 mM-EDTA (pH 9.0) for 15 min, cooled to RT where after sections were blocked in 2% BSA in TBS. Sections were then incubated o/n at 4 °C with primary monoclonal antibodies raised in mice against rainbow trout Serum amyloid A (SAA) diluted 1:5 in TBS with 1% BSA, according to previous descriptions [42]; Heinecke and Buchmann, 2013). Negative control slides were prepared by incubation in 1% BSA with no primary antibody. Staining of primary antibody binding sites was amplified using the UltraVision Quanto Detection System HRP (Thermo Scientific) and AEC Chromogen kit (Sigma). Finally, slides were counterstained in Mayer's hematoxylin (Dako), and mounted under cover glass using Aquatex (Merck). Slides were qualitatively assessed by visual examination.

### 2.5. Total RNA purification, cDNA synthesis and qPCR

Total RNA was isolated following a combined Tri reagent (Sigma Aldrich) and RNeasy (Qiagen) protocol as follows: each skin sample was added 1 ml TRI reagent and homogenized using 5 mm stainless steel beads (Qiagen) and a TissueLyserII (Qiagen) for 3 min at 30 Hz. Thereafter, samples were incubated for 5 min at room temperature, added 200 μl of chloroform (Sigma–Aldrich), and then vortexed and centrifuged at 4 °C for 15 min. The water phase was withdrawn and mixed with one volume of 70% ethanol, and RNA was further extracted using the RNeasy protocol. Samples were DNase treated on column, and total RNA was stored at −80 °C until further use. cDNA synthesis was carried out using the AffinityScript qPCR cDNA Synthesis Kit (Stratagene) according to the supplier's recommendations, adding 1 μg total RNA and oligo (dT) (10 ng/μl) primers in a total volume of 10 μl.

**Table 1**
Sequences of primers and TaqMan probes used in qPCR, and accession numbers of the targeted gene transcript. *Acidic ribosomal protein* (*arp*), *beta-actin* (*β-actin*), *elongation factor 1alfa* (*elf1α*), *complement component 3–4* (*c3-4*), *cathelicidin 2* (*cath2*), *cluster of differentiation 4* (*cd4*), *cluster of differentiation 8 alpha* (*cd8α*), *GATA binding protein 3* (*gata3*), *immunoglobulin M* (*igm*), *immunoglobulin D membrane bound* (*igdm*), *immunoglobulin D secreted* (*igds*), *immunoglobulin T* (*igt*), *interleukin 1beta* (*il1β*), *interleukin 4/13A* (*il4/13a*), *interleukin 6* (*il6*), *interleukin 8* (*il8*), *interleukin 10* (*il10*), *inducible nitric oxide synthase* (*inos*), *major histocompatibility complex class I* (*mhc1*), *major histocompatibility complex class II* (*mhc2*), *serum amyloid A* (*saa*), *transforming growth factor beta* (*tgfβ*), *tumor necrosis factor alfa* (*tnfα*).

| Name | Forward primer | Reverse primer | Probe | Accession no |
|---|---|---|---|---|
| *arp* | GAAAATCATCCAATTGCTGGATG | CTTCCCACGCAAGGACAGA | CTATCCCAAATGTTTCATTGTCGGCGC | AY505012 |
| *β-actin* | ACATCAAGGAGAAGCTGTGCTAC | TACGGATGTCCACGTCACAC | CCTCTCTGGAGAAGAGCTACGAGCTG | AB196465 |
| *elf1α* | ACCCTCCTCTTGGTCGTTTC | TGATGACACCAACAGCAACA | GCTGTGCGTGACATGAGGCA | AF498320 |
| *c3-4* | ATTGGCCTGTCCAAAACACA | AGCTTCAGATCAAGGAAGAAGTTC | TGGAATCTGTGTGTCTGAACCCC | AF271080 |
| *cath2* | AAAGATTCCAAGGGGGGT | CAAAGGGTGTGTTGTGCTGT | GCTCTCGTCCTGGGTTTGGCTCC | AY360356 |
| *cd4* | CATTAGCCTGGGTGGTCAAT | CCCTTTCTTTGACAGGGAGA | CAGAAGAGAGAGCTGGATGTCTCCG | AY973028 |
| *cd8α* | ACACCAATGACCACAACCATAGAG | GGGTCCACCTTTCCCACTTT | ACCAGCTCTACAACTGCCAAGTCGTGC | AF178054 |
| *gata3* | TCCTGGAGAGAGGGATGAAA | AGCCCGAGACCTATAGCACA | GGCCTTCACTTTCGCCTGCT | FM863826 |
| *igm* | CTTGGCTTGTTGACGATGAG | GGCTAGTGGTGTTGAATTGG | TGGAGAGAACGAGCAGTTCAGCA | BT059185 |
| *igdm* | CAGGAGGAAAGTTCGGCATCA | CCTCAAGGAGCTCTGGTTTGGA | CCACACCACACAGACTCTGGCCCTGAA | JQ003979 |
| *igds* | TGGCACGCCAGGATTTGAC | TCAGAATTGAGTGAACGGACAGACA | CCACACCACACAGACTCTGGCCCTGAA | AY870262 |
| *igt* | AGCACCAGGGTGAAACCA | GCGGTGGGTTCAGAGTCA | AGCAAGACGACCTCCAAAACAGAAC | GQ907004 |
| *il1β* | ACATTGCCAACCTCATCATCG | TTGAGCAGGTCCTTGTCCTTG | CATGGAGAGGTTAAAGGGTGGC | AY617117 |
| *il4/13a* | ATCCTTCTCCTCTCTGTTGC | GAGTGTGTGTGTATTGTCCTG | CGCACCGGCAGCATAGAAGT | AB574337 |
| *il6* | GGCAGACAGGTCCTCCACTA | GGCAGACAGGTCCTCCACTA | CCACTGTGCTGATAGGGCTGG | DQ866150 |
| *il8* | AGAATGTCAGCCAGCCTTGT | TCTCAGACTCATCCCCTCAGT | TTGTGCTCCTGGCCCTCCTGA | AJ279069 |
| *il10* | CGACTTTAAATCTCCCATCGAC | GCATTGGACGATCTCTTTCTTC | CATCGGAAACATCTTCCACGAGCT | AB118099 |
| *inos* | ACCAGAAGGAGGGTCACTT | TGGGTGAGGGTGATGCCAA | ATGTGTGTGGGGGTGTGAACATGG | AJ300555.1 |
| *mhc1* | TCCCTCCCTCAGTGTCT | GGGTAGAAACCTGTAGCGTG | CAGAAGACCCCCTCCTCTCCAGT | AY523661 |
| *mhc2* | TGCCATGCTGATGTGCAG | GTCCCTCAGCCAGGTCACT | CGCCTATGACTTCTACCCCAAACAAAT | AF115533 |
| *saa* | GGGAGATGATTCAGGGTTCCA | TTACGTCCCCAGTGGTTAGC | TCGAGGACACGAGGACTCAGCA | AM422446 |
| *tgfβ* | TCTGAATGAGTGGCTGCAAG | GGTTTCCCACAATCACAAGG | CTGGAGAGGAGCAGGGATTCCAAT | X99303 |
| *tnfα* | GGGGACAAACTGTGGACTGA | GAAGTTCTTGCCCTGCTCTG | GACCAATCGACTGACCGACGTGGA | AJ277604 |

Samples were diluted 1:10, and stored at −20 °C until use. qPCR was performed in an ArialMx Real-Time PCR system (AH Diagnostics). All assays (except *il6*) were run in 12.5 µl reactions containing 6.25 µl ready master mix (Brilliant III Ultra- Fast master mix, AH Diagnostics), 1 µl of a mixture consisting of forward primer (10 µM), reverse primer (10 µM) and TaqMan Probe (5 µM), 2.75 RNase/DNase free $H_2O$ and 2.5 µl template. Analysis of *il6* was run in 20 µl reaction with the same concentrations of reactants as above apart from the template, where 8 µl was added. Primers and probes employed are listed in Table 1.

Cycling conditions for qPCR were 94 °C for 10 min, followed by 40 cycles of 94 °C for 10 s and 60 °C for 15 s. In addition to experimental samples, all assays were also tested using RT-minus, no template and negative (RNase free $H_2O$) controls. The mRNA level was analyzed according to the simplified $2^{-\Delta\Delta Ct}$ method [43] as all qPCR assays had efficiencies of 100 ± 5%. Three reference genes were applied, *β-actin*, *elongation factor- 1α* (*elf1a*) and *acidic ribosomal protein* (*arp*). The reference gene stability was analyzed with the Normfinder software [44]; stability values obtained were 0.027, 0.019, 0.024 and 0.004 for *β-actin*, *elf1a*, *arp* and the geometric mean of the 3 genes, respectively (low value, high stability). Results are presented as fold increase/decrease in infested group compared to control group at each sampling time point. Changes in the threshold cycles (ΔCt) value were calculated as differences between RNA levels of the gene of interest and the geometric mean of the three reference genes. ΔΔCt values were calculated as differences between the ΔCt of the samples in the two groups from infested fish (non-attachment and attachment site) and the average ΔCt of the uninfested control group. Minimum two-fold difference in mRNA level were considered substantial and differences between the groups were tested with a two-tailed *t*-test ($p < 0.05$). Differences of fold regulations were only considered significant if both requirements were fulfilled. Tank effects were observed for a few samples and then tanks were treated statistically as independent units and only considered significant if both tanks were significantly different. This only affected *il8* results in non-attachment samples at 24 dpi. Here expression of *il8* was not significantly different in infested fish in attachment and non-attachment sites in one tank and hence was not considered significant.

## 3. Results

### 3.1. Progression of infestation

Due to their small size and the pigmentation of fish epidermis, copepodids and chalimi are difficult to count. Accordingly, early lice counts were mainly performed to confirm similar infestation levels on individual fish. Hence, an accurate count was first performed when the lice reached the preadult I stage. The registered numbers of preadult I lice were similar in both tanks (mean no. of 32 parasites per fish) and reflected a copepodid infestation success of approximately 40% (as a dose of 80 copepodids per fish was used for infection). At the last sampling, a mean no. of 11 adult lice per fish remained (Fig. 1). Salmon lice developed at a similar rate as on Atlantic salmon [45], and lice from all parasitic stages were collected during the trial (Table 2).

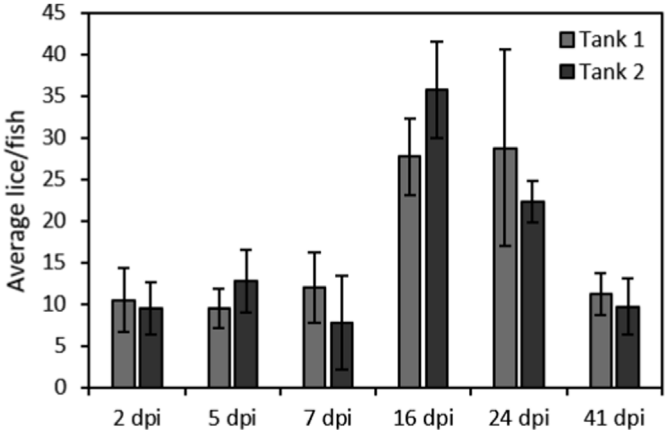


**Fig. 1.** Average number of salmon lice per fish ( ± SD) in each tank (1 and 2) at different days post infestation (dpi).

**Table 2**
The time points sampled (days post infestation – dpi) with the corresponding life stage reached by the majority of the collected salmon louse. At 24 dpi, female lice were in the preadult II stage and male lice in the adult stage. At 41 dpi, the adult female lice were fully mature carrying their first set of egg strings.

| | 2 dpi | 5 dpi | 7 dpi | 10 dpi | 16 dpi | 24 dpi | 41 dpi |
|---|---|---|---|---|---|---|---|
| Stage | copepodite | chalimus I | chalimus I | chalimus II | preadult I | preadult II, adult | adult |

Figure 1

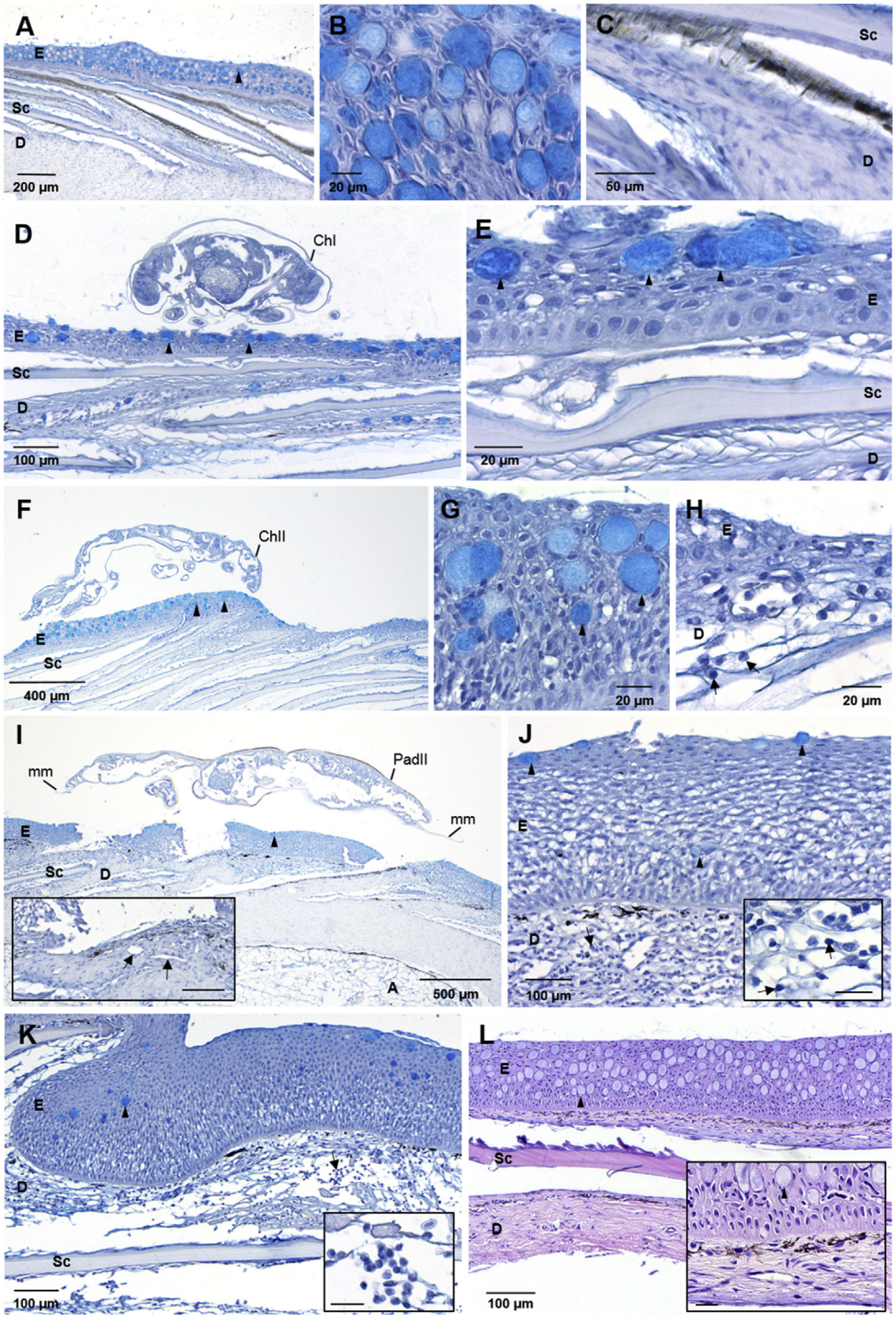


**Fig. 2.** Histopathology of rainbow trout skin during salmon louse infestation. Micrographs are from paraffin sections. (A–K) stained with hematoxylin and Alcian Blue, (L) stained with hematoxylin and eosin. (A) Skin from control fish at 10 days post infestation (dpi) with normal histological features. (B) Epidermis of control fish exhibiting numerous mucuos cells. (C) Dermis of control fish displaying normal connective tissue. (D) Louse attachment site (7 dpi). An irregular and rough epithelial surface with cell debris indicates erosion of the epidermis beneath the louse. Note that mucuos cells are still present at this stage of infestation. (E) Louse attachment site (7 dpi) displaying epidermal erosion, presence of mucous cells and a normal dermis, lacking immune cell infiltration. (F) Louse attachment site (10 dpi). Erosion of the epidermis is apparent anterior to the chalimus. (G) Epidermis at louse attachment site (10 dpi) showing mucuos cells. (H) Epidermis and outer dermis at louse attachment site (10 dpi) showing considerable epidermal erosion and some sparse immune cells in dermal connective tissue. (I) Louse attachment site (24 dpi) with a preadult II louse. In areas beneath the marginal membranes, by which the louse attaches, epidermal erosions are evident. A more severe lesion is seen where the mouth tube of the louse has penetrated the epidermis. The insert with higher magnification shows dermal ulceration and underlying veins (arrows). Scale bar 100 µm. (J) Epidermis and dermis (stratum spongiosum) at louse attachment site (24 dpi). Note immune cell infiltration in dermis. Epidermis displays edema and moderate hyperplasia. A few mucuos cells are present. The insert with higher magnification shows infiltration of immune cells within the stratum spongiosum of the dermis. Scale bar 20 µm. (K) Louse attachment site (41 dpi). Histopathological lesions include epidermal hyperplasia, epidermal edema, low density of mucous cells, and immune cell infiltration in dermis. Insert shows immune cells. Scale bar 20 µm. (L) Non-attachment site of infested fish (41 dpi). Note the abundance of mucous cells. Insert shows epidermis and dermis, with no apparent immune cells. Scale bar 20 µm. Abbreviations: A (Adipose tissue), D (Dermis), E (epidermis), Sc (Scales), ChI, II (chalimus I, II), PadII (preadult II), mm (marginal membrane). Arrow heads indicate mucus cells, arrows indicate immune cells.

### 3.2. Skin histology and immunohistochemistry

Sections of skin at louse attachment sites were compared to skin from control fish at all time points. In addition, sections obtained from non-attachment sites of infested fish were analyzed at 41 dpi. Hematoxylin staining revealed no changes during the trial in control fish or in non-attachment sites of infested fish (Fig. 2A, L). Distinct histopathological lesions were observed in skin below parasites, being more pronounced at the later sampling points (Fig. 2B–K). Skin sampled at 2–7 dpi exhibited minor erosions of the epidermis and minimal infiltration of immune cells (Fig. 2D and E). From 10 dpi, the epidermal erosion became more evident, but still a minimal infiltration of immune cells was seen and no evident epithelial hyperplasia (Fig. 2F–H). When the lice reached the mobile stages, erosions became less evident. Instead, shallow ulcerations were detected where the louse mouth tube and marginal membrane had been in contact with the skin (Fig. 2I). A local depletion of mucus producing goblet cells was observed in sections from 24 to 41 dpi (Fig. 2I–K). Moreover, at the three last sampling points the dermis showed a more evident but still moderate local infiltration of immune cells, in addition to moderate epidermal hyperplasia and epidermal edema. Gross lesions with scale loss and hemorrhages were not observed. Non-parasitized skin samples from infested fish resembled control fish skin, exhibiting numerous goblet cells and no immune cell infiltration (Fig. 2L).

Sections from samples taken at 7 to 24 dpi incubated with antibodies against SAA revealed strong immunostaining at the site of parasite attachment (Fig. 3). Staining was observed in the dermis, near the epidermal basement membrane (Fig. 3A–E), while staining at a lower intensity was detected within the epidermis (Fig. 3E and F).

### 3.3. Transcript patterns during salmon louse infestation

Transcript levels of immune genes in skin from control fish were compared with those of the two sample types taken from infested fish, non-attachment and attachment sites. Comparisons were also made between the two sample types from infested fish. Of the 20 transcripts analyzed (Table 1), 16 transcripts showed a significant increase or decrease when samples from infested fish were compared with control fish (Figs. 4 and 5). Significant differences were mainly observed between controls and infested fish when measured directly under the louse. At all sampling points one or more of the respective immune gene mRNA levels showed a significant increase, with the highest number observed seen early at 16 dpi, and the lowest at the latest sampling point at 41 dpi (Fig. 6). For the respective immune gene mRNA levels that showed a significant decrease, the opposite temporal pattern was observed. The lowest numbers were recorded early in the trial, while the highest towards the end of the trial at 24 and 41 dpi.

#### 3.3.1. Transcripts with increased levels

When comparing the skin mRNA levels in both sample types from infested fish to those of control fish, 10 transcripts were found to be significantly increased: *cathelicidin 2* (*cath2*), *immunoglobulin* (*ig*) *m*, *igd secreted* (*igds*), *interleukin* (*il*) *1β*, *il4/13a*, *il6*, *il8*, *saa, transforming growth factor beta* (*tgfβ*), and *tumor necrosis factor α* (*tnfα*) (Figs. 4 and 5). These increases were most prominent at samples from lice attachment sites. Here, a significant increase of *cath2, il1β, il6,* and *il8* was detected. For *il1β,* the increase was of highest magnitude (up to 80-fold) and significant at all sampling points. The increase of *cath2, il6,* and *il8* was more modest but significant at all sampling points, except for the last when fish were carrying adult lice only. At attachment sites, *saa* and *tnfα* levels varied over time with a significant increase at specific sampling points, while for *igds*, *il4/13a* and *tgfβ* a significant increase was detected at a single sampling point only.

In general, the mRNA levels at the non-attachment sites were similar to those of controls. Correspondingly, when a significant increase was observed at the attachment site compared to controls, there was also a significant increase when attachment sites were compared with non-attachment sites. Exceptions here were single random time points

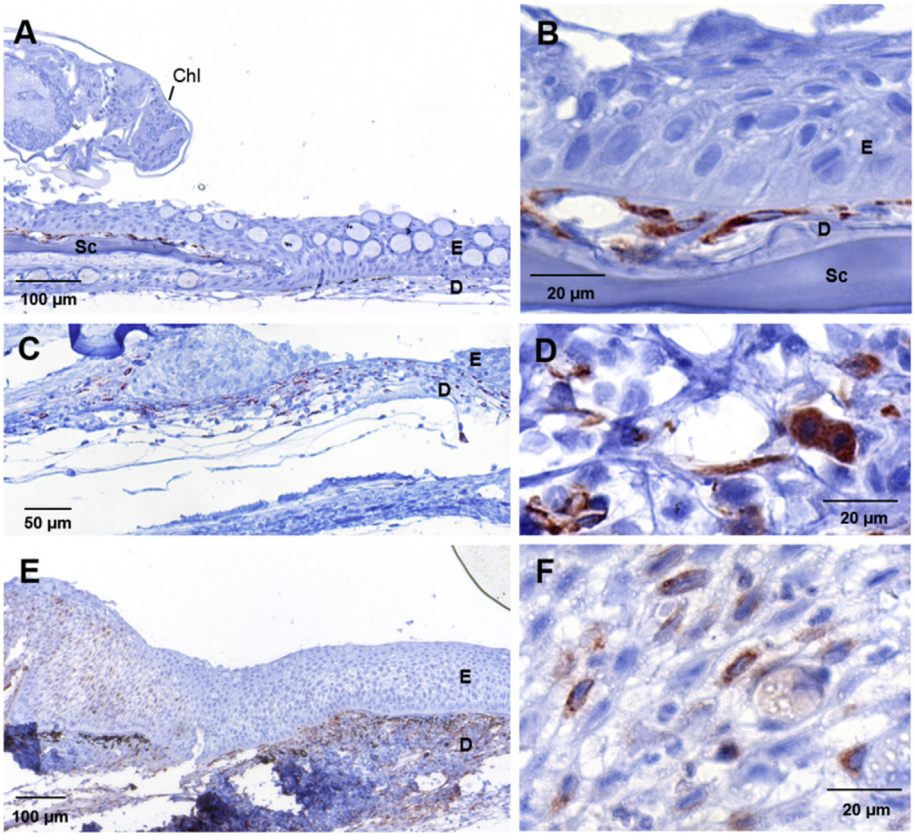


**Fig. 3.** Immunohistochemical staining of SAA in paraffin sections of skin from salmon louse infested rainbow trout employing primary antibodies raised against SAA and counterstained with hematoxylin. (A) 7 days post infestation (dpi), with positive immunostaining seen in dermis. (B) higher magnification of 7 dpi. (C) 10 dpi, with immunostaining mainly in dermis. (D) higher magnification of 10 dpi. (E) 24 dpi, with immunostaining in both epidermis and dermis. (F) higher magnification of epidermis, 24 dpi. Abbreviations: ChI (Chalimus I), D (Dermis), E (epidermis) and Sc (Scales). Scale bar is indicated.

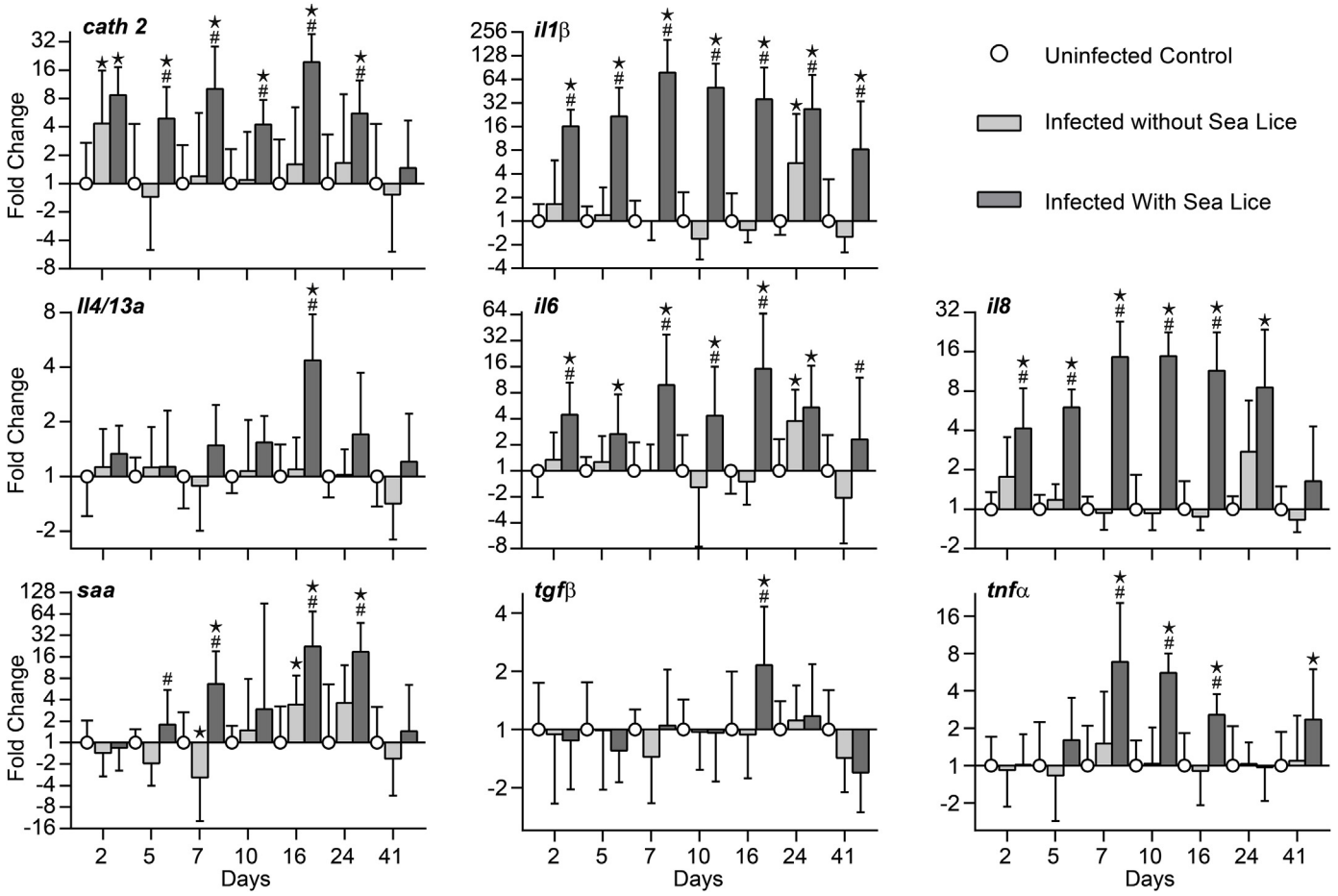


**Fig. 4.** Fold change ( ± SEM) of mRNA transcript levels of selected immune genes showing a significant increased expression compared to controls during salmon lice infestation (N = 8). Expression of immune genes was related to *acidic ribosomal protein* (*arp*), *beta-actin* (*β-actin*), and *elongation factor 1 alpha* (*elf1α*). * indicate significant differences when compared to skin from non-infested fish. # indicates significant differences when compared to skin from non-attachment sites of infested fish.

where levels of *cath2, il1β, il6* and *saa* were significantly higher both at non-attachment and at attachment sites compared to those of controls. Furthermore, a significant increase of mRNA levels at non-attachment sites compared to levels both at attachment sites and in controls was detected for *igds* and *igm* at 16 and 24 dpi, respectively.

### 3.3.2. Transcripts with decreased levels

Infested fish showed significant decrease of 10 transcripts when compared to those of control fish: *complement component 3–4* (*c3-4*), *cluster of differentiation 8α* (*cd8α*), *gata3, igm, igds, igd membrane bound* (*igdm*), *il10, inducible nitric oxide synthase (inos), major histocompatibility complex* (*mhc*) *1* and *saa* (Fig. 5). Except for *inos*, the reductions in levels were modest (2–4 fold) and mainly significant at attachment sites sampled at the later time points. For *inos*, the decrease was of greater magnitude (up to 10-fold) at attachment sites compared to controls and significant both at early and late samplings, though with a large variation between individuals at 7 to 16 dpi. While *gata3* showed a pattern of overall decrease in mRNA levels at the attachment sites when compared to controls and non-attachment sites, the decrease in levels of the remaining transcripts varied with time. Here, minor non-significant decreases were typical at early samplings, followed by significant decreases at 24 and/or 41 dpi.

Generally, no significant decreases were found at non-attachment sites, except for *igm* at 41 dpi where a significant decrease was detected in both sample types from infested fish when compared to controls. In a similar manner, non-significant trends were observed for *cd8α*, *igdm*, *igds* at 24 and/or 41 dpi.

## 4. Discussion

In the present study, rainbow trout was exposed to salmon louse copepodids and the infestation monitored from the initial challenge with copepodids to after the development of adult lice. The infestation was established employing the primary infective stage, the copepodid, in order to simulate the natural infestation occurring both in wild and farmed salmonids. A 66% reduction in the number of parasites from the preadult I stage and until the parasite reached the adult stage was recorded here. This may be interpreted as a host immune response, but factors related to host behavior and parasite biology may also be involved. During lice ontogenesis, both the mode of attachment and feeding activity changes [16]. Concomitantly, there is development in the composition, number and activity of exocrine glands that may cause distinct differences in the modulation of the host immune responses between lice stages [26]. Our observations reflect the above through both the successive increase in the severity of lice-induced lesions, and the modulation of immune gene transcript levels. The transition from an initial more intense immune response to a more dampened response associated with appearance of mobile lice may imply immunomodulation conveyed by secreted louse effector molecules. The present study also demonstrates that the cutaneous immune response of rainbow trout towards all stages of salmon lice is restricted mainly to the site of infestation, as indicated by previous studies of other salmonids [29,32,33,40].

When assessing the rainbow trout innate immune response through analysis of the transcript levels for proinflammatory cytokines and

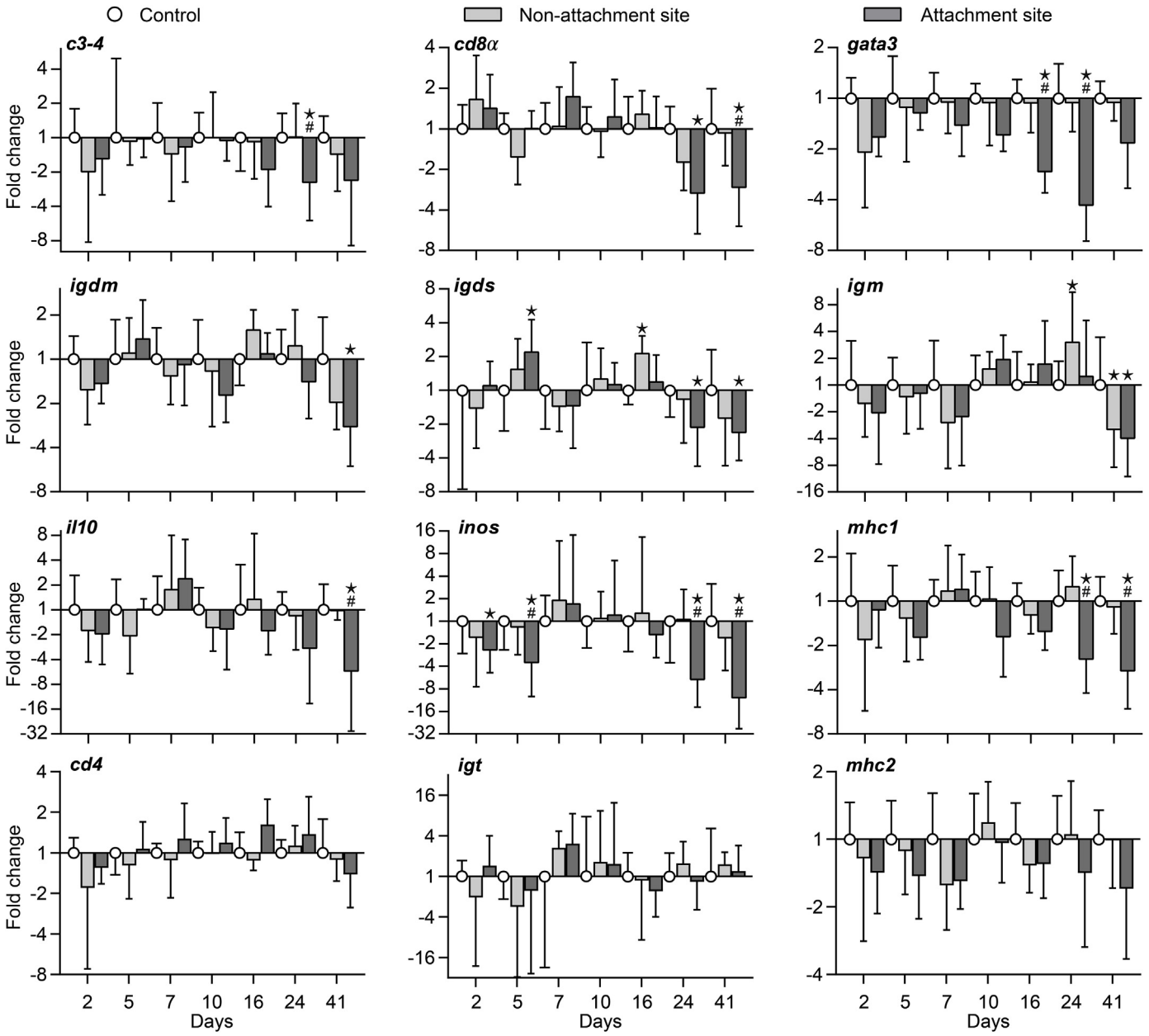


**Fig. 5.** Fold change ( ± SEM) of selected immune gene transcripts showing a significant decreased transcript level compared to controls or no significant regulation during salmon lice infestation (N = 8). Transcript levels of immune genes were related to transcript levels of *acidic ribosomal protein* (*arp*), *beta-actin* (*β-actin*), and *elongation factor 1 alpha* (*elf1α*). The degree of decrease was calculated as negative reciprocal fold. * indicate significant differences when compared to skin from non-infested fish. # indicates significant differences when compared to skin from non-attachment sites of infested fish.

acute phase proteins, a constitutive elevation was seen at the site of attachment, though with a decrease at the latest sampling points. The proinflammatory cytokines *il1β*, *il6* and *il8* were elevated already at the first sampling, like that observed in Atlantic salmon infested with copepodids [33]. Teleost IL8 is chemotactic for neutrophils, macrophages and lymphocytes [46–50]. Nevertheless, the influx of immune cells at the attachment site of early lice stages was not found to be prominent in rainbow trout, as previously observed in Atlantic salmon [17,51]. Expression of the proinflammatory cytokine *tnfα* was not induced before parasites molted to the chalimus stage, when *il1β* and *il8* was at its highest. The chalimus attaches via a frontal filament, which, in Atlantic salmon attracts a limited number of immune cells [17,51]. Epidermal erosion, however, increases as the chalimus movement and feeding is restricted around the point of attachment [17]. Correspondingly, we observed increased severity of erosions in rainbow trout during the chalimus stages. Such lesions may trigger inflammation, reflected by the constitutive increase of *il1β*, *il6*, and *il8*. Immune cells, especially activated macrophages are considered the major source of these cytokines, and indeed IL1β+ lymphocyte-like cells have been detected both in epidermis and dermis at adult salmon louse attachment sites (Braden et al., 2015). IL1β was, however, also found to be expressed by epidermal epithelial cells of Atlantic, coho (*O. kisutch*) and sockeye (*O. nerka*) salmon. Similarly, human keratinocytes produces IL1, IL6 and IL8 that can be released upon injury or environmental stimuli [52,53]. In addition to their key role in inflammation, IL1, IL6 and IL8 may be involved in tissue repair through a proliferative and chemotactic effect on keratinocytes [52]. In the present study, early and constitutive levels of *il1β*, *il6*, and *il8* were detected without a clear correlation to dermal immune cell infiltration. From onset, louse infestation is associated with continuous damage to the epidermis; however, further research is

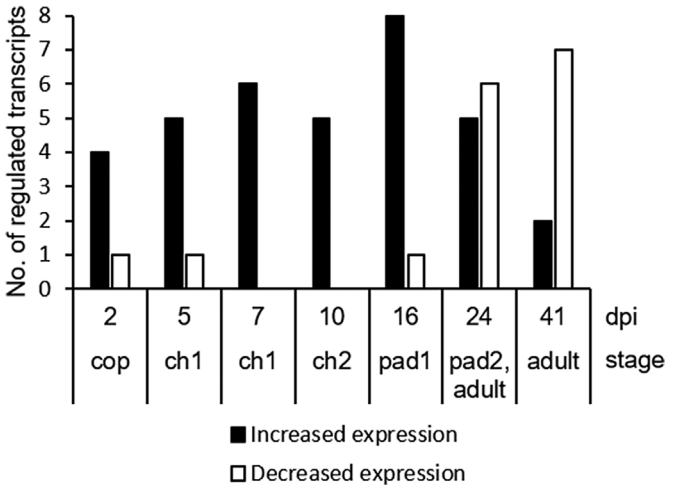


**Fig. 6.** Number of regulated transcripts in rainbow trout skin at all sampling days. Transcripts with a significantly increased (black) or decreased (white) mRNA level at the attachment site compared to non-infested fish on days past infestation (dpi). Louse stages collected on fish from day 2–10 were copepodids (cop) and the attached chalimus stages (ch1, ch2), whereas fish from day 16–41 carried mobile preadult (pad1, pad2) and adult lice.

warranted to elucidate the role of teleost epidermal epithelial cells in inflammation and potential regulatory roles of these inflammatory cytokines in tissue repair responses.

The transcript levels of the acute phase proteins CATH2 and SAA were also increased in lice infested rainbow trout. Whereas the increase of *cath2* occurred already at the copepodid stage, *saa* showed a slower response with significantly elevated levels from the first chalimus stage. In Atlantic salmon infested with adult salmon louse, an increase of *saa* has also been detected, whereas the louse resistant coho salmon exhibits an earlier and stronger expression of *saa* [29]. Being a general marker of inflammation, *saa* is upregulated both in response to viral, parasitic and bacterial infections [54–58]. Moreover, it is a prominent acute phase reactant within the pentraxin group, which can opsonize and/or block functions of invading pathogens. Hence, high expression of *saa* in salmon lice infested coho salmon may indicate a role in lice resistance, as do the correlation between lice loss and local *saa* expression seen in the present study. Further studies should address whether the inclusion of SAA positive cells within the rainbow trout epidermis and dermis, as shown here by immunohistochemistry, is limiting lice infestation, or if the decrease of *saa* is due to lice immune modulation.

C*ath2* is constitutively expressed in rainbow trout skin, where the peptide is likely to be produced by epidermal cells acting mainly as an antimicrobial peptide (AMP) [59]; and references therein). Moreover, rainbow trout IL6 has been shown to induce a sustained upregulation of *cath2* in both cell line (RTS-11) and primary head kidney derived macrophages [60]. In the present study, both the level of *Il6* and *cath2* were significantly increased following a similar expression pattern at the attachment site, indicating an interplay between the two also in louse infested skin. This suggests a dual function of CATH2 in rainbow trout, acting both as an AMP, and playing a role in inflammatory processes, as suggested by others [61]. Interestingly, recent studies have suggested that CATH2 also act as a cue that triggers sea lice copepodids to settle on a suitable host. *In vitro* experiments have shown that CATH2 increases the swimming activity and expression of chemosensory receptor transcripts in salmon louse copepodids [62], and stimulates *Caligus rogercresseyi* frontal filament extrusion and expression of molting associated genes [63]. Thus, if CATH2 is a signal molecule that triggers physiological processes important for the salmon louse, the fast and constitutive increase of *cath2* seen here in rainbow trout, could be important for settlement and in maintaining the infestation.

Host immunosuppression may be a vital mechanism that enables the louse to chronically infest susceptible salmonids. The local significant decrease of *c3-4*, *cd8α*, *igd*, *igm*, *gata3* and *inos*, as observed in the present study, may imply suppression of the complement system, adaptive immunity and the production of nitric oxide. The decrease in *inos* levels were of significant magnitude (up to 10-fold) both initially in the infestation and again during the presence of preadult II and adult lice. Macrophages and other phagocytes produce *inos* that catalyzes the formation of nitric oxide, which plays a role both in the innate and adaptive immune response [64]. Nitric oxide may react by enzyme catalysis with superoxide to form peroxynitrite [65], a molecule that is an important cytotoxic reactive oxygen species (ROS) in the respiratory burst. Moreover, iNOS may contribute to upregulate macrophage phagocytosis [66]. Macrophage phagocytic activity, in addition to respiratory burst capacity, is reduced both in louse infested rainbow trout and Atlantic salmon when lice reach the preadult stage [4,67]. In *Gyrodactylus derjavini* infested rainbow trout, an upregulation of *inos* was detected in fish that eventually rid themselves of the parasite [68]. Thus, low levels of *inos* may reflect attenuation of these cellular processes or loss/reduced recruitment of phagocytes that produce it, both of which may promote to immunosupression and, in extension, louse survival on their host.

The transcription factor GATA binding protein 3 (GATA3) is a key regulator of mammalian T helper 2 cell (Th2) responses, and acts by stimulating their production of IL4, IL13, and IL5. Genes that encode IL4 and IL13 are highly expressed by Th2 cells, and, by positive feedback, IL-4 enhances and sustains Th2 differentiation [69]. Teleosts have two cytokines evolutionary related to both IL4 and IL13, namely IL4/13A and IL4/13B [70,71]. As for mammalian IL4, the zebrafish IL4/13A gene has a GATA3 binding motif, and, in turn, recombinant IL4/13A promotes zebrafish Th2-type immune responses by increasing B-cell proliferation and antibody production [72]. In rainbow trout, probably through the same pathway, IL4/13A augments B-cell IgM secretion [71]. Hence, GATA3 and IL4/13A play key roles in the adaptive immunity of teleosts. In healthy rainbow trout skin, there is a constitutive expression of both *il4/13a* and *gata3*, which is indicative of a Th2-skewed environment [73]. Moreover, a Th2-skewed response to salmon louse in Atlantic and coho salmon have been indicated [29,32]. In the present study, however, levels of *gata3* were overall decreased at lice attachment sites, with a significant decrease in samples taken 16 and 24 dpi, when lice were at preadult stages. Reversely, *il4/13a* levels were increased, with a significant peak at 16 dpi. This indicates a suppression of Th2-immunity, and the presence of an alternative *il4/13a* source at the lice attachment site. In mammals, for instance, other leukocytes populations such as NKT cells, eosinophils, basophils and mast cells may produce IL4 and IL13 [74,75]. Furthermore, the significant reduction of transcripts typical of an adaptive immune response, *cd8α*, *mhcI*, *igm*, *igdm* and *igds*, indicate an overall low number of T- and B-cells at the site of attachment. Supporting this notion is the observed down modulation of *cd8α* and *igm* in Atlantic and sockeye salmon infested with adult lice for only 72 h [29]. Regardless of this, an apparent influx of immune cells to the rainbow trout dermis was observed in the presence of mobile lice, an influx that also has been observed in Atlantic salmon [76]. Whether cells with a major function in adaptive immunity are not attracted to the site of infestation or present with their cellular functions suppressed by substances secreted from louse exocrine glands, remains to be elucidated.

At the feeding sites of preadult and adult lice, an apparent depletion of epidermal mucous cells was observed in the present study. This is similar to that seen in other Pacific salmonids (*O. gorbuscha*, *O. nerka* and *O. kisutch*), where experimental infestations with adult salmon louse placed onto the fish also induces a decrease in mucous cell numbers already after 48 h [29,77]. An equivalent reaction has been reported to be less distinct in Atlantic salmon [29,76]. The mechanisms behind the exhaustion and/or inhibition of mucous cell differentiation are unknown. It may be a response to inhibitory effector molecules

secreted from exocrine glands of mobile lice to create a less slippery, roughened surface for sturdy adhesion to their host. However, irritation of the rainbow trout skin initially causes mucous cell hyperplasia, but during continued stimulation, exhaustion occurs [78]. Additionally, as part of the wound healing process, epidermal hyperplasia and high epithelial turnover is likely to be induced during lice infestation. These processes have not been comprehensively studied in salmon louse infested rainbow trout, but are likely to be dynamic as wound size and depth varies in accordance with lice stage and infestation intensity. In the present study, histopathological lesions comprising epidermal erosion were initially seen as a result of the feeding activity of attached lice stages, and later beneath the marginal membrane and appendages of mobile lice. In addition, mobile lice gain access to blood [79], and to do so, they break through the epidermal basement membrane to create small ulcers with hemorrhage in dermal connective tissue [76]. Correspondingly, ulcerations and increased influx of immune cells to the dermis were observed in the present study.

Development from the preadult I stage until the adult stage was also associated with a large loss of lice. Other studies have also shown marked decreases of lice numbers on Atlantic salmon skin in this developmental phase [4,18,80–82]; however, lower losses have also been reported [45,83]. Retention of lice is highly dependent on the size and density of host fish and tank environment [41], and will therefore vary between experiments. Whether lice loss is related to the increased lice size and mobility, which both augment the probability for detachment in the artificial tank environment, and/or fish immune responses, is not possible to discern based on the data obtained in the present study. However, our results indicate that immune responses may play a lesser role as the levels of immune gene transcripts showed an overall decreasing trend after the appearance of mobile lice stages. Given the higher abundance of immune cells detected after lice became mobile, compared to when lice were attached, the decrease in transcript levels could point towards suppression or disruption of cellular immune processes, possibly induced by immunomodulatory proteins or other effector molecules secreted into the dermis from louse exocrine glands [26]. On the other hand, chronic stress can increase cortisol secretion in teleosts, which again may induce immunosuppression [84]. Increased plasma cortisol levels have been detected in Atlantic salmon with high burdens of mobile salmon lice [19,34,67]. However, both rainbow trout and Atlantic salmon that were infested with comparable numbers of lice as in the present study displayed normal cortisol levels concomitant with decreased phagocytic and respiratory burst activity in isolated head kidney macrophages [4]. Wheter the latter reflects immunosupression caused by lice effector molecules remains unknown. Also, it cannot be excluded that the decrease in immune gene transcripts levels observed towards the end of the trial in the present study, may reflect a slightly lower proportion of dermal tissue relatively to epidermal tissue in the samples due to the moderate epidermal hyperplasia seen locally.

In sum, the results show that in rainbow trout infested with salmon lice, skin pathology and the characteristics of the local immune response change over time. In the early phase with stationary lice, lesions comprised of epidermal erosions were evident and probably caused by continuous grazing on the epithelium. The initial immune gene transcript profile was indicative of early inflammation with a characteristic innate immune response probably activated by release of chemical factors from damaged epidermal cells, and possibly irritants from the louse. As the infestation progressed and lice became mobile, and shallow ulcers where observed where lice had penetrated the epidermis to reach dermal vasculature. Concomitantly, there was a successive decrease in levels of transcripts associated with adaptive immunity and *inos*. When lice reached the adult stage, moderate epidermal hyperplasia and loss of mucuos cells was clearly visible in sections from the site of attachment. Furthermore, transcript levels of genes reflecting an innate immune response had markedly decreased. Further studies should address whether hitherto unknown immunosuppressive molecules secreted by mobile lice induce the fall in transcript levels of many immune genes as observed in the present study. Moreover, the mechanisms behind the apparent loss of epidermal mucuos cells need elucidation; here, substances produced by tegumental type 3 glands, which empty their products directly onto the epidermis [26], are of interest. Identification of such substances, especially those with a presumptive immunomodulatory effect, may reveal proteins, exposed antigens, which could be utilized in vaccine development. Finally, the present study clearly shows that studies of salmon louse infestations addressing immune responses should include skin samples from louse attachment sites, since skin from non-attachment sites display a limited response.

## CRediT authorship contribution statement

**Sussie Dalvin:** Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing, Funding acquisition. **Louise v.G. Jørgensen:** Methodology, Writing - review & editing. **Per W. Kania:** Formal analysis, Visualization, Validation, Writing - review & editing. **Sindre Grotmol:** Investigation, Writing - original draft, Writing - review & editing. **Kurt Buchmann:** Conceptualization, Writing - review & editing, Funding acquisition. **Aina-Cathrine Øvergård:** Conceptualization, Methodology, Investigation, Formal analysis, Visualization, Validation, Writing - original draft, Writing - review & editing.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2020.05.014.

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