Accepted refereed manuscript of: Fernandez-Senac C, Monaghan SJ, Mascolo D, Baily JL, Betancor M, Chalmers L, Paladini G, Adams A, Fridman S & Bron JE (2022) Investigating the impacts of H(2)O(2) treatment on gills of healthy Atlantic salmon reveals potential changes to mucus production with implications on immune activity. *Fish and Shellfish Immunology*, 128, pp. 74-81. https://doi.org/10.1016/j.fsi.2022.06.063

Title: Investigating the impacts of H_2O_2 treatment on gills of healthy Atlantic salmon reveals potential changes to mucus production with implications on immune activity

Authors: Carolina Fernandez-Senac[a], Sean J. Monaghan[a]., Dario Mascolo[a], Johanna L. Baily[a], Monica Betancor[a], Lynn Chalmers[a], Giuseppe Paladini[a], Alexandra Adams[a], Sophie Fridman[a], James E. Bron[a]

[a]Institute of Aquaculture, University of Stirling, Stirling, UK, FK9 4LA

Corresponding author: Carolina Fernandez-Senac (carolina.fdez91@gmail.com); Institute of Aquaculture, University of Stirling, Stirling, UK, FK9 4LA

Sean J. Monaghan: s.j.monaghan@stir.ac.uk
Dario Mascolo: dario.mascolo@mowi.com
Johanna L. Baily: j.l.baily@stir.ac.uk
Monica Betancor: m.b.betancor@stir.ac.uk
Lynn Chalmers: lynnallan@lateraldx.com
Giuseppe Paladini: paladini2000@yahoo.it
Alexandra Adams: adams.alexandra58@gmail.com
Sophie Fridman: fridmansophie@gmail.com
James E. Bron: j.e.bron@stir.ac.uk

Abstract

Current treatment strategies for relevant infectious diseases in Atlantic salmon (*Salmo salar* L.) include the use of low salinity or freshwater bathing. However, often availability is restricted, and hydrogen peroxide (H_2O_2) is used as an alternative. The potential impacts of H_2O_2 on fish mucosal tissues, especially the gills therefore need to be considered. In this study the mucosal and immunological effects of H_2O_2 treatment on the gills of healthy Atlantic salmon were examined by gene expression (qPCR) and immunohistochemistry (IHC) investigating T-cell, B-cell, and mucin activity. Healthy fish were treated with H_2O_2 and sampled at different times: 4 h, 24 h and 14 days post-H_2O_2 treatment (dpt) (total n = 18) to investigate the effect of holding time and H_2O_2 treatment. Treatment with H_2O_2 resulted in up-regulation of markers for T-cell activity and anti-inflammatory response and down-regulation of mucin expression in the gills at 14 dpt compared to fish sampled prior to treatment (0h; n=5 fish). These findings were supported by IHC analysis, which despite being highly variable

between samples, showed an increase in the number of $CD3^+$ T cells at 14 dpt in 50% of treated fish compared to pre-treatment fish. The results from this study suggest that H_2O_2 treatment does not immune compromise healthy Atlantic salmon after 14 dpt (*i.e.,* post-recovery) but modulates gill immune activity and disrupts the mucus covering of the gills. However, further studies are required to determine whether the effects observed are related to H_2O_2 treatment in isolation or other variables such as holding time or environmental factors.

1. Introduction

Gill diseases have become a consistent problem in worldwide salmonid aquaculture through the exposure to non-infectious [1] and infectious agents [2-10] following aquaculture intensification and climate change.

Low salinity or freshwater bath treatments have been applied to treat parasitic diseases, such as amoebic gill disease (AGD) or sea lice infection, where freshwater is readily available or where resources exist to produce low salinity water from seawater [11,12]. However, when fresh- or low salinity water is not easily obtainable, chemotherapeutants may be applied to fish stocks as an alternative strategy to mitigate the impacts of pathogenic agents. Worldwide, hydrogen peroxide (H_2O_2) is the most used chemical due to its anti-pathogen efficacy [13] while being readily decomposed into oxygen and water. While this treatment successfully kills or removes pathogens, potential adverse effects on the gills of treated fish have also been reported [14, 15].

Treatment with high concentrations of H_2O_2 provoked intense signs of respiratory distress and accelerated mortality of affected turbot (*Scophthalmus maximus* (Linnaeus, 1758)) [15]. Similar acute effects were observed on kingfish (*Seriola lalandi* Valenciennes, 1833), although the implications of H_2O_2 treatment were significantly less severe than the effects on the fish following chronic infection with the monogean parasite *Zeuxapta seriolae* (Meserve, 1938), being targeted by treatment [16]. Although commonly used as a treatment, increasing

concentration of H_2O_2 has been correlated with increased fish mortalities [17,18]. Side effects on mucous cells and lysozyme in gills has been noted in olive flounder *Paralichthys olivaeceus*, which exhibited innate immune response modulation due to treatment [19]. Lysozyme activities were observed to decline gradually in gill mucus of treated fish, potentially due to the strong oxidising nature of H_2O_2 which causes peroxidation of lipid and cellular membranes, inhibition of DNA replication and inactivation of enzymes [20].

H_2O_2 treatment has been found to cause physiological stress in Atlantic salmon, whereby various stress markers (*e.g., glucose, lactate, cortisol gpx1, cat, Mn-sod* and *hsp70*) were upregulated post-treatment when 1500 mg/L of H_2O_2 was applied for 20 min at six different times of the day during a 24-h cycle [21]. However, the implications of using H_2O_2 in the context of mucosal and immune responses has never been determined. Mucosal tissues include mucosal epithelia with epithelial and mucous cells. The latter produce mucus, which is composed of mucins, that are known to play a key role in innate immunity. They are gel-forming glycoconjugates which produce a protein matrix that accommodates the natural commensal flora within mucosal tissues and restrain infectious disease [22]. Immune and mucosal responses of Atlantic salmon have been broadly investigated in the context of diseases such as AGD [24] and during sea lice infection [25, 26], but not in apparently healthy fish that will also be exposed to H_2O_2 during treatment of a farmed population.

The current study was conducted to investigate the potential mucosal and immunological effects of H_2O_2 on the gills of healthy Atlantic salmon through the evaluation of three different mucin types, in addition to eleven genes related to T-cell, B-cell, and Th1/Th17 and Th2 pathways. The objective of this study was to inform further of the safety margins with regards to applying H_2O_2 as a treatment for farmed Atlantic salmon, highlighting potential side effects on the mucosal coat and immune responses of gills from healthy Atlantic salmon over a period of 14 days post-treatment recovery, by investigating the effect of timing of the treatment.

2. Material and Methods

2.1. Experimental fish, H_2O_2 treatment and sampling

A total of 24 fish were netted out from a naïve stock and randomly allocated into 4 x 250 L tanks (n=6 fish per tank) at the Marine Environmental Research Laboratory (MERL) (Machrihanish, Scotland). Tanks were held at a temperature of 11±1°C, in full-strength seawater from wells located 50 m from the shore (ca. 35 ‰) in a flow-through system, and at a concentration of dissolved oxygen (DO) ranging between 8.6 and 8.8 ppm. Fish were fed daily with commercial salmon pellets (Inicio Plus, BioMar, UK) at 1% of their body weight. H_2O_2 treatment was performed by decreasing tank volume to 200 L and administering the treatment as a bath at a concentration of 1250 mg L^{-1} for 15 min (exposure time). Water chemistry parameters such as oxygen concentration and pH were monitored and logged every 3 min during the entire duration of the H_20_2 treatment. As mentioned, water temperature was monitored and kept maintained at 11±1°C. Due to logistical limitations, and the preference to not interfere with fish and avoid stress-induced responses during sampling procedures, only 1 tank was used per H_2O_2 treatment exposure group. The study therefore investigated the effetv of timing of H_2O_2 exposure on fish gills. When treatment was completed, weights (kg) and lengths (cm) of all fish were registered (Table 1).

Table 1. Collection of the weight (kg), fork length (cm) (± s.e.m) of each fish used during the H_2O_2 treatment experiment (n = 24). Table shows data from the treated Atlantic salmon from time points 4 h, 24 h and 14 d (including pre-treatment group).

Group	Fish (n)	Weight (kg)	Length (cm)
Time 0 h group (pre-treated)	6	0.177 ± 0.014	23.3 ± 2.1
Time 4 h group	6	0.155 ± 0.011	25.3 ± 0.677
Time 24 h group	6	0.166 ± 0.011	26.16 ± 0.54
Time 14 d group	6	0.190 ± 0.008	26.8 ± 0.360

Fish were sampled from each tank at 0 h (pre-treated group), 4 h, 24 h and 14 days post treatment (dpt) (total n=24). Fish were subject to anaesthetic overdose using MS-222 (100 mg L^{-1}) and destruction of the brain according to UK Home Office Schedule 1i methods.

For gill collection, samples were taken from the second left gill arch for further processing. One eighth was preserved in RNA preservation solution (0.45 M ammonium sulphate, 2 mM EDTA, and 25 mM sodium citrate, pH 5.2) for RNA extraction and subsequent qPCR analysis for assessing gene expression. A record of the fish fork lengths and weights was taken immediately following euthanasia.

Additional gill arches (third and fourth) were excised and fixed in different fixatives, including Modified Davidson's (standard - with buffered PBS solution) and methacarn fixative [27].

All experimental procedures were all approved by the Animal Welfare and Ethical Review Body (AWERB) of the University of Stirling and were conducted under UK Government Home Office project licence 60/4189.

2.2. SYBR® green qPCR analysis for gene expression on gills

2.2.1. RNA extraction from gills and cDNA synthesis

Gills were sampled from every time point post H_2O_2 treatment (4h, 24h and 14 dpt) and from time point 0 h (pre-treated group) and were processed for RNA extraction and subsequent cDNA synthesis. First, gills were cut into small pieces and 1 mL of TRI Reagent was added (approx. per 100 mg of gill (maximum of 1.5 mL in screw cap tubes)). Samples were incubated on ice for 60 min.

Homogenised samples were incubated at RT for 5 min and centrifuged at 12,000 x *g* for 10 min at 4°C. The supernatant was combined with 100 µL 1-Bromo-3-chloropropane (BCP) and shaken vigorously by hand for 15 s. Tubes were incubated at RT for 15 min, followed by centrifugation at 20,000 × g for 15 min at 4ºC.

For precipitation of the RNA, RNA precipitation solution (1M NaCl, 1M $C_6H_6Na_2O_7$) and isopropanol were added at 50% volume to the aqueous phase. Then, the samples were gently inverted 4-6 times and incubated for 10 min at RT. Samples were centrifuged at 20,000 × g for 10 min at 4°C and the RNA pellet was retained.

The RNA pellet was washed for 15 min at RT with 1 mL of 75% ethanol. The pellet was then re-suspended and centrifuged at 20,000xg for 5 min at RT. The ethanol was removed, and the RNA re-suspended in 100 µL of RNase free water and the concentration measured using a NanoDrop 1000 Spectrophotometer. Dilutions of the RNA samples (1:10) were made for a final total RNA concentration of 2 µg in 10 µL. The remainder of RNA samples were stored at -70°C.

DNase treatment of the samples was performed prior to cDNA synthesis with Ambion ® DNA-free™ DNase Treatment and Removal Reagents (ThermoFisher, UK) according to the manufacturer's instructions.

cDNA synthesis was performed using the High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Cheshire, UK) according to the manufacturer's protocol.

cDNA samples were diluted by pipetting 10 µL from the stock solution to a volume of 90 µL of ddH_2O (1:10 dilution). Dilutions and stock cDNA samples were stored at -20 ºC. RNA samples were visualised via electrophoresis through 1% agarose/tris–borate EDTA buffer and bands were visualized by staining with a final concentration of 0.5 µg mL^{-1} from a 10 mg mL^{-1} ethidium bromide stock. After cDNA synthesis was performed, conventional PCR was performed with the samples with housekeeping transcript ELF-1α primers (FW: 5' CTGCCCCTCCAGGACGTTTACAA 3' and RV: 5' CACCGGGCATAGCCGATTCC 3'; NCBI accession number: AF321836) for Atlantic salmon to assess viability. Cycle conditions were 95ºC for 5 min; 95ºC for 30 s, 58ºC for 30 s and 73ºC for 2 min, for 35 cycles; and 73ºC for 8 min. The PCR reaction products were subjected to electrophoresis as described before.

2.2.2. qPCR and data analysis

qPCR was performed on a qTOWER^3 (Analytik Jena, Germany) using SYBR green chemistry to measure the differential expression of the target genes and primer sequences listed in Table 2. Each PCR reaction consisted of 15 µL of the SYBR® master mix (Thermo Scientific, Epsom, Surrey, UK) along with the forward and reverse primers (final concentration 0.2 µM each) and 5 µL cDNA template in molecular grade water to a final volume of 20 µL. Samples were assayed in duplicates and cycling conditions consisted of an initial activation of DNA polymerase at 95 °C for 3 min, followed by 40 cycles of 5 s at 95 °C, 10 s at 60 °C, and 10 s at 72 °C. The mRNA transcripts / gene expression was calculated relative to the geometric mean of three reference genes ELF1-α, β-actin and β-tubulin which were previously described as valid reference genes in Atlantic salmon [28].

167 **Table 2.** List of primers (5' → 3') used for the immune and mucin gene expression analysis in pre-treated fish treated with H_2O_2.

Gene target name	Accession number (NCBI)	Oligonucleotides (5' → 3')	Product size (bp)	Tm (°C)	Efficiency (%)
Housekeeping					
ELF-1α	AF321836	FW: CTGCCCCTCCAGGACGTTTACAA	176	60	97.57
		RV: CACCGGGCATAGCCGATTCC		60	
β-actin	XM_014194537	FW: CCCATCTACGAGGGTTACGC	112	60	86.21
		RV: TGAAACTGTAACCGCGCTCT		61	
β-tubulin	NM_001140841	FW: CCGTGCTTGTGGACTTGGAG	144	60	91.92
		RV: CAGCGCCCTCTGTGTAGTGC		62	
Immune response					
CD3γδ-B	NM_001123721	FW: CCGGCAAGAAAACATCTACCAAA	81	59	98.15
		RV: GCTGATAGTGGCCAATGGGG		61	
CD4-2α	XM_014163618	FW: GCCCCTGAAGTCCAACGA	79	61	88.58
		RV: AGGCTTCTCTCACTGCGTCC		63	
CD8α	XM_014167443	FW: ACTTGCTGGGCCAGCC	96	62	81.76
		RV: CACGACTTGGCAGTT		58	
IL-4/13 β2	HG794525	FW: GCATCATCTACTGAGGAGGATCATGAT	63	60	95.07
		RV: GCAGTTGCAAGGGTGAAGCATATTGT		63	
IL-10	XM_014186180	FW: GGGTGTCACGCTATGGACAG	118	61	80.17
		RV: TGTTTCGGATGGAGTCGATG		57	
	HQ664669	FW: CCAGACATCGATACTAAAAAGAACCACA	110	59	99.24
IL-22		RV: TGTGGTGGTGGTCAGTGTAGTGTT		63	
IFN-γ	NM_001171804	FW: TCTCCCTCTAACGGTGAAGGT	148	60	99.7
		RV: TGGCCAGTTGAGGCATTTTGT		62	
IgT	ACX50291	FW: CAACACTGACTGGAACAACAAGGT	121	60	99.8
		RV: CGTCAGCGGTTCTGTTTTGGA		61	

m IgM	AAB24064	FW: TGCGCTGTAGATCACTTGGAA	134	59	86.21
		RV: ATGGTGTTGCTGCATGGACA		60	
TCRα	XM_014140002	FW: AACTGGTATTTTGACACAGATGC	146	56	88.89
		RV: ATCAGCAGGTTGAAAACGAT		54	
TNF-α2	NM_001123590	FW: ACTGGCAACGATGCAGGATGG	144	64	98.25
		RV: GCGGTAAGATTAGGATTGTATTCACCCTCT		62	
Mucin response					
Muc1	XM_014160723	FW: TCACGTCCAGAAACCAGGAAG	101	60	82.52
		RV: GTCGCAGGCTGAGAAAACCT		61	
Muc17	XM_014171406	FW: TTTCCCGACTTCCCAGTTTCC	163	60	89.16
		RV: CTGGCATCTTGATTAACCGCTG		59	
Muc5ac	XM_014189016	FW: TTTTCTCAGTTGCCGCTTTT	92	58	82.37
		RV: AGTCGGAGCCCATAAGACGT		61	

2.5. Mucous cell semi-quantitative analysis

Gill samples from all time points were treated following a previously validated and developed fixation technique using a Modified Davidson's solution [27] and all sections were stained using a combined Alcian blue-PAS technique [29, 30]. Then, preparations were analysed to quantify the presence of mucous cells.

All sections (3 slides per fish; n=6) were scanned for any signs of histopathological events. Davidson's fixed gill sections were scanned using a 10x objective, selecting an area with at least 3 whole primary lamellae, and an image of ~ $1mm^2$ was acquired from each sample. On each of the 3 primary lamellae present in the micrograph, one mid-section comprising 10 inter-secondary lamellar spaces on each side of the primary lamellae was chosen and used for standardised mucous cell counts.

Selected fields of primary lamellae were limited to only primary lamellae that appeared to be equally transversally sectioned with limited cutting or folding secondary lamellar artefacts. The 3 resulting counts from each section were exported and a mean count for each sample was obtained. In addition, a mean was also calculated for each sampling group.

2.6. Immunohistochemistry (IHC) for evaluation of CD3+ cells expression and localisation in gill sections

Sections obtained from samples fixed in methacarn [27], with the modification of using SuperFrost Plus™ Adhesion slides (Fisher scientific, UK) were dewaxed in 2 steps of xylene for 5 min each, then in 100% ethanol for 5 min and 70% ethanol for 3 min. After dewaxing, sections were rinsed in TBS (2.42 g L^{-1} Tris Base (10 mM), 24.24 g L^{-1} NaCl (0.5 mM), pH 7.5 in distilled water). A wax circle was drawn around the gill section with a PAP pen (Merck, UK) and sections were transferred to a humidifying chamber. DAKO Peroxidase block (DAKO EnVision System kit, Agilent, US) was added, just enough to cover the fixed gills, and slides

were incubated for 5 min. After incubation, a rinse was performed for 5 min with TBST (same as TBS recipe by adding 0.5 mL/ L Tween-20). Following this, sections were processed for antigen retrieval; this procedure was carried out by immersing the slides in 500 mL of tri-sodium citrate solution (2.94 g L^{-1} Tri-sodium citrate, pH 6) and heating twice at 900W in a microwave for 2 min, with a cooling step of 5 min in between.

Non-specific antibody blocking was performed by covering the fixed gill with 2% bovine serum albumin (BSA) in TBST. Sections were incubated for 30 min at RT in a humidifying chamber. After this, the BSA-TBST blocker was dabbed off and sections were covered with 10% goat serum diluted in the TBST. After 30 min incubation RT primary antibodies (CD3g/d, clone T1 - mouse IgG monoclonal antibody (Vertebrate Antibodies Ltd., Aberdeen, UK)) and negative controls (TBS only and an isotype matched monoclonal antibody control raised to Koi herpesvirus antigen; KHV [31]) were prepared by preparing 1/5 dilutions of antibodies in 1% BSA in TBS. Without washing the slides, the primary antibodies and controls were added to the sections by covering the fixed gills. An overnight incubation was followed at 4°C in the humidifying chamber.

The following day, sections were washed in TBST three times for 3 min. DAKO Labelled polymer HRP Anti-mouse (DAKO EnVision System kit, Agilent, US) was added to the sections at sufficient volumes to cover the gill section and then incubated for 30 min at RT. Sections were then washed 3x 3 min in TBST. After this, DAKO AEC+ Substrate chromogen (DAKO EnVision System kit, Agilent, US) was added the same way as before and sections were incubated between 5-30 min until a signal was evident in the positive control without any background in the negative controls. The reaction was stopped by dipping of the slides in distilled water.

Slides were then counterstained by immersing in haematoxylin for 3-4 min. Excess stain was washed away by submerging in a running tap water bath for 10 min. Sections were cover slipped and left to dry for 1h or overnight.

2.7. Image analysis for CD3+ cell expression quantification

Quantification of the expression of CD3+ cells in the gill section was undertaken using ImageJ 1.8v software. Twelve randomised fields of view of 10 inter-secondary lamellar spaces in the mid-section of the primary lamella (n=6 pre-treated fish (0h) and n = 6 14 dpt fish) were assessed, one section per fish and six different images taken within the section. Gill images were processed splitting the colour channels. Blue channel was selected because it provided best highlighting of the $CD3^+$ marked cells. This image was then adjusted to a threshold of 0 – 121. The same parameters were used for all images. The threshold adjustment masked labelled cells which belonged to the $CD3^+$ cell population. After this, the analysis feature was used to measure the area of the image that was stained with colour red (IHC staining previously explained in section 2.6). The expression ratio was calculated following the equation shown below.

$$\boldsymbol{CD3+\ cell\ expression\ ratio} = \frac{\%\ \textbf{experimental (0h and 14dpt) slides area stained}}{\%\ \textbf{TBS buffer only slides area stained}}$$

2.8. Statistical analysis

All the results obtained from the semi-quantitative analysis and image analysis for CD3+ cell expression quantification was exported to IBM SPSS statistical analysis software (v23, IBM Corporation) and were all processed and tested to determine significant differences between mucous cell counts and cell expression within the different time points and fish. Kolmogorov-Smirnov test was first performed on the data to verify normality. As a result of non-normalised data, a Kruskal-Wallis was performed on the data, to examine the significance between

medians (time-point after treatment vs semi-quantification of mucous cells; time 0 fish vs time 14d fish for the $CD3^+$ cell expression quantification). Mann Whitney test was performed between the two sets of data from time 0 fish and 14d fish to investigate significant differences between fish.

Regarding the qPCR results, Kolmogorov-Smirnov test was performed on the data, to verify normality again. Data were then subjected to a one-way ANOVA to examine the significance between means (pre-treatment fish *vs* different time points post-treatment) for the gene expression. A further *post-hoc* Tukey HSD test was conducted to confirm the differences between groups.

3. Results and Discussion

3.1. Gill pathology

Pre-treated fish presented no pathological signs like the H_2O_2 treated fish when screened at 4h, 24h and 14d after treatment.

3.2. Mucous cells semi-quantitative analysis

Different distributions were observed in the number of mucous cells when ANOVA testing was performed on the data ($p < 0.05$). Semi-quantification of mucous cells showed a significant decrease in mucous cells numbers 14 dpt compared to the pre-treated group (0h) (post-hoc Tukey HSD test; $p = 0.00027$; n = 6) (Fig. 1).

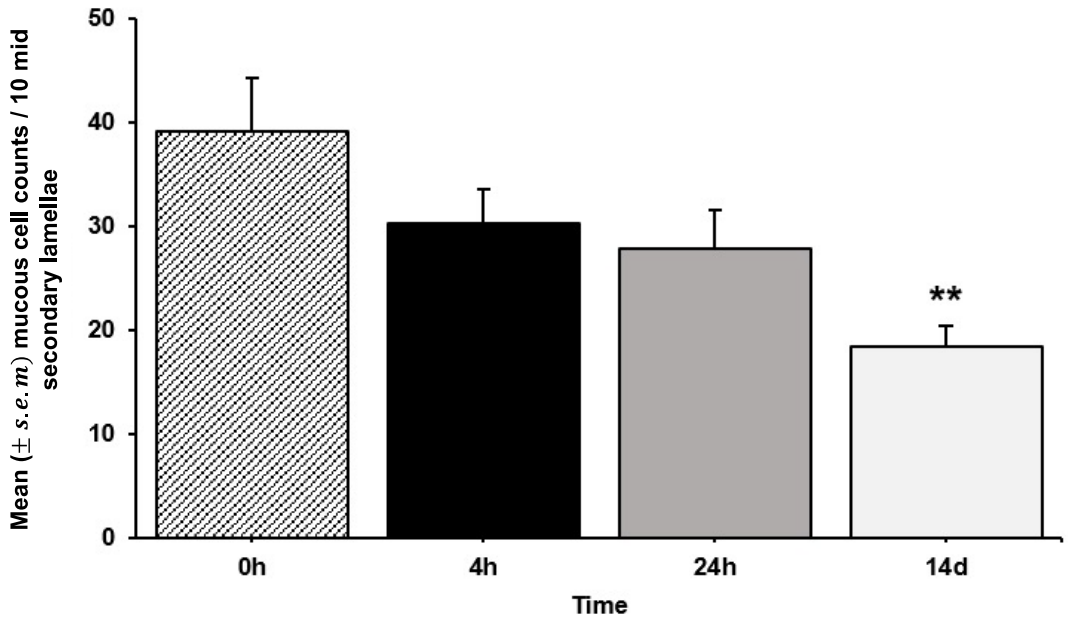


Figure 1. Semi-quantitative analysis of Atlantic salmon gill mucous cells. Graph showing the mucous cell counts across all the time points 0 h, 4 h, 24 h and 14 d post-H_20_2 treatment. Bars represent mean of mucous cell counts ± s. e. m, n = 6, 3 random fields of 10 interlamellar spaces; post-hoc Tukey HSD test: $p < 0.001$**).

Results indicated that the lowest number of cells were observed at 14 dpt. Thus, it could be speculated that H_2O_2 had an impact on the ability of gills to regenerate mucous cells over a 14-day period. Previously, gill mucus lysozyme activity was found to gradually decline in H_2O_2 treated olive flounder, *Paralichthys olivaeceus*, where a decrease in mucous cell numbers was also observed after 12 days with a H_2O_2 treatment at a dose of 500 mg L^{-1} [19]. Skin mucus lysozyme activity has also been related to changes in epidermal thickness and mucus production/composition caused by H_2O_2 treatment [32].

3.4. Immunohistochemistry for $CD3^+$ cell expression quantification

Two of the 6 H_2O_2-treated fish (F1 and F2) had a $CD3^+$ cell expression ratio between 2–5 fold greater than the highest $CD3^+$ cell expression ratio of non-treated fish (Fig. 2). However, the use of a limited gill arch area and a small gill size resulted in high variability amongst the gill sections from the pre-treated and 14 dpt fish. For future work, a greater number of replicates may need to be assessed on different gill arches to determine whether the trend observed is statistically significant.

T-cells are found to be distributed in many tissues of the fish; however, accumulations of these cells are greater in the thymus, spleen and, more recently, reported in the gill epithelium where lymphoid structures were characterised [33]. As the development of the CD3 monoclonal antibody applied for IHC in the current study was not undertaken until after this trial, and the targeted T cell analysis was only decided following qPCR analysis of crude gill samples, thus specific sampling of the interbranchial lymphoid (IBL) tissue was not performed. Therefore, the distribution of the $CD3^+$ cells were assessed along the primary and secondary lamellae through image analysis (ImageJ software), but this may have resulted in high variation due to vast differences between lamellar tissues compared to more immunologically active tissue (e.g. IBL).

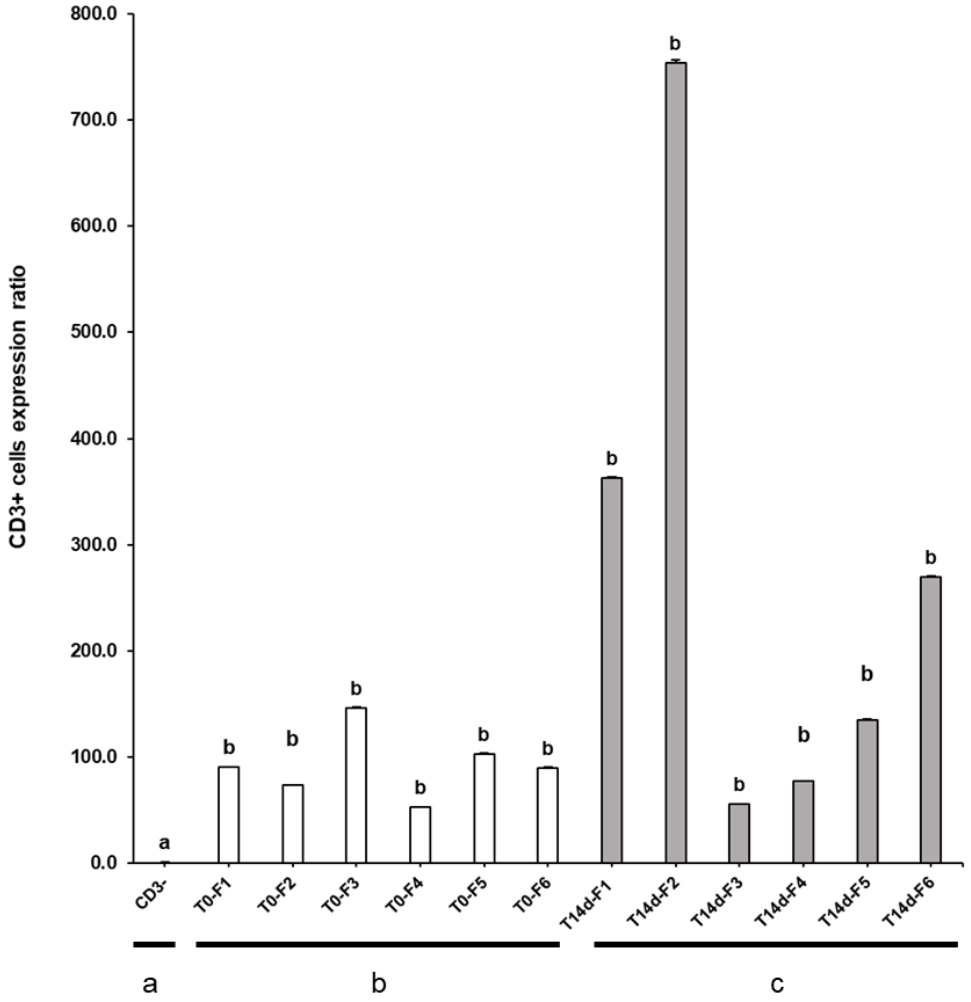
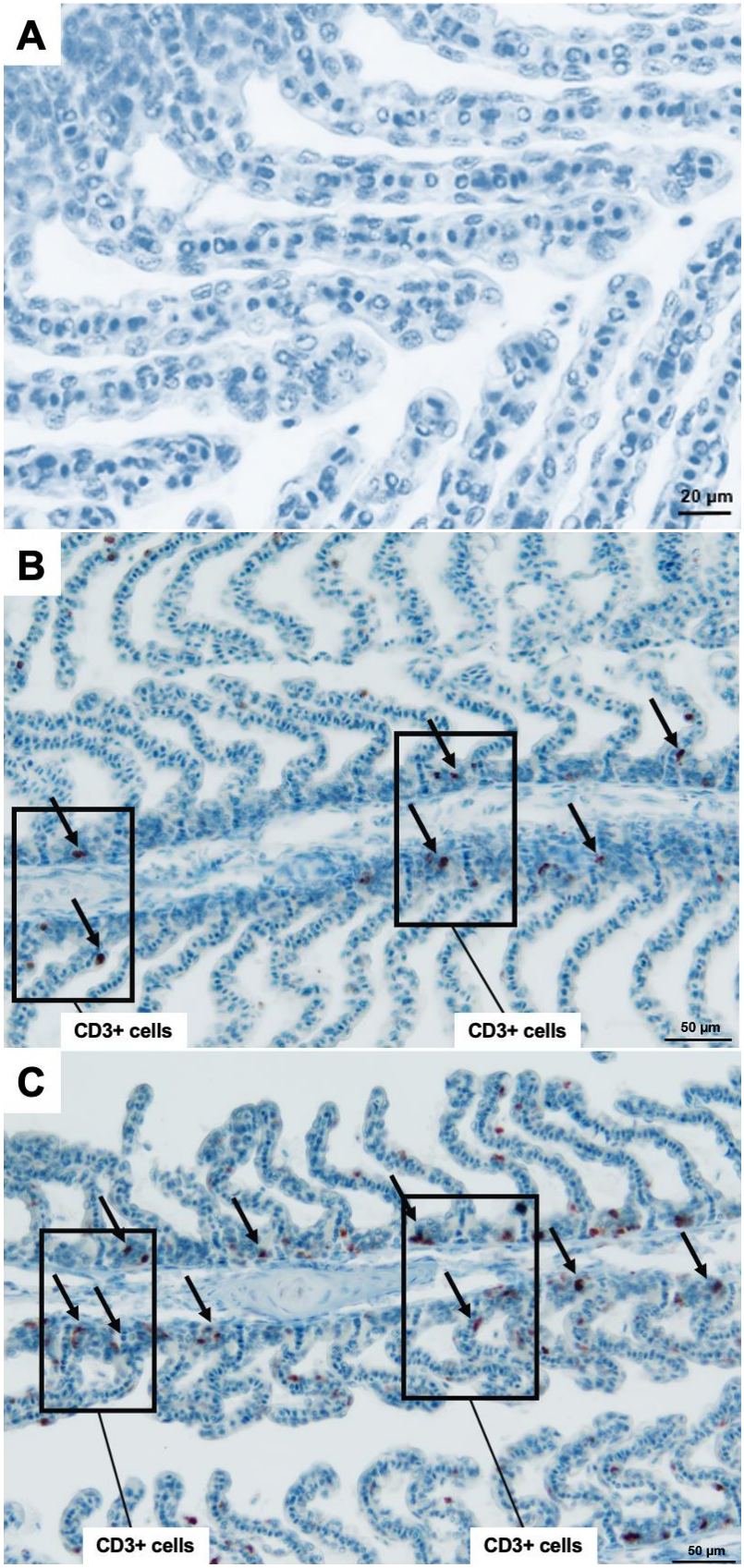


Figure 2. Quantification of the presence of $CD3^+$ cells within the gills of Atlantic salmon in time 0h fish and 14 dpt fish treated with H_2O_2: a. $CD3^-$ is the pre-treated slides with TBS buffer only; b. T0 pre-treated fish 1 (F1) to fish 6 (F6); c. T14d is the time point 14 dpt from fish 1 (F1) to fish 6 (F6). Error bars show s.e.m. Different letters on top of the bars represent statistical differences ($p < 0.05$). Statistical differences only between pre-treated group ($CD3^-$) and the different time points with $CD3^+$ cells.

TBS buffer only pre-treated slides with no anti-CD3 antibody can be observed in Figure 3A, where no red colouration was observed. For the time 0h fish, red stained cells ($CD3^+$ cells) can be observed (Fig. 3B) but fewer than observed in the 14dpt fish (Fig. 3C). However, not all fish displayed obvious colouration, with a lot of variation observed between gill sections and fish. This could be due to the different responses to the treatment between fish or more likely to individual animal / lamellae variability.



318

319 **Figure 3.** Representative images from (A) Atlantic salmon gills with: TBS buffer only; (B) Atlantic salmon gills
320 from time point 0h (pre-treated fish) with antibody added; (C) Atlantic salmon gills from time point 14 dpt with
321 antibody added. Boxes show the area with CD3+ cells along the primary and secondary lamellae (arrows).

3.5. Gill gene expression

Gene expression was quantified in relation to the geometric mean of the three reference genes EF1-α, β-actin and β-tubulin, in the fish gill after H_2O_2 treatment. Of the 6 fish that were sampled, one provided very poor quality of RNA, therefore only 5 fish was used for the gene expression analysis.

Quantitative PCR results showed that T-cell activity appeared significantly up-regulated 14 d post-H_2O_2 treatment, in TCRα chain ($p = 0.00058$, $n = 5$), CD8α ($p < 0.00021$, $n = 5$) and CD3γδ-B ($p = 0.013$, $n = 5$) genes; up-regulation of a cytokine indicative of an anti-inflammatory response, IL-4/13β2 gene, was observed after 14 d post-treatment ($p = 0.017$, $n = 5$) (Figure 4A&C). Meanwhile, significant down-regulation was observed in IL-22 ($p = 0.0003$, $n = 5$) across all time points (Figure 4C) and in the three mucin genes after 14 d post-treatment ($p = 0.00026$, $n = 5$) (Figure 5).

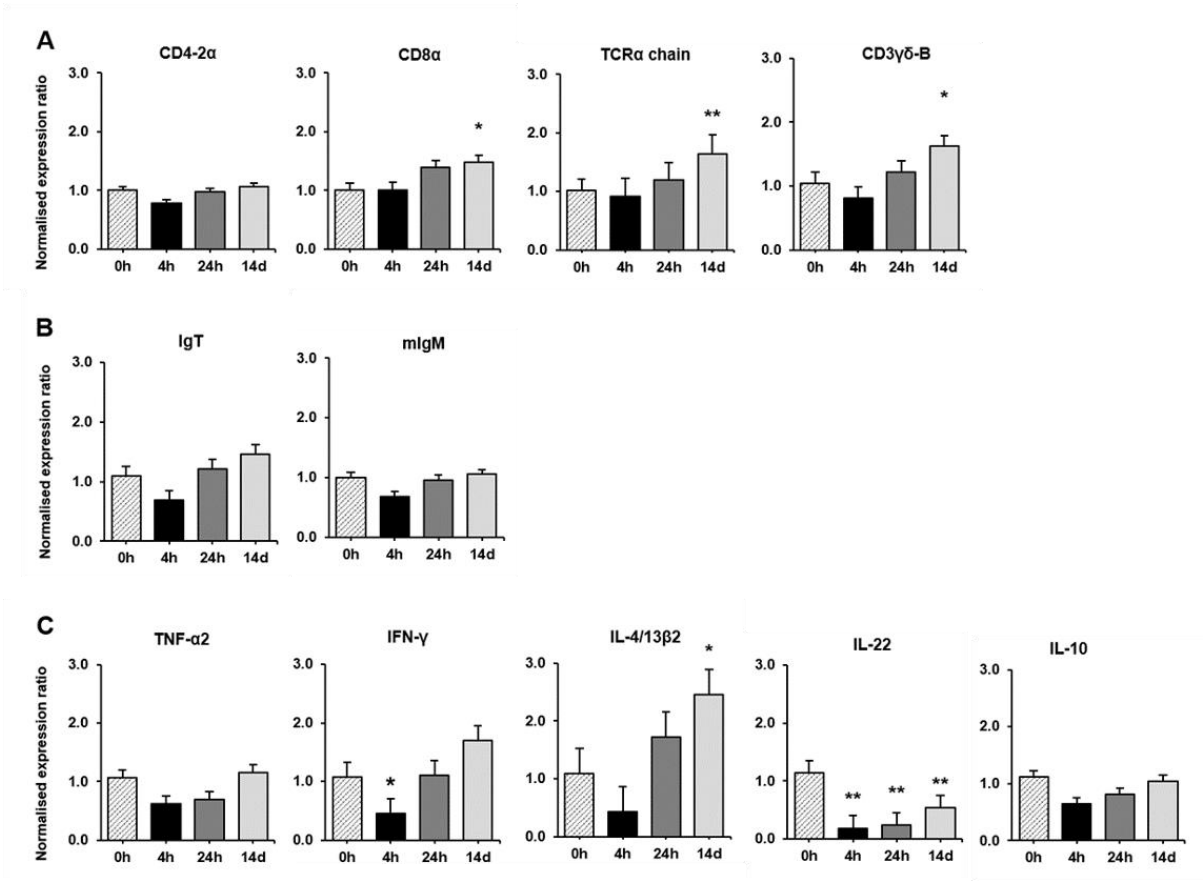


Figure 4. Quantitative RT-PCR analysis of (A) T-cell, (B) B-cell, and (C) Th1/Th17 and Th2 pathway related gene expression in healthy gill samples from Atlantic salmon after H_2O_2 treatment within different time points (0h, 4h, 24h and 14 d). Statistical differences were determined by a *post-hoc* Tukey HSD test. Results are normalised expression ratios (average ± s.e.m, n = 5) of the expression of these genes in relation to pre-treatment time point (0h). Asterisk (*) denotes statistically significant regulation in target gene expression relative to the pre-treated fish ($p < 0.05$) while double asterisk (**) represents highly significant regulation ($p < 0.001$).

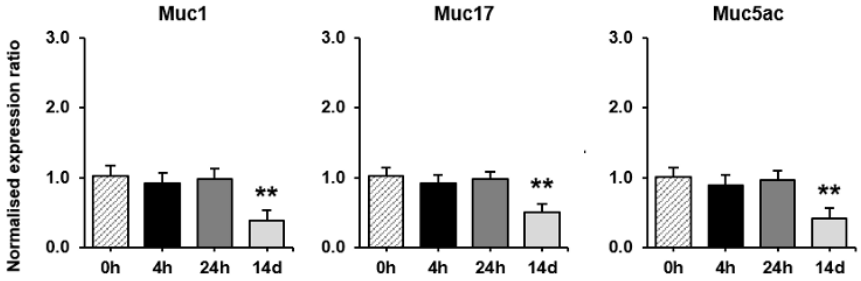


Figure 5. Quantitative PCR analysis of mucin related gene expression in healthy gill samples from Atlantic salmon after H_2O_2 treatment within different time points (0h, 4h, 24h and 14 d). Statistical differences were determined by a a post-hoc Tukey HSD test. Results are normalized expression ratios (average ± s.e.m, n = 5) of the expression of these genes in relation to pre-treatment time point (0h). Asterisk (*) denotes statistically significant regulation in target gene expression relative to the pre-treated fish ($p < 0.05$) while double asterisk (**) represents highly significant regulation ($p < 0.001$).

The down-regulated mucin gene expression could be related to a potential reduction in mucus production which can lead to the stimulation of immune responsiveness in the gills to external environmental antigens (*e.g.*, pollutants, chemicals, other insults), when fish are exposed with less protective mucus covering the gill epithelium [22]. There have been studies investigating the role of certain insults in the aquatic environment in wild populations [37, 38]. This has also been a problem in the aquaculture industry, where the accumulation of high fish stocks can provoke fluctuations in the environment (*e.g.*, algal blooms, temperature oscillations, hypoxia, supersaturation, chemical, predation, escapees, and infectious diseases) [39]. The most common routes of exposure to these insults are through gill surfaces [40, 41, 42]. Because of their large surface, they become the first targets of insults in the water causing histopathological changes [43] which translate in a potential shift in the immune response as was observed in the current study.

This translated in significant up-regulation of T-cell markers (*i.e.*, CD8α, TCRα chain and CD3γδ-B) at 14 dpt, which could mean that there is infiltration and involvement of a cellular response [44]. The up-regulation of the CD3γδ-B marker also correlates with the higher presence of $CD3^+$ cells along the primary and second lamellae of the 14 dpt gill as observed with IHC in gill sections (Fig. 3C). Even though these differences were not statistically significant between the pre-treated fish and the 14 dpt fish, a tendency of higher presence of $CD3^+$ cells in the fish sampled 14 dpt was noted with 2/6 treatment fish exhibiting 2-5-fold greater numbers of $CD3^+$ cells compared to the highest $CD3^+$ cell count of pre-treatment gills.

Lastly, the up-regulation of the Th2 cytokine IL-4/13β2, which is known to have an anti-inflammatory capacity [45], may be induced to prevent extensive inflammatory responses that may occur beyond pathogen/agent clearance. This prevents further damage to healthy gills by chronic inflammation, causing down-regulation of the immune response until homeostasis is reached [46, 47]. In addition to this cytokine, IL-22 transcripts were down regulated, even after

4-24 h post-treatment, which has been hypothesised to play a role in activating antimicrobial peptide genes and antibacterial immunity [47–50]. Hence, its down regulation may have implications on the presence of bacterial pathogens in the gill and the ability of salmon to resist a potential pathogen/agent.

However, the experimental design of this study could be improved. During this study, the only pre-treated fish were from the time point 0 h. As a result, there were no pre-treated fish sampled over time and thus, time and treatment being confounded. It must be considered that there might be a potential difference between time 0 and treated fish on day 14 post-treatment due to changes caused by the holding time and conditions during those two weeks. Nonetheless, treatment-induced immune modulation was certainly likely considering the rapid significant down-regulation of certain cytokines, *i.e.*, IFNγ and IL-22 after only 4-24 h post-H2O2 treatment compared to untreated fish from the same stock.

4. Conclusions

This study highlighted H_2O_2 impacts on immunological activity in the gills of healthy treated Atlantic salmon. This immune modulation may be due to the decrease in mucous cell numbers up to 14 dpt, translating into a potential reduction of the protective mucosal coat normally found in untreated fish during a 14-day period of recovery after exposure to H_2O_2. Understanding the safety margins of applying this important chemical is key for monitoring health of treated stocks, including healthy fish within the treated population.

Ultimately, this study suggests that H_2O_2 treatment does not immunocompromise Atlantic salmon but does result in modulation of immunity and disruption of the mucus covering the gills. This provides a platform for future research focusing on the mucosal health in salmon. Future work must study the effects of holding time and hydrogen peroxide separately.

5. Acknowledgements

The authors would like to thank Mr. Chessor Matthew, Dr. William Roy and Dr. David Bassett at MERL for provision of tanks and maintenance of fish during the study. The authors would like to thank Ms. Debbie Faichney for her technical advice and support with histology. This study has received funding from the European Union's Horizon 2020 Research and innovation programme under Grant Agreement no. 634429 (ParaFishControl). This output reflects only the author's view, and the European Union cannot be held responsible for any use that may be made of the information contained herein.

6. References

1. Rodger H.D., Henry L. & Mitchell S.O. (2011). Non-infectious gill disorders of marine salmonid fish. *Reviews in Fish Biology and Fisheries,* 21, 423–440. doi: 10.1007/s11160-010-9182-6.
2. Mitchell, S. O. & Rodger, H. D. (2011). A review of infectious gill disease in marine salmonid fish. *Journal of Fish Diseases*, 34(6), 411-432. doi: 10.1111/j.1365-2761.2011.01251.x.
3. Gjessing, M. C., Thoen, E., Tengs, T., Skotheim, S. A. & Dale, O. B. (2017). Salmon gill poxvirus, a recently characterized infectious agent of multifactorial gill disease in freshwater-and seawater-reared Atlantic salmon. *Journal of Fish Diseases*, 40(10), 1253-1265. doi: 10.1111/jfd.12608.
4. Gunnarsson, G. S., Karlsbakk, E., Blindheim, S., Plarre, H., Imsland, A. K., Handeland, S. & Nylund, A. (2017). Temporal changes in infections with some pathogens associated with gill disease in farmed Atlantic salmon (*Salmo salar* L). *Aquaculture*, 468, 126-134. doi: 10.1016/j.aquaculture.2016.10.011.
5. Young, N. D., Crosbie, P. B. B., Adams, M. B., Nowak, B. F., & Morrison, R. N. (2007). *Neoparamoeba perurans* n. sp., an agent of amoebic gill disease of Atlantic salmon (*Salmo salar*). *International journal for parasitology*, *37*(13), 1469-1481. doi:10.1016/j.ijpara.2007.04.018
6. Herrero, A., Thompson, K. D., Ashby, A., Rodger, H. D. & Dagleish, M. P. (2018). Complex gill disease: an emerging syndrome in farmed Atlantic salmon (*Salmo salar* L.). *Journal of Comparative Pathology*, 163, 23-28. doi: 10.1016/j.jcpa.2018.07.004.
7. Nylund, A., Watanabe, K., Nylund, S., Karlsen, M., Saether, P. A., Arnesen, C. E. & Karlsbakk, E. (2008). Morphogenesis of salmonid gill poxvirus associated with proliferative gill disease in farmed Atlantic salmon (*Salmo salar*) in Norway. *Archives of Virology*, 153(7), 1299-1309. doi: 0.1007/s00705-008-0117-7.
8. Fringuelli E., Savage P.D., Gordon A., Baxter E.J., Rodger H.D. & Graham D.A. (2012). Development of a quantitative real-time PCR for the detection of *Tenacibaculum maritim*um and its application to field samples. *Journal of Fish Diseases*, 35, 579–590. doi: 10.1111/j.1365-2761.2012.01377.x.

9. Ruane N., Rodger H., Mitchell S., Doyle T., Baxter E. & Fringuelli E. (2013). GILPAT: An Investigation into Gill Pathologies in Marine Reared Finfish. Marine Research Sub-Programme (NDP 2007–2013). Marine Institute, Oranmore.
10. Apablaza, P., Frisch, K., Brevik, Ø. J., Småge, S. B., Vallestad, C., Duesund, H. & Nylund, A. (2017). Primary isolation and characterization of *Tenacibaculum maritimum* from Chilean Atlantic salmon mortalities associated with a *Pseudochattonella* spp. algal bloom. *Journal of Aquatic Animal Health*, 29(3), 143-149. doi: 10.1080/08997659.2017.1339643.
11. Parsons, H., Nowak, B., Fisk, D. & Powell, M. (2001). Effectiveness of commercial freshwater bathing as a treatment against amoebic gill disease in Atlantic salmon. *Aquaculture*, 195(3-4), 205-210. Doi: 10.1016/S0044-8486(00)00567-6
12. Powell, M. D., Reynolds, P. & Kristensen, T. (2015). Freshwater treatment of amoebic gill disease and sea-lice in seawater salmon production: Considerations of water chemistry and fish welfare in Norway. *Aquaculture*, 448, 18-28. doi: 10.1016/j.aquaculture.2015.05.027.
13. Adams, M. B., Crosbie, P. B. B. & Nowak, B. F. (2012). Preliminary success using hydrogen peroxide to treat Atlantic salmon, *Salmo salar* L., affected with experimentally induced amoebic gill disease (AGD). *Journal of Fish Diseases*, 35(11), 839-848. doi: 10.1111/j.1365-2761.2012.01422.x.
14. Kiemer M.C.B. & Black K.D. (1997). The effects of hydrogen peroxide on the gill tissues of Atlantic salmon, *Salmo salar* L. *Aquaculture*, 53, 181–189. doi: 10.1016/S0044-8486(97)00037-9.
15. Avendaño-Herrera, R., Magariños, B., Irgang, R. & Toranzo, A. E. (2006). Use of hydrogen peroxide against the fish pathogen *Tenacibaculum maritimum* and its effect on infected turbot (*Scophthalmus maximus*). *Aquaculture*, 257(1-4), 104-110. doi: 10.1016/j.aquaculture.2006.02.043
16. Mansell, B., Powell, M. D., Ernst, I. & Nowak, B. F. (2005). Effects of the gill monogenean *Zeuxapta seriolae* (Meserve, 1938) and treatment with hydrogen peroxide on pathophysiology of kingfish, *Seriola lalandi* Valenciennes, 1833. *Journal of Fish Diseases*, 28(5), 253-262. doi: 10.1111/j.1365-2761.2005.00625.x.
17. Martinsen, K. H., Thorisdottir, A. & Lillehammer, M. (2018). Effect of hydrogen peroxide as treatment for amoebic gill disease in Atlantic salmon (*Salmo salar* L.) in different temperatures. *Aquaculture Research*, 49(5), 1733-1739.
18. Overton, K., Samsing, F., Oppedal, F., Dalvin, S., Stien, L. H. & Dempster, T. (2018). The use and effects of hydrogen peroxide on salmon lice and post-smolt Atlantic salmon. *Aquaculture*, 486, 246-252. doi: 10.1016/j.aquaculture.2017.12.041.
19. Hwang, B. O., Kim, Y. K. & Nam, Y. K. (2016). Effect of hydrogen peroxide exposures on mucous cells and lysozymes of gill tissues of olive flounder *Paralichthys olivaeceus*. *Aquaculture Research, 47*(2), 433-444. doi: 10.1111/are.12504.
20. Sies, H., Berndt, C. & Jones, D. P. (2017). Oxidative stress. *Annual Review of Biochemistry*, 86, 715-748. doi: 10.1146/annurev-biochem-061516-045037.
21. Vera, L. M. & Migaud, H. (2016). Hydrogen peroxide treatment in Atlantic salmon induces stress and detoxification response in a daily manner. *Chronobiology International*, 33(5), 530-542. doi: 10.3109/07420528.2015.1131164.
22. Linden, S. K., Sutton, P., Karlsson, N. G., Korolik, V. & McGuckin, M. A. (2008). Mucins in the mucosal barrier to infection. *Mucosal Immunology*, 1(3), 183. doi: 10.1038/mi.2008.5.

23. Sveen, L. R., Grammes, F. T., Ytteborg, E., Takle, H. & Jørgensen, S. M. (2017). Genome-wide analysis of Atlantic salmon (*Salmo salar*) mucin genes and their role as biomarkers. *PloS one*, 12(12). doi: 10.1371/journal.pone.0189103.
24. Marcos-López, M., Calduch-Giner, J. A., Mirimin, L., MacCarthy, E., Rodger, H. D., O'Connor, I. & Piazzon, M. C. (2018). Gene expression analysis of Atlantic salmon gills reveals mucin 5 and interleukin 4/13 as key molecules during amoebic gill disease. *Scientific Reports*, 8(1), 1-15. doi: 10.1038/s41598-018-32019-8.
25. Braden, L. M., Monaghan, S. J., & Fast, M. D. (2020). Salmon immunological defence and interplay with the modulatory capabilities of its ectoparasite Lepeophtheirus salmonis. *Parasite Immunology*, *42*(8), e12731. doi.org/10.1111/pim.12731.
26. Gallardi, D., Xue, X., Hamoutene, D., Lush, L. & Rise, M. L. (2019). Impact of origin (wild vs. farmed) and sea lice (Lepeophtheirus salmonis) infestation on expression of immune-relevant genes in Atlantic salmon (Salmo salar L.) skin. *Aquaculture*, 499, 306-315. doi: 10.1016/j.aquaculture.2018.09.036.
27. Fernandez, C., Mascolo, D., Monaghan, S. J., Baily, J. L., Chalmers, L., Paladini, G. Adams, A., Bron, J.E. & Fridman, S. (2019). Methacarn preserves mucus integrity and improves visualization of amoebae in gills of Atlantic salmon (*Salmo salar* L.). *Journal of Fish Diseases*, 42(6), 883-894. doi: 10.1111/jfd.12988.
28. Ingerslev, H. C., Pettersen, E. F., Jakobsen, R. A., Petersen, C. B. & Wergeland, H. I. (2006). Expression profiling and validation of reference gene candidates in immune relevant tissues and cells from Atlantic salmon (Salmo salar L.). *Molecular Immunology*, 43(8), 1194-1201. doi: 10.1016/j.molimm.2005.07.009.
29. Mowry, R.W. (1956) Alcian blue techniques for the histochemical study of acidic carbohydrates. *Journal of Histochemistry and Cytochemistry*, 4, 403-407.
30. Chalmers, L., Taylor, J. F., Roy, W., Preston, A. C., Migaud, H. & Adams, A. (2017). A comparison of disease susceptibility and innate immune response between diploid and triploid Atlantic salmon (*Salmo salar*) siblings following experimental infection with *Neoparamoeba perurans*, causative agent of amoebic gill disease. *Parasitology*, 144(9), 1229-1242. doi: 10.1017/S0031182017000622.
31. Monaghan, S. J., Thompson, K. D., Bron, J. E., Bergmann, S. M., Jung, T. S., Aoki, T., ... & Adams, A. (2016). Expression of immunogenic structural proteins of cyprinid herpesvirus 3 in vitro assessed using immunofluorescence. *Veterinary Research*, *47*(1), 1-14. doi: 10.1186/s13567-015-0297-6.
32. Fast M.D., Sims D.E., Burka J.F., Mustafa A. & Ross N.W. (2002) Skin morphology and humoral non- specific defence parameters of mucus and plasma in rainbow trout, coho and Atlantic salmon. *Comparative Biochemistry and Physiology*, A132, 645–657. doi: 10.1016/s1095-6433(02)00109.
33. Koppang, E. O., Fischer, U., Moore, L., Tranulis, M. A., Dijkstra, J. M., Köllner, B. & Hordvik, I. (2010). Salmonid T cells assemble in the thymus, spleen and in novel interbranchial lymphoid tissue. *Journal of Anatomy*, 217(6), 728-739. doi: 10.1111/j.1469-7580.2010.01305.x.
34. Nakanishi, T., Toda, H., Shibasaki, Y. & Somamoto, T. (2011). Cytotoxic T cells in teleost fish. *Developmental and Comparative Immunology*, 35(12), 1317-1323. doi: 10.1016/j.dci.2011.03.033.
35. Fallon, P. G., Jolin, H. E., Smith, P., Emson, C. L., Townsend, M. J., Fallon, R. & McKenzie, A. N. (2002). IL-4 induces characteristic Th2 responses even in the combined absence of IL-5, IL-9, and IL-13. *Immunity*, 17(1), 7-17. doi: 10.1016/s1074-7613(02)00332-1.

36. Vigano, S., Utzschneider, D. T., Perreau, M., Pantaleo, G., Zehn, D. & Harari, A. (2012). Functional avidity: a measure to predict the efficacy of effector T cells? *Clinical and Developmental Immunology*, 2012. doi: 10.1155/2012/153863.
37. Newman, M. C. (2001). *Population Ecotoxicology*. Wiley.
38. Calow, P., & Forbes, V. E. (2003). Peer reviewed: Does ecotoxicology inform ecological risk assessment? doi: 10.1021/es0324003.
39. Geffard, O., Geffard, A., His, E., & Budzinski, H. (2003). Assessment of the bioavailability and toxicity of sediment-associated polycyclic aromatic hydrocarbons and heavy metals applied to *Crassostrea gigas* embryos and larvae. *Marine Pollution Bulletin*, *46*(4), 481-490. doi:10.1016/S0025-326X(02)00451-4.
40. Grosell, M., Hansen, H. J. M., & Rosenkilde, P. (1998). Cu uptake, metabolism and elimination in fed and starved European eels (*Anguilla anguilla*) during adaptation to water-borne Cu exposure. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, *120*(2), 295-305. doi: 10.1016/s0742-8413(98)10009-9.
41. Handy, R. D. (1993). The accumulation of dietary aluminum by rainbow trout, *Oncorhynchus mykiss*, at high exposure concentrations. *Journal of Fish Biology*, *42*(4), 603-606.
42. Mallatt, J. (1985). Fish gill structural changes induced by toxicants and other irritants: a statistical review. *Canadian Journal of Fisheries and Aquatic Sciences*, *42*(4), 630-648. doi: 10.1139/f85-083.
43. Van Anrooy, R. (2006). *Review of the current state of world aquaculture insurance* (No. 493). Food & Agriculture Org.
44. Wang, Z. J., Liu, X. H., Jin, L., Pu, D. Y., Huang, J. & Zhang, Y. G. (2016). Transcriptome profiling analysis of rare minnow (*Gobiocypris rarus*) gills after waterborne cadmium exposure. *Comparative Biochemistry and Physiology Part D: Genomics and Proteomics*, 19, 120-128. doi: 10.1016/j.cbd.2016.05.003.
45. Liang, S. C., Tan, X. Y., Luxenberg, D. P., Karim, R., Dunussi-Joannopoulos, K., Collins, M. & Fouser, L. A. (2006). Interleukin (IL)-22 and IL-17 are coexpressed by Th17 cells and cooperatively enhance expression of antimicrobial peptides. *Journal of Experimental Medicine*, 203(10), 2271-2279. doi: 10.1084/jem.20061308.
46. Aujla, S. J., Chan, Y. R., Zheng, M., Fei, M., Askew, D. J., Pociask, D. A. & Kolls, J. K. (2008). IL-22 mediates mucosal host defense against Gram-negative bacterial pneumonia. *Nature Medicine*, 14(3), 275-281.
47. Sang, Y. & Blecha, F. (2008). Antimicrobial peptides and bacteriocins: alternatives to traditional antibiotics. *Animal Health Research Reviews*, 9(2), 227-235. doi: 10.1017/S1466252308001497.
48. Khader, S. A., Gaffen, S. L. & Kolls, J. K. (2009). Th17 cells at the crossroads of innate and adaptive immunity against infectious diseases at the mucosa. *Mucosal Immunology*, 2(5), 403. doi: 10.1038/mi.2009.100.
49. Monte, M. M., Zou, J., Wang, T., Carrington, A. & Secombes, C. J. (2011). Cloning, expression analysis and bioactivity studies of rainbow trout (*Oncorhynchus mykiss*) interleukin-22. *Cytokine*, 55(1), 62-73. doi: 10.1016/j.cyto.2011.03.015.
50. Qi, Z., Zhang, Q., Wang, Z., Zhao, W., Chen, S. & Gao, Q. (2015). Molecular cloning, expression analysis and functional characterization of interleukin-22 in So-iny mullet, *Liza haematocheila*. *Molecular Immunology*, 63(2), 245-252. doi: 10.1016/j.molimm.2014.07.006.