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In vivo and *in vitro* assessment of Atlantic salmon skin exposed to hydrogen peroxide

Christian Karlsen *, André Sture Bogevik, Aleksei Krasnov, Elisabeth Ytteborg

Nofima, Muninbakken 9-13, Breivika, Tromsø, Norway

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ABSTRACT

The salmon louse (*Lepeophtheirus salmonis*) is a significant problem in Atlantic salmon aquaculture. The industry is forced to use harsh antiparasitic treatments, including hydrogen peroxide (H_2O_2) baths, to treat the fish. We have utilized *in vivo*, semi *in vivo,* and *in vitro* methods to study the effects of exposures to H_2O_2. Assessment of external welfare indicators and plasma levels of cortisol, glucose, lactate, and ions did not reveal systemic impacts. Transcriptome analyses showed acute changes in the skin, with transient activation of several transcription factors known as generic stress markers of Atlantic salmon. Post-treatment (24 h), genes involved in the neutralization of free radicals were induced. Expression changes of regulators of cell cycle and cell differentiation were linked to microscopic observations in tissue sections and scale explants cultures. Histology showed adverse effects of H_2O_2 on skin morphology followed by compensatory recovery, which was not completed after 24 h. Decreased proliferation of cells was observed in keratocyte cultures. Exposure to H_2O_2, lowered pH (6.8), and elevated temperature (16 °C) markedly decreased the migration of keratocytes, which may indicate a reduced ability to repair skin and compound effects with H_2O_2. The study adds to the understanding of the adverse effects of hydrogen peroxide and promotes new models in Atlantic salmon skin research.

1. Introduction

Infections with the ectoparasite salmon lice (*Lepeophtheirus salmonis*) are among the greatest Atlantic salmon aquaculture problems, affecting production, health, welfare, and the public reputation of the industry (Torrissen et al., 2013; Liu and Bjelland, 2014). The situation has worsened in recent years due to the development of lice resistance to drugs used as feed additives (Aaen et al., 2015a). At present, fish producers are forced to apply harsh treatments such as thermal and mechanical de-lousing and bathes with chemotherapeutants, which negatively affect salmon health and increase mortality (Overton et al., 2019; Sommerset et al., 2020). Welfare scoring has been developed to assess damages from farming operations, including antiparasitic treatments (Noble et al., 2018; Bui et al., 2020). Hydrogen peroxide (H_2O_2) has been widely used as a de-lousing chemotherapeutants in salmon aquaculture (Johnson et al., 1993; Treasurer and Grant, 1997; Aaen et al., 2015b). Following a drastic increase in the first part of 2010, the use of H_2O_2 has reduced in the northern hemisphere due to development of resistance to this treatment in lice (Helgesen et al., 2015). However, this agent may still be included as a pharmaceutical alternative or reintroduced in comparative treatment regimens (Valenzuela-Muñoz et al., 2020). The extended use has also made H_2O_2 one of the most studied chemotherapeutants. H_2O_2 removes pre-adult and adult lice by mechanical paralysis induced by bubble development in the hemolymph (Aaen et al., 2014). It affects lice reproduction and viability of their offspring (Valenzuela-Muñoz et al., 2020). The adverse effects on salmon include oxidative and respiratory stress, which can eventually cause mortality (Johnson et al., 1993; Vera and Migaud, 2016; Overton et al., 2018; Valenzuela-Muñoz et al., 2020). The treatment can induce both local stress in gills and skin, and systemic responses (reviewed in (Sadoul and Geffroy, 2019)). The latter is mediated with the endocrine system, and cortisol is most commonly used as a marker of acute stress. The secondary responses are associated with the metabolic changes manifested with increased levels of plasma glucose and lactate (Barton, 2002).

In Norway, farmed Atlantic salmon suffer from skin damages (Sommerset et al., 2020), and a conservative estimate of losses due to ulcerations is from 1.1 to 2.5%. In addition, 0.7 to 3.8% of the products are down-classified at harvest due to skin-related disorders and following reduced market quality (Takle et al., 2015). Susceptibility to

* Corresponding author.
E-mail address: christian.karlsen@nofima.no (C. Karlsen).

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pathogens, damages, and importance for health and welfare explain continually increasing interest in research in Atlantic salmon skin. Skin is a barrier tissue essential for integrity, health, and welfare, characterized by exceptional complexity, reactivity, and the ability for regeneration and repair. In contrast to most land animals that have a dead layer of keratinized cells as the outer surface, the fish epidermis consists of live cells (reviewed in Sveen et al., 2020). These cells are responsive to external stimuli, which also may modulate wound healing (Jensen et al., 2015; Sveen et al., 2018). For superficial wounds or early stage of repair, re-epithelialization by rapidly migrating keratocytes restores the epithelial barrier (Quilhac and Sire, 1999; Richardson et al., 2016). The keratocyte model system has been assessed for studies of skin processes and responses (McDonald et al., 2013; Rapanan et al., 2014).

In the current work, we report an integrative study of effects of H_2O_2 applied in a therapeutic dose, which included welfare scores, plasma metabolites, skin morphological changes, including cellular responses, transcriptomics and cultures of scale explants. Two experiments were performed at different research stations. The work aimed to develop further methods and models for studies of Atlantic salmon skin. Studying the effects of exposure to H_2O_2 using *in vivo*, semi *in vivo,* and *in vitro* methods, we focused on linking external indicators, physiological stress parameters, cellular processes, tissue morphology, and transcriptome.

2. Material and methods

2.1. Experiment 1. In vivo H_2O_2 treatment

The experiment was conducted at the Research Station for Sustainable Aquaculture, Sunndalsøra, Norway. The experimental trial was approved by the Norwegian Animal Research Authority (NARA) and was conducted under regulations controlling experiments and procedures in live animals in Norway. Atlantic salmon smolts ($n = 180$) were obtained by artificial photoperiod and transferred to seawater 7 months prior to study start. Fish received commercial feed (Skretting, Norway, 4 mm). Thirty Atlantic salmon were randomly netted and distributed in eight experimental tanks 13 days prior to exposure (average bulk weight 1112.1 ± 24.6 (g); mean ± SD). Tanks contained a water volume of 1 m^3 with a temperature of 8 °C and the seawater flow-through rate set to 33–37 L min^{-1}. Tanks for H_2O_2 treatment were randomly allocated. Fish were starved for 3 days to mimic industry procedures. H_2O_2 (hydrogen peroxide 35%, Fybikon, Norway) was added in four tanks to a final tank concentration of 1.0 g L^{-1}, which is commonly used by the industry for de-lousing (Treasurer and Grant, 1997; Overton et al., 2018). The chemical was prediluted ten times to prevent any short time exposure to high H_2O_2 concentration. A recirculation pump was used in each tank. Water flow was turned off, and H_2O_2 was added in front of the recirculation pump that was used to ensure an even distribution of the chemical. The water levels were measured to ensure equal volumes; the oxygen levels and pH were controlled. Four control tanks were run in parallel to take into account the netting effect of repeated sampling. The treatment was terminated after 20 min by refilling the tank with seawater and readjusting the flow rate to 33–37 L min^{-1}. All sampled fish were netted and euthanized with an overdose of benzocaine (ACD Pharma), and sampling finished within 15 min post netting. Five fish from tanks 1–3 in both treatment and control were sampled 24 h prior to treatment, and 4 h, and 24 h post-treatment. Five fish from the fourth tank in both treatment and control were sampled immediately (0 h) post-treatment.

2.2. Welfare assessment and sampling

Fish were visually inspected and evaluated scoring the condition of the fins, snout, skin hemorrhaging, and scale loss falling into one of four categories (Noble et al., 2018): no, minor, moderate, and severe damage/effect.

Two pieces of skin were aseptically removed by a scalpel from the left side of each fish in the area posterior of the dorsal fin and above the lateral line. Samples for histology were added into 20 mL pots containing 10% buffered formalin (CellStor™ pots, CellPath) and stored at 4 °C. Additional samples for microarray analysis were stored in RNAlater™ (Invitrogen) at −80 °C until RNA extraction. The blood sample was taken within 15 min post netting by caudal puncture utilizing serum tubes (BD Vacutainer®). Tubes stored at 4 °C were centrifuged at 5000*g* for 10 min, serum was aspirated and stored at −20 °C. Serum samples were analyzed using the chemical benchtop analyzer Pentra C400 (Horiba), measuring cortisol, glucose, lactate, potassium (K^+), sodium (Na^+), and chloride (Cl^-) according to the manufacturer's protocol.

2.3. Imaging of skin tissue sections

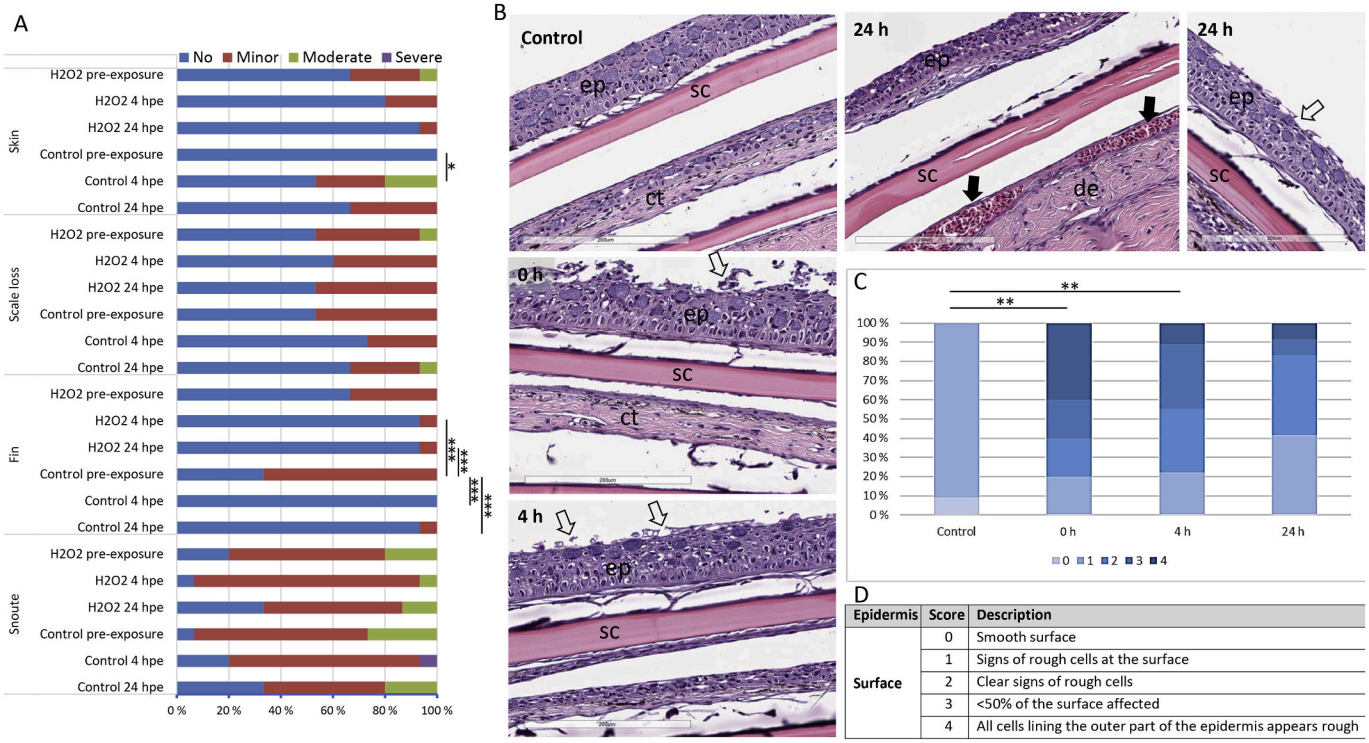
Skin samples ($n = 5$ time 0 and $n = 15$ at time points pre-treatment, and 4 h and 24 h post-treatment) fixed in buffered 4% formalin were carefully dissected, orientated, and placed in tissue embedding cassettes (Simport, Quebec, Canada). To decalcify the skin, samples were incubated in 10% EDTA (Merck KGaA, Darmstadt, Germany), pH 7, for 4 days. Tissue processing was performed using an automated tissue processor (TP1020, Leica Biosystems, Nussloch GmbH, Germany) where the samples were dehydrated through to 100% alcohol and then a clearent xylene bath before infiltration in melted 60 °C paraffin (Merck KGaA, Darmstadt, Germany). Paraffin-embedded tissue samples were cut in 5 μm sections using a microtome (Leica RM 2165), mounted on polysin-coated slides (VWR, Avantor, Pennsylvania, USA), and dried overnight at 37 °C. The sections were deparaffinized and rehydrated, and staining was performed using an automated special stainer (Autostainer XL Leica Biosystems, Nussloch GmbH, Germany). Parallel sections ($n = 2$ per fish) were stained with hematoxylin-eosin (HE) (Sigma-Aldrich) and Alcian blue (Sigma) and periodic acid-Schiff (Merck) (AB-PAS). Overall morphology was examined in HE-stained sections, numbers of mucus cells were counted in AB-PAS images. Total numbers of magenta (neutral) and blue (acidic) mucus cells were counted, and the ratios basic:acidic mucus cells calculated. The epidermis and dermis thickness was measured ($n = 10$ measurements per sample) using scanned sections and the Aperio Image Scope software (Leica Microsystems, Wetzlar, Germany). An in-house scoring system was used to evaluate the overall skin morphology. This system includes *e.g.*, the outer epidermis, mucous cells, and scale loss and has a scoring system ranging from 0 to 4, where 0 is considered no morphological changes and 4 is considered severely affected (Fig. 1).

2.4. RNA isolation and microarray

Total RNA was extracted from skin samples using the automated Biomek 4000 (Beckman Coulter) including an on-column DNase treatment according to the manufacturer's protocol. 2100 Bioanalyzer and RNA Nano Chips (Agilent Technologies) were used to verify the integrity of the RNA samples (RIN values > 7.5). RNA purity and concentration were measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies). Total RNA samples were stored at −80 °C until prepared for multiple gene expression profiling using Nofima's 15 K Atlantic salmon oligonucleotide microarray SIQ-6 (Agilent Technologies).

2.5. Experiment 2. Semi in vivo exposure and primary cell culture

The semi *in vivo* exposure investigated the effects of H_2O_2 and pH in combination with temperature. Six 1.3 kg sized Atlantic salmon from Norwegian Institute for Water Research (NIVA, Solbergstrand, Norway) reared in flow-through full strength seawater were netted and immediately killed by a blow to the head, individually placed in containers filled with tank seawater of 8 °C, transported to the laboratory and utilized within an hour post-mortem. Fish were treated individually using a system modified from Karlsen and colleagues (Karlsen et al., 2012),



Epidermis	Score	Description
Surface	0	Smooth surface
	1	Signs of rough cells at the surface
	2	Clear signs of rough cells
	3	<50% of the surface affected
	4	All cells lining the outer part of the epidermis appears rough

Fig. 1. A) Welfare scoring of skin (total of 90 fish, $n = 15$ per group per time point). B) Histology of skin samples stained with AB/PAS from control and 0, 4, and 24 h post H_2O_2 exposure. Notice the rougher outer border of keratocytes in the exposed samples, and empty mucous cells (white arrow), and occurrence of blood cells after 24 h (black arrow). Scale bar indicated. Ep, epidermis; sc, scale; de, dermis. C) Scoring results of outer epidermal border ($n = 15$ control, 4 h and 24 h, $n = 5$, 0 h). D) Scoring system used to evaluate the outer border. Differences between groups are shown as lines, * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$ (Kruskal-Wallis and Dunn's multiple comparisons test).

where 3 polypropylene cups (height: 7.3 cm, diameter: 5.7 cm) sealed by elastic bands are attached to the skin of each fish. Two treatments of skin were performed. Each treatment used three fish placed in trays with either 8 °C seawater (rearing temp) or 16 °C seawater (three centigrade above recommended temperature for H_2O_2 treatment of Atlantic salmon (Overton et al., 2018)). The skin area limited by the cups was either kept untreated (seawater control, pH 7.8), exposed to 1.5 g L^{-1} H_2O_2, or treated with seawater with a lowered pH at 6.8 by HCl (Sigma) for 20 min at both temperatures. Control and exposed parts of the skin were sampled for histological evaluations, as described for the first experiment.

The semi *in vivo* skin model is powerful by reducing the need for research on live animals as several treatments can be applied simultaneously by compartmentalizing the skin surface. In terms of complexity, responses involving many cell types are close to those in living fish, which is impossible to reach in *in vitro* systems. To test the vitality and use of skin as a semi *in vivo* model, we evaluated the skin morphology through a time-series, where samples were taken from dead fish 20 min after killing, and after 1, 2 and 5 h ($n = 3$). Results showed no morphological changes after 1 h, but minor damage such as loose keratocytes could be found in one sample after 2 h. After 5 h, three out of three samples had loose keratocytes, emptied mucous cells in the outer epidermal region, and edema was observed in the red muscle (Supplementary file 1).

Scales for *in vitro* studies were carefully picked using forceps and placed in 12 well tissue culture plates (Falcon Multiwell™ Becton Dickinson, NJ, USA.), 3 scales per well, 3 wells per fish. Each well contained L-15 supplemented with fetal bovine serum (FBS) 10%, 25 μg amphotericin B, 10 mL/L antibiotics, antimycotics and 0.01 M HEPES (Sigma). Plates were incubated at 8 °C or 16 °C in a cell incubator without CO_2. The cells were microscopically analyzed every day (Leica). Migration effect was measured as the ratio between scales with migrating cells and scales without migrating cells. Cells for immunohistochemistry were grown on glass-slides, and fixated in 4% paraformaldehyde (PFA, Sigma).

2.6. Immunohistochemistry

Fixed cells were washed twice in FBS, incubated 10 min in 50 mM NH_4Cl, washed twice in PBS, and permeabilized in 0,1% Triton X-100 for 10 min before blocking with 1% BSA for 30 min. Slides were incubated with primary rabbit anti-mouse iNOS from Affinity Bioreagents Inc. (Golden, CO, USA) diluted 1:2000 for 1 h and washed before secondary antibody (anti-rabbit IgG) diluted 1:50 (Dako, Glostrup, Denmark) and 4′, 6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific Inc.) was added (diluted in 1% BSA) and incubated 1 h. Slides were washed 3× in PBST, mounted and microscopically examined. Images and measurements were done using Zeiss Axio Observer Z1 equipped with an AxioCam MRc5 camera and AxioVision software (Carl Zeiss Microimaging GmbH, Göttingen, Germany). Live cells were stained with Mitotracker and EdU (Invitrogen) according to the manufacturer's protocols.

2.7. Statistical analyses

Statistical analysis was performed using JMP®Pro v13.1.0. Data were assessed for normal distribution using normal quantile plots evaluated by the Shapiro-Wilk normality test. For comparisons of parametric data, group means were tested by two-way ANOVA with *post hoc* pairwise multiple comparisons using Tukey honest significant difference (HSD). Nonparametric data, including welfare scoring, were analyzed by the Kruskal–Wallis test followed by Dunn *post hoc* tests for all pairs by ranking to compare the groups ($p < 0.05$). Gene expression data were processed and analyzed with the aid of Nofima's

bioinformatics package (Krasnov et al., 2011). In brief, global normalization was performed by equalizing the mean intensities of all microarrays. Next, the individual values for each feature were divided to the mean value of all samples, thus expression ratios (ER) were calculated. The log2-ER were normalized with the locally weighted non-linear regression (Lowess). Differentially expressed genes (DEG) were selected by criteria: 1.75-fold and $p < 0.05$.

3. Results

3.1. Welfare status after H_2O_2 exposure

In total, of 90 fish ($n = 15$ per group at each sample time point, Fig. 1A) were scored for the welfare of fin, snout, skin and scale loss during the H_2O_2 treatment. Differences between the time-points were observed within fin and skin status, but there was no significant contribution of H_2O_2. Occurrence and categorization of snout damage were higher in comparison with other indicators.

3.2. Effects of H_2O_2 exposure on skin morphology

The morphology of the skin sampled 24 h pre-exposure was in concordance to what is normally observed in healthy farmed salmon: a smooth outer epidermis with intact mucous cells, and evenly distributed mucous cells in the deeper epidermis (Fig. 1B). The outermost layer of keratocytes makes the continuous outer border, which is important for the skin's barrier properties. Skin sections from the *in vivo* trial sampled at 0 h, 4 h, and 24 h post H_2O_2 exposure were compared to the control. The epidermis surface was most affected immediately after treatment (0 h): rough cells in the outer epidermis and empty mucous cells were observed in all samples (Fig. 1B). The severity and surface of damaged area decreased with time, but full recovery was not observed within 24 h. 5 out of 15 samples had the occurrence of hematomas 24 h post-treatment compared to 2 out of 15 samples 4 h after exposure. In the control group, 1 out of 15 fish had blood cells in the loose dermis, but this sample also had missing scales and damaged epidermis. Most of the blood cells were located under the epidermis or between the dense dermis and the scales.

3.3. Transcriptional changes in Atlantic salmon skin in response to H_2O_2 exposure

The H_2O_2 treated group was compared with the control and differences steadily increased with time (Fig. 2), the number of DEG (>1.74-fold, $p < 0.05$) being 34, 76, and 121 at respectively 0 h, 4 h and 24 h post-exposure. The earliest stress sensors activated at 0 h include *gadd45* together with *small inducible cytokine a13*, *lectins*, several proteases and protease inhibitors, *matrix GLA* (ECM protein), water channel *aquaporin,* and two growth factors. A transient up-regulation at 4 h was shown for a cluster of genes including mainly transcription factors that control the expression of other genes including *junb*, *junc*, *immediate early response 2* and *c/ebp beta* and *kruppel* factors. Of further note was the up-regulation of motor proteins (*troponin* and *tropomyosin*), *glutamate synthase* and several immune genes including enzymes of amine metabolism (*arginase II* and *ornithine decarboxylase*). A cytoprotective system based on glutathione and chaperones was activated at 24 h. An increase of hemoglobin-related transcripts together with other blood-associated gene products was also detected. Several cell cycle regulator genes were significantly down-regulated in addition to expression changes of a small number of immune genes.

3.4. Biochemical blood parameters

Serum cortisol levels increased at 4 h post-exposure and restored at 24 h after with no significant difference between the control and H_2O_2 exposed groups (Fig. 3). The glucose levels mirrored the cortisol pattern (transient increase at 4 h was followed with recovery at 24 h), but lactate did not change. The concentrations of ions (K^+, Na^+, Cl^-) at 24 h were lower in comparison with the pre-exposure level in both treatment groups.

3.5. Semi in vivo skin model

The semi *in vivo* skin model was used to study the effect of H_2O_2 and pH combined with temperature on skin morphology. In the control fish kept at a constant temperature, the epidermal layer of skin was characterized by an intact, smooth outer surface formed by flattened keratocytes, as described for the control fish in the *in vivo* trial. This structure was disrupted when exposed to H_2O_2 and lowering of pH, with flattened keratocytes missing, mucous cells bursting and the rounded, inner keratocytes exposed to the external environment. Both treatments worsened with the combined effect of high temperature (Fig. 4). The abrupt increase in temperature from 8 °C to 16 °C also severely affected the skin's epidermal surface, as a rougher outer border was observed. Cells in the epidermis of pH-exposed fish also had a more condensed appearance compared to the control.

Keratocytes were cultured from whole scale explants and used to investigate the effect of the stressors on migration (Fig. 5). The size and shape of cell sheets were different in cultures from scales of control fish and fish exposed to H_2O_2, and low pH in the semi *in vivo* model (Fig. 5A-I). Sheets of cells from the control fish migrated more actively, covered a larger surface, and exhibited a smoother leading edge compared to treated fish (Fig. 5BC and EF). The initial rates of migration were assessed by the percentage of scales with advancing cell sheets. Control fish scales reached the highest percentage within day 1, while in cultures from H_2O_2 treated skin, an increase was observed until day 3 with ~20% less scales compared to the control. Cell sheet migration was impaired in cultures from salmon exposed to low pH. Cells started dying at day 5, with a reduction from 40% of scales having migrating cells at day 3, to only 20% at day 5 (Fig. 5Q). Cells had less continuous leading-cell edges, with less lamellipodia in front and more cells stretching out. The rapid advancement of cell sheets is not caused by cell proliferation, as suggested by fluorescent labeling of EdU (Fig. 5JK). However, more cells showed evidence of cell division in explants from control scales compared to scales from H_2O_2 and low pH treated fish. Cultures from treated scales also had more intense staining of iNOS (Fig. 5LM), indicating more stress, and reduced staining of mitotracker, a marker for mitochondrial activity (Fig. 5NO).

4. Discussion

This study highlights the importance of combining multiple methods in research on fish health and welfare. The combination of methods applied in this study may improve our understanding of treatments and testing of new handling procedures before they are further exploited in large-scale commercial settings.

The transcriptional changes in Atlantic salmon skin after H_2O_2 treatment suggest compensatory responses. The immediate response includes upregulation of the stress sensor growth arrest and DNA damage gene, *gadd*45. The *gadd*45 gene is involved in responses to physiological stressors, which include cyclin kinases regulating the cell cycle. Several cell cycle master regulatory genes were down-regulated 4 h post H_2O_2 exposure, and further reduced 24 h post exposure. In response to H_2O_2, several transcription factors that control expression of various genes were up-regulated 4 h post-exposure. Observing the explant keratocyte culture suggested reduced vitality as observed as reduced migration potential, and less incorporating of the EdU marker, demonstrating that one of the responses to H_2O_2 treatment is reduced proliferation.

Exposing Atlantic salmon to H_2O_2 induces expression of oxidative stress genes in the skin (Valenzuela-Muñoz et al., 2020) and other tissues (Vera and Migaud, 2016; Chalmers et al., 2018). Our *in vitro* assay

Gene	0 h	4 h	24 h
GADD45 beta	*1.02*	*2.33*	*1.13*
C-C motif chemokine 13	*1.09*	0.06	0.24
Small inducible cytokine A13	*1.47*	0.09	0.34
Mannose-specific lectin	*3.69*	*3.19*	*4.39*
Lectin	*3.23*	*2.58*	*4.10*
Serine protease 23	*1.66*	*1.50*	*2.00*
Matrix metalloproteinase-20	*1.26*	0.75	0.43
Leukocyte elastase inhibitor	*1.25*	0.71	0.50
Aquaporin-1	*0.91*	0.35	0.47
Matrix Gla protein	*1.50*	*0.97*	*1.33*
Angiopoietin 7	*0.81*	0.51	*1.05*
Bone morphogenetic protein 8B	*0.87*	0.34	0.56
Jun B-1	-0.09	*1.28*	-0.17
Jun C	0.01	*1.01*	0.20
Jun C	0.32	*1.14*	0.71
Immediate early response 2	0.07	*1.22*	-0.18
DNA damage-inducible 4	0.12	*0.82*	0.20
C/EBP beta-1	0.08	*2.04*	0.51
C/EBP beta-2	0.02	*1.54*	0.26
C/EBP delta-1	0.41	*1.26*	0.49
C/EBP delta-2	0.52	*1.25*	0.44
Kruppel factor 9	0.37	*1.19*	0.63
Kruppel factor 4b	0.77	*1.97*	*0.99*
Hairy-related 6	-0.22	*-1.07*	-0.24
Glutamine synthetase	0.36	*0.80*	*1.10*
Tropomyosin alpha-3 chain	0.04	*0.90*	0.10
Troponin T2d, cardiac	0.59	*1.09*	0.89
Ornithine decarboxylase	0.08	*1.25*	0.60
MHC class I antigen	0.61	*0.97*	0.35
C-C chemokine receptor 9	0.43	*1.10*	0.62
Defensin beta 4	1.28	*1.37*	*1.81*
Arginase, type II	0.94	*0.99*	0.59
CD276 antigen	0.24	*1.96*	0.28
Glutathione reductase	0.07	-0.12	*1.23*
Glutathione S-transferase A	0.38	-0.07	*0.96*
Glutathione S-transferase A	0.39	-0.05	*0.93*
Glutathione S-transferase A	0.26	-0.08	*0.98*
Heat shock cognate 70	0.01	0.30	*0.86*
Heat shock protein 60 kDa	0.04	0.01	*0.83*
Transferrin-a	0.33	-0.14	*0.83*
Methionine adenosyltransferase	0.03	0.55	*1.23*
Glutamate-cysteine ligase	0.06	-0.10	*0.95*
Glutamine synthetase	0.30	0.37	*1.13*
G2/mitotic-specific cyclin-B1	-0.13	-0.48	*-0.80*
Cyclin-dependent kinase inhibitor 1C	-0.30	*-0.80*	*-0.93*
Ubiquitin-conjugating enzyme E2 C	-0.07	-0.46	*-0.81*
Protein regulator of cytokinesis 1	-0.07	-0.50	*-0.84*
MHC class I antigen	0.19	0.19	*-0.91*
MHC class II antigen	-1.00	-0.78	*-0.92*
Antigen peptide transporter	-0.72	-0.56	*-0.87*
C-C motif chemokine 19-2	-0.77	0.15	*-1.37*
complement C4-B	0.44	0.41	*0.80*
Complement component C7	0.65	0.38	*0.81*
Complement C3	0.20	0.01	*1.15*

Fig. 2. Selected differently regulated transcripts in Atlantic salmon skin in response to H_2O_2. Data are $\log_2$ ER relative to unexposed. Differentially expression is indicated with underlined italics. Up-regulated and down-regulated genes are highlighted with red and blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

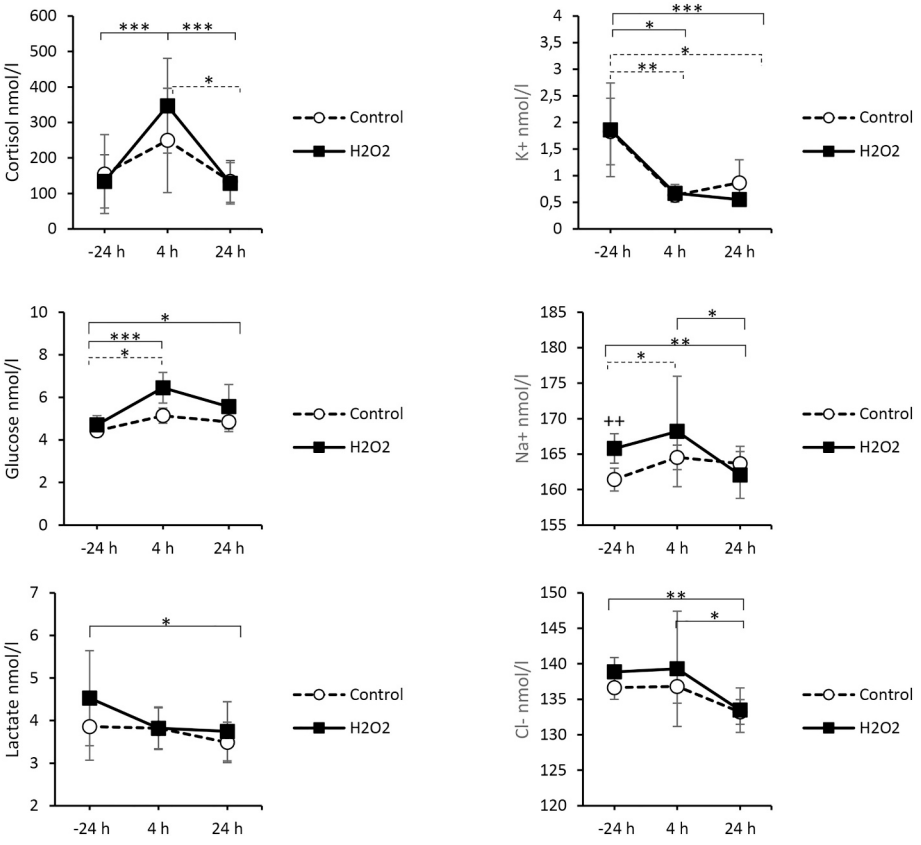


Fig. 3. Serum levels of cortisol, glucose, lactate, and the electrolyte concentration of potassium, sodium, and chloride (mean ± SD) over time in control (open) and H_2O_2 (black) groups. A Kruskal-Wallis test followed by Dunn's *post hoc* tests for all pairs by ranking were used to show significant difference for an experimental group between time-points as indicated by $*p < 0.05$, $**p < 0.001$, $***p < 0.0001$. Significant differences between experimental groups are indicated by $+p < 0.05$, $++p < 0.001$, $++p < 0.0001$. Time-points – 24 h and 24 h, $n = 15$. Time-point 4 h, $n = 10$.

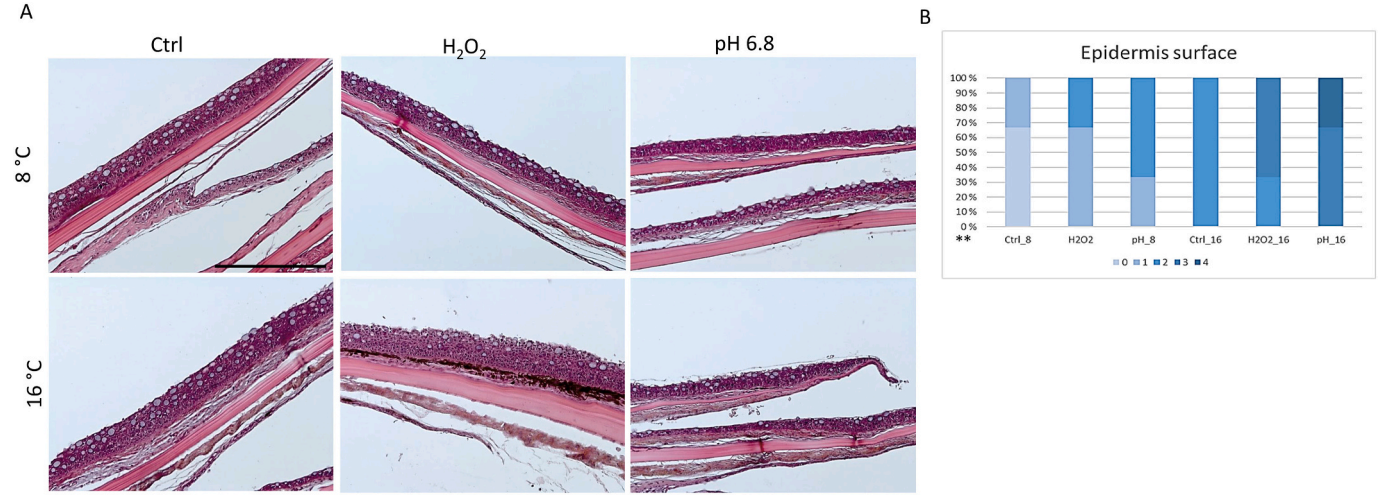


Fig. 4. Epidermis from the skin from Atlantic salmon used in the semi *in vivo* exposure model. Histological images of AB-PAS stain skin of A) control (seawater, pH 7.8), and skin exposed to H_2O_2 (1.5 g L^{-1}), and low pH 6.8, at 8 °C and 16 °C. B) Scoring results of the outer epidermal border ($n = 3$ per group).

suggested an increase of iNOS, an enzyme generating free radicals. Increased iNOS response was also observed in the skin of chronically stressed fish (Sveen et al., 2016). Our findings were further strengthened by the skin 24 h post-exposure transcription profile, which showed activation of a cytoprotective antioxidant system based on glutathione and chaperones (Forman et al., 2009; Takada et al., 2010). The transcription data indicate that glutathione synthesis could be mediated by *junb* and *junc* (Daniel, 1993) which were found up-regulated 4 h post-exposure and further stimulated by the increased glutamate and methionine metabolism (Gould and Pazdro, 2019). A role of glutathione in the oxidative stress response observed in tissues following exposure to H_2O_2 is corroborated with previous findings from Atlantic salmon (Chalmers et al., 2018) and other fish species (Tort et al., 2005; Sinha et al., 2020). The elevated abundance of hemoglobin transcripts indicates increased blood circulation at the recovery phase. This is further supported by a more pronounced detection of capillary formation and blood cells in skin 24 h post-exposure.

In addition to modulating cell proliferation and differentiation, H_2O_2 can induce other changes in the skin. In mammals, low doses of H_2O_2 can stimulate wound healing by epithelial cell adhesion and migration (Pan et al., 2011). Our results show that high doses used for the anti-parasitic treatment of Atlantic salmon inhibit various cellular and tissue

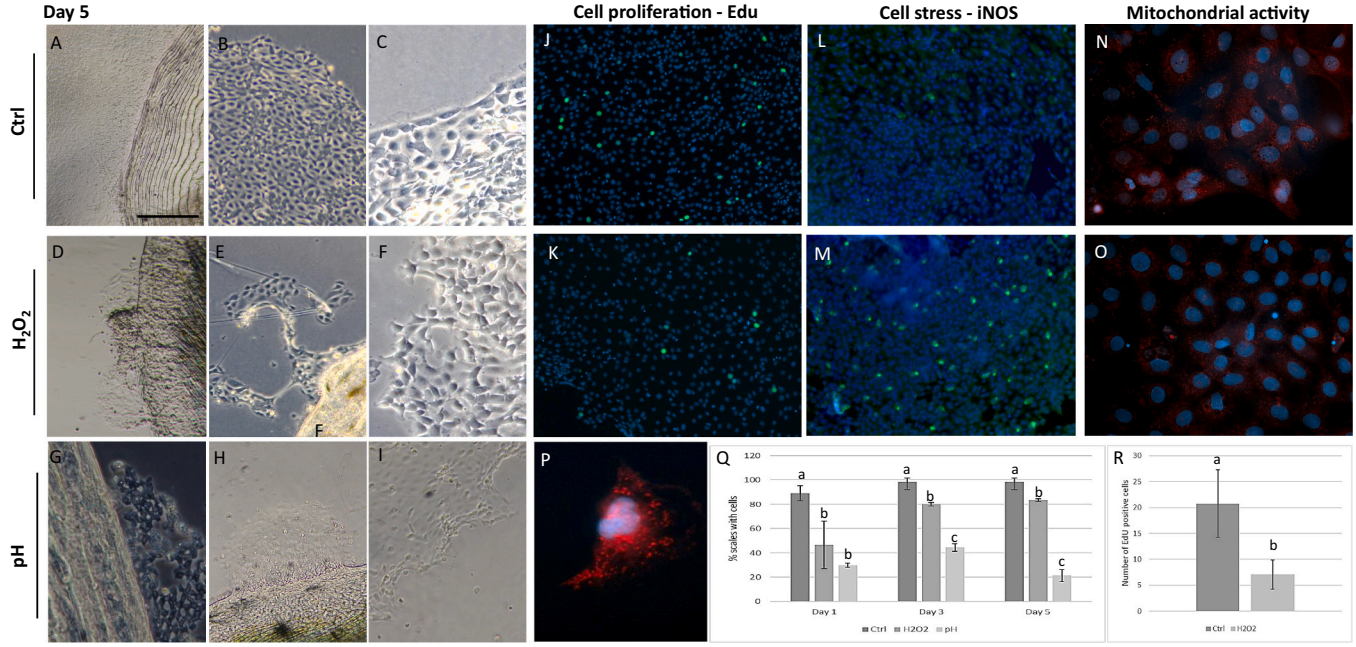


Fig. 5. Primary Atlantic salmon keratocytes after 5 days in culture. A) Confluent sheets of migrating cells with (B) continuous cell edges and (C) leader cells with lamellipodia in front were observed from control scales. From scales isolated from fish exposed to H_2O_2, cell sheets were (D) smaller and cell sheets less confluent, with (E) spacing between individual cells and (F) distorted frontline cells. Similar morphology was found in cells migrating from pH-treated scales, (G-H) reduced cell sheets, and (I) disturbed pattern of frontline cells. J-K) IHC with EdU shows reduced activity in H_2O_2 treated cells. L-M) increased activity of iNOS and (N-O) reduced activity of mitotracker. P) Details of mitotracker in a keratocyte, showing mitochondrion distributed around the nuclei and extending to the lamellipodia. Q) Percentage of migrating cells from the scales after 1, 3, and 5 days. R) Difference in EdU positive cells between control and H_2O_2 treated cell cultures.

processes. Histology and transcriptome analyses showed that skin damages and responses to H_2O_2 treatment persist for at least 24 h. Semi *in vivo* and *in vitro* models added to the understanding of effects on the cellular level. The trial confirmed adverse effects from single and joint exposures to H_2O_2, low pH, and increased water temperature. Reduced migration from scales exposed to H_2O_2, pH, or increased temperature might reflect a weaker adhesion to the substratum and between cells that disrupts motility (Farooqui and Fenteany, 2005; Rapanan et al., 2014). The explant model suggested that the treated cells have a reduced size of the cell lamellipodium, proliferation potential, and energy metabolism in addition to the impaired migratory ability. The lamellar extension plays a key role in cellular migration (Farooqui and Fenteany, 2005) and is also a key feature for migration of fish skin keratocytes (Rapanan et al., 2014) with a homogeneous front in migrating epithelial sheets (Richardson et al., 2016). Our data support that leader cells may play an important role in advancing cell sheets, as our treated cells showed retarded frontline cells, with reduced lamellipodia and increased spacing between migrating cells. Reduced proliferation would likely affect the wound re-epithelialization process due to decreased epidermis thickness in adjacent regions of the wound (Richardson et al., 2016) and the total epidermis restoration potential. Thus, H_2O_2 treatment may indirectly enhance susceptibility to infections and weaken skin regeneration, as keratocytes are an important part of the innate immune system of fish (Karlsen et al., 2012; Lindell et al., 2012) and wound healing (Sveen et al., 2020).

In addition to direct impacts that may lead to mortalities in commercial production (Overton et al., 2018; Overton et al., 2019), treatments with H_2O_2 can also affect fish due to crowding, increase of temperature to >13 °C, and lowering of pH. Combined stressors can cause gill damage and increase mortality, especially of already compromised fish (Bruno and Raynard, 1994; Overton et al., 2019; Wynne et al., 2020). As shown in this trial, higher temperatures alone decrease the keratocyte migration potential and severely affect the outer border of the epidermis, morphological changes that may reduce the resistance of the skin barrier properties. Industrial de-lousing prevention strategies are reported to have no effect on the overall welfare status of salmon (Bui et al., 2020), which is in concordance with the lack of effect on external welfare indicators in this study. However, microscopic and gene expression analyses suggested possible negative consequences, which are not detected with a visual inspection.

5. Conclusions

Skin is a responsive tissue that allows combinations of *in vitro* and semi-*in vitro* trials and provides live cells for many hours *post mortem*. We could not find any adverse outcome of the treatment from assessing external welfare indicators and blood metabolites. However, transcriptome analyses detected stress in the skin, and our *in vitro* models demonstrated adverse effects on keratocyte migration. Thus, this study emphasizes the benefit of combining different methods which complement each other and improve understanding of biological responses. The study suggests secondary or long-term effects not directly associated with acute toxicity of H_2O_2 treatment, which can interfere with re-epithelializing of wounds and increase the susceptibility to infections. These results add to the evidence that the salmon farming industry must be cautious when treating with H_2O_2, as well as more attentive in the post treatment period.

Author contributions

CK: Conceptualization, Methodology, Formal analysis, Investigation, Writing – Original Draft. ASB: Conceptualization, Investigation, Resources. AK: Formal analysis, Data curation. EY: Conceptualization, Methodology, Formal analysis, Investigation. All co-authors reviewed, edited, and approved the final manuscript.

Declaration of Competing Interest

Authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2021.736660.

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