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Oxidant-induced modifications in the mucosal transcriptome and circulating metabolome of Atlantic salmon

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ABSTRACT

Here we report the molecular networks associated with the mucosal and systemic responses to peracetic acid (PAA), a candidate oxidative chemotherapeutic in Atlantic salmon (*Salmo salar*). Smolts were exposed to different therapeutic doses (0, 0.6 and 2.4 mg/L) of PAA for 5 min, followed by a re-exposure to the same concentrations for 30 min 2 weeks later. PAA-exposed groups have higher external welfare score alterations, especially 2 weeks after the re-exposure. Cases of fin damage and scale loss were prevalent in the PAA-exposed groups. Transcriptomic profiling of mucosal tissues revealed that the skin had 12.5 % more differentially regulated genes (DEGs) than the gills following PAA exposure. The largest cluster of DEGs, both in the skin and gills, were involved in tissue extracellular matrix and metabolism. There were 22 DEGs common to both mucosal tissues, which were represented primarily by genes involved in the biophysical integrity of the mucosal barrier, including *cadherin*, *collagen I α 2 chain*, *mucin-2* and *spondin 1a*. The absence of significant clustering in the plasma metabolomes amongst the three treatment groups indicates that PAA treatment did not induce any global metabolomic disturbances. Nonetheless, five metabolites with known functions during oxidative stress were remarkably affected by PAA treatments such as citrulline, histidine, tryptophan, methionine and trans-4-hydroxyproline. Collectively, these results indicate that salmon were able to mount mucosal and systemic adaptive responses to therapeutic doses of PAA and that the molecules identified are potential markers for assessing the health and welfare consequences of oxidant exposure.

1. Introduction

Chemotherapeutics are still used to treat fish diseases, however, stricter rules for their application have been implemented in different aquaculture-producing countries and many have shifted to more environmentally friendly options (Buchmann, 2015; Burridge et al., 2010; Quesada et al., 2013). Nonetheless, chemotherapeutics remain the only available alternatives in some cases (Holan et al., 2017; Overton et al., 2019). There are persistent apprehensions regarding the use of chemical disinfectants for disease treatment, especially against ectoparasitic infections, which may be partly related to their excessive use and the lack of experimentally verified data on how they impact the fish and the environment. The use of hydrogen peroxide (H_2O_2) in Atlantic salmon farming is a good example (Bechmann et al., 2019; Hjeltnes et al., 2019). It is imperative that an integrative approach is adopted for streamlining the use of chemicals (e.g., parasiticides) in aquaculture (Pedersen et al., 2009).

Peracetic acid (CH_3CO_3H, *hereafter referred to as PAA*) is a potent oxidative organic compound that has gained prominence in the last ten years as a sustainable disinfectant in aquaculture (Gesto et al., 2018; Pedersen et al., 2013). It is the peroxide of acetic acid and is commercially available in an equilibrium mixture with acetic acid, H_2O_2 and water. PAA has high oxidising potential and fat solubility (Luukkonen and Pehkonen, 2017), in which both properties are contributory to its broad potency against numerous fish pathogens including *Ichthyophthirius multifiliis*, *Aeromonas salmonicida*, *Flavobacterium columnare*, *Yersinia ruckeri*, *Saprolegnia* spp., *Aphanomyces* spp., and infectious salmon anaemia virus (Meinelt et al., 2015; Straus et al., 2018a). The antimicrobial activity of PAA is based on the formation of highly reactive free radicals and the release of active oxygen atoms that eventually disrupt

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the chemiosmotic function of the lipoprotein cytoplasmic membrane and transport through the dislocation or rupture of cell walls (Kitis, 2004; Straus et al., 2012a). PAA has stronger disinfecting power than H_2O_2 because of the former's fat solubility (Block, 1991). The combination of PAA and H_2O_2 is synergistic (Alasri et al., 1992).

While earlier studies documented the potency of PAA against numerous pathogens and toxicity towards a number of farmed fish species (Straus et al., 2012a, b; Straus et al., 2018a), there is a major gap in our understanding of the physiology behind the adaptations of fish to this strong oxidant. This is a crucial aspect that must be addressed in order to fully substantiate the claim that PAA is a more eco-friendly and safe alternative peroxide for fish. As an oxidative disinfectant with free radicals and reactive oxygen as intermediate products, PAA may trigger oxidative stress. In both rainbow trout and Atlantic salmon (*Salmo salar*), singular and repeated exposure to PAA could trigger oxidative stress as indicated by an increase in systemic antioxidant levels, as well as in other stress-related indicators such as cortisol and glucose (Liu et al., 2020; Soleng et al., 2019). Nevertheless, the recovery was quick and there was no lasting impact, demonstrating that the changes observed were likely physiological adaptations to the therapeutic doses of PAA (Gesto et al., 2018; Liu et al., 2017a, b). However, the extent to which PAA impacts fish physiology remains unknown. To our knowledge, no global response study has been conducted in fish exposed to PAA. Elucidating the physiological response at the molecular level will provide a better understanding of the biological mechanisms of PAA and identify markers that may be valuable for health monitoring following oxidant treatment.

Here we investigated the health and welfare impact of exposing salmon smolts to therapeutic doses of PAA. This oxidant is currently being explored as a chemotherapeutic for amoebic gill disease (AGD) in salmon. There are no available data on the tolerance of salmon to PAA, therefore, we identified the test concentrations used in this study based on their earlier applications against key fish pathogens (Straus et al., 2018b), reported in another salmonid species (rainbow trout) (Davidson et al., 2019; Hushangi and Hosseini Shekarabi, 2018; Liu et al., 2020), and biocidal activity against *Neoparamoeba perurans,* the causative agent of AGD (Breiland et al., 2019). An ideal chemotherapeutic in aquaculture should be effective against the pathogen, have minimal environmental risk and not pose substantial health and welfare issues to the fish (Pedersen and Lazado, 2020). The first two characteristics have been explored to some extent with regards to the application of chemical disinfectants in aquaculture, whereas our knowledge on the third feature is fragmentary. This manuscript addresses this knowledge gap by employing global transcriptomic and metabolomic profiling techniques.

2. Materials and methods

2.1. Ethics statement

All fish handling procedures employed in this study were in accordance with national and EU legislation (2010/63/EU) on animal experimentation.

2.2. Fish and husbandry conditions

The experimental fish were sourced from a local land-based RAS supplier (Danish Salmon, Hirtshals, Denmark) and transported to the experimental recirculation aquaculture facility of DTU Aqua (Hirtshals, Denmark). The fish had not been exposed to any oxidant prior to this trial. Sixty fish (~ 100 g) were stocked in each of the 6 1-m^2 holding tanks (volume approximately 600 L) connected to a common recirculation system with seawater (33–34 ppt) at 15 ± 1 °C. Details of the RAS and other system parameters are described in a previous publication (Soleng et al., 2019). The experimental hall was illuminated following a 16L:8D (0600-2200) photoperiod cycle. Regular production feed (Biomar, EFICO Enviro, 4.5 mm, Brande, Denmark) was provided via a belt feeder at a provision of 1–1.5 % total biomass per day. The experimental fish were allowed to acclimate to the experimental conditions for 3 weeks.

2.3. Exposure to therapeutic doses of peracetic acid (PAA)

Feeding was temporarily ceased 24 h prior to PAA exposure. The exposure protocol is described in detail in an earlier publication (Soleng et al., 2019). Briefly, fish were netted out from the holding tank and immediately transferred to an exposure tank with similar volume and water quality parameters. Thereafter, fish were exposed to three nominal doses of PAA (Divosan® Forte, Lilleborg AS, Oslo, Norway): 0 (control), 0.6 and 2.4 mg/L. The actual PAA concentration in the trade product (*ca* 18 % PAA) and its degradation during the trial had been experimentally verified and previously reported (Pedersen and Lazado, 2020). Two replicate tanks were allocated for each treatment group. After 5 min, the fish were immediately netted out of the PAA-exposure tanks and returned to their respective holding tanks. Following a two-week recovery from the initial exposure, the fish were re-exposed to the same concentration of PAA using the same protocol as in the initial exposure, except that the duration lasted for 30 min. The fish were then allowed to recover for 2 weeks. The experiment was designed to reduce the number of fish used in a trial involving live animals but still robust to answer key questions on how salmon respond to singular exposure and re-exposure of PAA. This adheres to the 3R (reduce, reuse, replace) principles in aquaculture research.

The choice for the age and size of fish in the trial was based on the eventual application of PAA, i.e. as a treatment for AGD. This disease affects salmon in the saltwater stage and the treatment (i.e. H_2O_2 or freshwater) is usually performed when the fish are between 100−500 g. The exposure protocol simulated a proposed method of treating AGD at an early stage (0.5–1 gill score) which requires a short contact time with the fast-acting, potent PAA oxidant, but when the disease further develops, a treatment at a longer duration is to be administered. The 30-min exposure time is a common exposure time in treating AGD infected fish (Hytterød et al., 2017).

2.4. Sample collection

Sample collection was performed at 48 h and 2 weeks after each exposure occasion. The results at 2 weeks post-exposure provided insights into the persistent consequences of the treatment. Five fish were taken from each replicate tank and were humanely euthanised with an overdose of benzocaine solution (n = 10 per group). After the length and weight of the fish were determined, the external welfare status was evaluated according to the FISHWELL handbook (Noble et al., 2018). The welfare scoring scheme includes 11 external welfare parameters (emaciation, eye damage, skin damage, operculum damage, snout damage, vertebral deformity, jaw deformity, dorsal fin damage, caudal fin damage, pectoral fin damage and pelvic fin damage) that are scored 0–3, with 0 as fully intact and 3 as severely compromised. To ensure objectivity and limit biases, only one person, who did not have prior knowledge about the treatments, evaluated all the fish throughout the experiment. Blood was withdrawn from the caudal artery using a heparinised vacutainer, centrifuged at 1000 x g for 10 min at 4 °C, plasma was collected and kept at −80 °C until analysis. Small portions of the dorsal skin and the second gill arch were collected, suspended in RNAlater™ (Thermo Fischer Scientific, City, MA, USA) incubated for overnight penetration at room temperature and stored at −80 °C until RNA isolation.

2.5. RNA extraction and microarray analysis

Total RNA was isolated from the skin and gill tissues using Agencourt RNAdvance™ Tissue Total RNA Purification Kit (Beckman Coulter Inc., CA, USA). The RNA concentration was determined using a NanoDrop

8000 spectrophotometer (Thermo Fischer Scientific) and the quality was further assessed with the Agilent® 2100 Bioanalyzer™ RNA 6000 Nano Kit (Agilent Technology Inc., Santa Clara, CA, USA). All samples had an RNA Integrity Number (RIN) above 9. Nofima's Atlantic salmon DNA oligonucleotide microarray SIQ-6 (custom design, GPL16555) contains 15 K probes for protein-coding genes involved in immunity, tissue structure, integrity and function, cell communication and junctions and extracellular matrix, among many others (Krasnov et al., 2011). Annotation of this microarray contains four major groups: **Tissue**, which includes genes with known functions in tissue structure, integrity, development and architecture; **Metabolism**, which includes genes with known functions in metabolic processes; **Immune**, which includes genes with known functions in innate and adaptive, cellular and humoral immune responses; **Cell**, which includes genes with known functions in cellular processes, development, communication and signalling. Agilent Technologies manufactured and supplied the microarrays, reagents and equipment used in the analysis. RNA amplification and Cy3 labelling were performed with the One-Color Quick Amp Labelling Kit with a 200-ng RNA template per reaction and Gene Expression Hybridization Kits were used for the fragmentation of labelled RNA. Hybridisation was carried out for 17 h in an oven at 65 °C with a constant rotation speed of 10 rpm. Thereafter, the arrays were washed successively with Gene Expression Wash Buffers 1 and 2 and were scanned using the Agilent SureScan Microarray Scanner. Data processing was carried out in Nofima's bioinformatics package STARS.

2.6. Plasma metabolomics

Plasma samples were reconstituted in 200 µL Eluent A and transferred to an HPLC vial. The analysis was carried out using a UPLC system (Vanquish, Thermo Fisher Scientific) coupled with a high-resolution quadrupole-orbitrap mass spectrometer (Q Exactive™ HF Hybrid Quadrupole-Orbitrap, Thermo Fisher Scientific). An electrospray ionisation interface was used as the ionisation source. The analysis was performed in negative and positive ionisation modes. The UPLC was performed using a slightly modified version of the protocol described by Doneanu et al. (Doneanu et al., 2011). Data-processing was carried out in MZmine 2 (Pluskal et al., 2010) followed by curation using a custom made in-house protocol of MS-Omics ApS (Denmark). Identification of compounds was performed using both peak retention times (compared against authentic standards included in the analytical sequence) and accurate mass (with an accepted deviation of 0.0005 Da). This targeted approach was used to extract the response of compounds included in the in-house standard list of MS-Omics ApS, covering 142 compounds. The relative concentrations are peak areas normalised using linear regression of the signal in QC samples to remove systematic variation throughout the sequence.

Compound identification was performed in two levels: *level one* included compounds identified based on accurate mass and retention times matched with those from authentic standards analysed in MS-Omics ApS laboratory, whereas *level two* was based on accurate mass and estimated retention times as inferred from the structural information of the compound. Compounds identified in level two are indicated with a question mark in front of their name.

2.7. Data handling

The overall welfare index was calculated by averaging the combined scores from the different welfare parameters. One-way ANOVA was used to identify inter-treatment differences for a welfare index and statistical significance was set at $p < 0.5$.

Microarray expression values were $\log_2$ transformed and further processed in R (version 3.5.2, https://www.r-project.org/). The values were normalised to the expression values of the control group and ANOVAs were calculated for each gene to identify significant differences between treatments and time points (package *HybridMTest*). Mean expression values for each group (treatment and time point) were calculated and the absolute difference between minimum and maximum was determined for each gene. Differentially expressed genes (DEGs) were defined as genes with a minimum absolute difference of 0.5 and a significant difference in the ANOVAs ($p < 0.05$). Groupwise mean DEGs were clustered according to their Euclidean distance with the complete linkage method (function *hclust* from package *stats*). The resulting dendrogram was cut into sub-clusters to achieve a sufficient separation without too much fragmentation (function *cutree* package *stats*). Dendrogram and group means were plotted with the function *heatmap.2* (package *gplots*). Mean values by group within the sub-clusters were plotted in custom bar graphs with the respective standard error of the mean added as +/- error bars. The STARS package provides a categorical annotation of gene functions, which is based on public gene annotations and experience from previous experiments. At the time of this analysis, 106 different categories were present on the used 15k microarray and approximately two-thirds of the represented genes were annotated with these categories. Functional gene categories were counted for each sub-cluster and enrichment analyses were computed using Fisher's exact test (function *fisher.test*). Results were filtered for at least one significantly enriched ($p < 0.05$) category within the sub-clusters and plotted as dots of different sizes, with bigger dots for lower p-values.

A PCA model of the plasma metabolome was generated using the reduced dataset. The change in metabolite concentration in the PAA-exposed groups (0.6 and 2.4 mg/L) was expressed as $\log_2$ ratio or the logarithmic value of the fold change relative to the control group (0 mg/L) at a specific time-point. The statistical change between two groups was determined by *t*-test. A compound regarded as a differentially modulated metabolite should pass the following condition: $P < 0.05$, $\log_2$ ratio > 0.3.

3. Results and discussion

3.1. PAA exposure does not elicit aberrant behavioural responses

PAA is recognised as a strong irritant and lacrimator in mice (Gagnaire et al., 2002), and, as such, may trigger sensory irritation. The narratives from earlier studies in rainbow trout suggest that erratic swimming, agitation and gasping for air are some of the typical behavioural responses observed following PAA exposure at concentrations from 0.5 to 2 mg/L. These are fundamental responses associated with sentient organisms' processing of a potential threat in their immediate environment. The present study observed no major behavioural changes in the experimental fish during the first exposure and the re-exposure trials, as well as during recovery. The fish remained calm and exhibited no apparent agitation during both exposure occasions. This suggests that the therapeutic doses of PAA used were not identified by the fish as a potential danger, hence, no escape behaviour was observed, though we cannot exclude the potential limitation of space in the tank. This was further supported by the zero-mortality record throughout the trial. The fish resumed feeding right after each treatment in all exposure trials. Weight at termination was not statistically different amongst treatment groups. Unaltered production performance after treatment corroborated earlier observations in rainbow trout (Hushangi and Hosseini Shekarabi, 2018; Liu et al., 2017b), indicating that the treatment did not interfere with the growth potential and metabolism of salmon, as supported in part by the metabolomics data (discussed in Section 3.4).

3.2. External welfare scores remain favourable after oxidant exposure

External welfare parameters are operative indices that may be used on farms as indications of the welfare status of salmon (Noble et al., 2018). Using semi-quantitative scoring, this rough evaluation can help farm operators gauge the impact of husbandry practices, for example, stocking density, handling and treatment, to name a few. We applied this

strategy in the present experiment to determine whether a simulated PAA treatment could affect the external welfare of smolts. Overall, oxidant exposure did not significantly affect the overall external welfare status of salmon (Fig. 1A), with composite scores below 1, on a scale of 0–3.

We further evaluated variations in the individual 11 external parameters in the scoring scheme (Fig. 1B,C). At 2 weeks post-exposure, the profile was similar on both occasions, though it was quite apparent that the scores were relatively lower after the re-exposure compared with the first exposure. Skin, pectoral fin, and dorsal fin damage amongst the parameters were considerably represented in both exposure trials and in all three treatment groups. Almost 90 % of the recorded skin damage was scale loss. This may be attributed to the handling and transfer of the fish from the holding to the exposure tank. The alterations in all the evaluated parameters in oxidant-exposed groups were not statistically different from the control. These results show that PAA at the tested concentrations did not compromise the external welfare of salmon. Moreover, re-exposure to the same dose at longer durations did not aggravate previously highly scored external parameters (i.e., fin and skin damages).

3.3. *Oxidant exposure orchestrates a network of adaptive responses at the mucosal surfaces*

Mucosal surfaces (i.e., skin, gills, gut, olfactory) are the interface between the fish and the surrounding aquatic environment and, hence, encounter constant stringent biological pressures (Cabillon and Lazado, 2019). One of these is the changing levels of environmental reactive oxygen species (ROS), which greatly impact the organism's key biological processes. In aquaculture, fish are often exposed to higher levels of environmental ROS using strong oxidative disinfectants either as a treatment for ectoparasitic infection or routine treatment of rearing water.

Global transcriptomic profiling revealed that there were 587 differentially expressed genes (DEG) in the gills following exposure to the oxidative disinfectant (Fig. 2). The full list of DEGs is provided in Supplementary File 1. We further subdivided these DEGs into 7 clusters based on their expression profiles. Cluster 1 represents the largest group with 329 DEGs, though the overall magnitude of response was lower compared with the other clusters (i.e., Clusters 3, 4, 5 and 6) (Fig. 2A). About 25 % of the DEGs in Cluster 1 are involved in mucosal tissue adhesion, differentiation, extracellular matrix and neural activity (Fig. 2B). In both PAA-exposed groups, the collective tendency was downregulation at 48 h after first exposure. Unlike in the 0.6 mg/L group, where a downregulation tendency was observed at 2 weeks after first exposure, the 2.4 mg/L group exhibited a striking upregulation of the genes in Cluster 1 the majority of which have known functions in cell autophagy, cell cytoskeleton, cell lysosome, cell stress, chemokine receptor and immune regulation, which may likely suggest recuperation from higher dose. Following re-exposure, both treatment groups demonstrated an identical branchial response after 2 weeks but not after 48 h of exposure in this gene cluster. Most of the DEGs that were substantially upregulated at this time point, particularly in the 0.6 mg/L group, were involved in the immune response especially chemokines, cytokines and other key immune response effectors. The considerable representation of genes involved in branchial immune signalling indicates that PAA elicited a strong immunological impact that likely initiated a series of immune responses as a protective mechanism at the gill mucosa. The degradation of PAA in the water results in the production of free radicals (Liu et al., 2020; Pedersen et al., 2009) and these radicals will eventually affect the oxidative state, triggering a cascade of immune effects to counter the physiological pressure (Biller and Takahashi, 2018; Srivastava and Pandey, 2015). The upregulation of these genes, especially those with immune regulatory functions, 2 weeks post-exposure may be related to establishing immunological homeostasis following a substantial downregulation during the early hours of exposure.

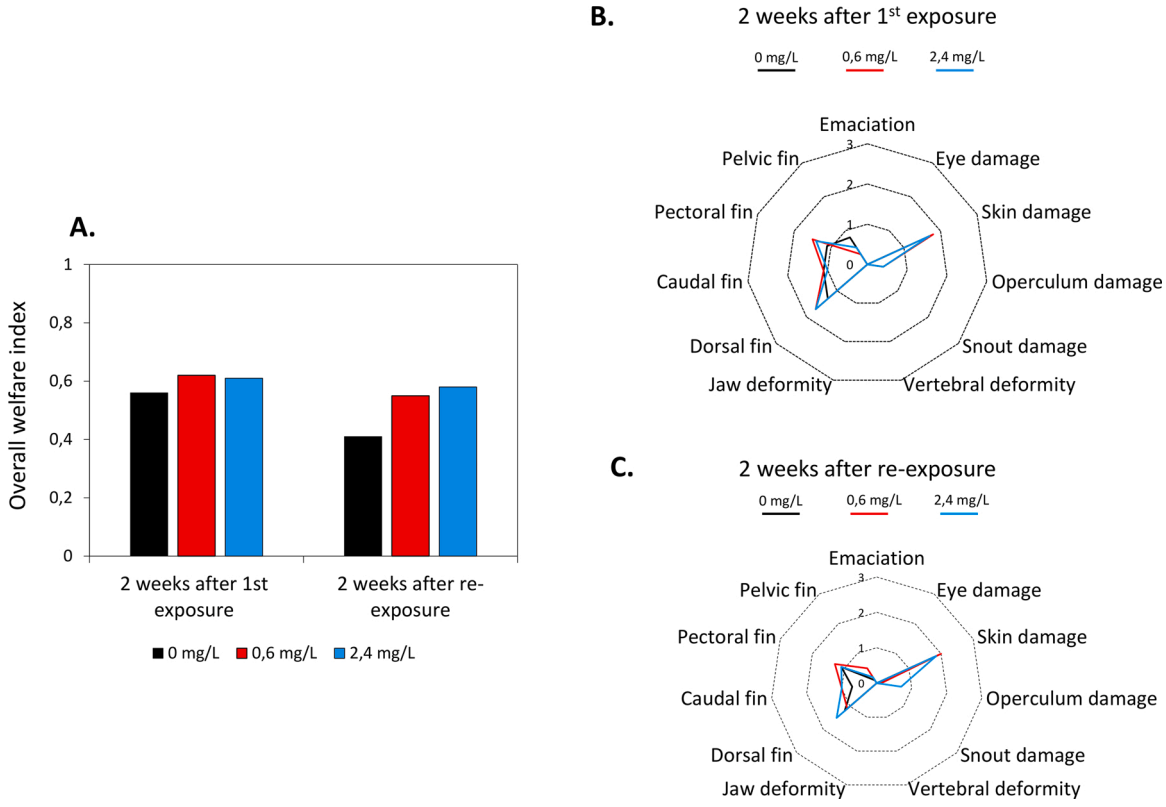

Fig. 1. External welfare status of Atlantic salmon exposed to therapeutic doses of peracetic acid. **A)** The overall external welfare index of each treatment group based on the average composite score of 11 external welfare indicators, as presented individually in panels B and C.

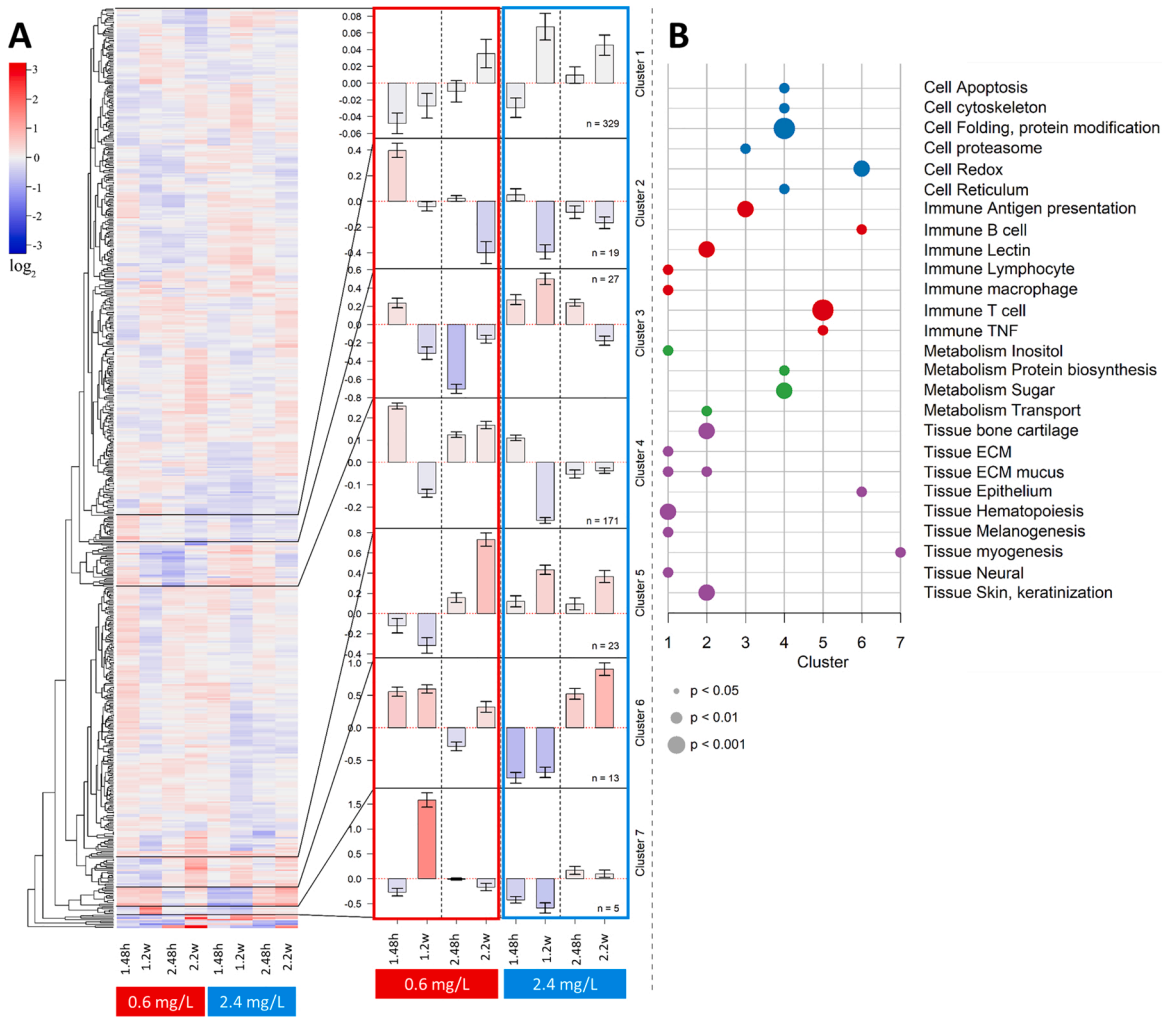

Fig. 2. Gill transcriptome of Atlantic salmon exposed to therapeutic doses of peracetic acid. **A)** The heatmap on the left shows the down- and upregulation of DEGs in a colour gradient from blue to red. The dendrogram was split into seven sub-clusters and the mean values for genes within these clusters were represented in bar plots (error bars show +/- standard error of the mean) in the centre. **B)** Enrichment analyses of the seven sub-clusters. The identified functional gene categories are shown along the Y-axis and the six clusters along the X-axis. Dots were coloured according to the super categories (cell, immune, metabolism and tissue) and the size indicates the respective p-value level according to Fisher's exact test. Sampling time notations: *1.48 h* = 48 h after initial exposure; *1.2 w* = 2 weeks after initial exposure; *2.48 h* = 48 h after re-exposure; *2.2 w* = 2 weeks after re-exposure.

The second overly represented group is Cluster 4 with 171 DEGs (Fig. 2A), where 40 % of the DEGs are involved in cell apoptosis, cytoskeleton, folding and signalling, whereas a similar percentage play roles in mitochondrial metabolism, sugar and xenobiotic metabolism and protein biosynthesis (Fig. 2B). In this cluster, the patterns of expression in the two PAA-exposed groups were identical after the first exposure – upregulation 48 h and downregulation 2 weeks after exposure. Moreover, the magnitude of change was considerably larger in the 0.6 mg/L than in the 2.4 mg/L group, indicating that the scale of the PAA impacts on gill cellular signalling and metabolism are not entirely dependent on dose, though the patterns of regulation are similar. Responding to an environmental challenge (e.g., elevated ROS) carries a strong metabolic demand. Hence, the upregulation of these genes is likely important in mustering a robust and coordinated response to a stimulus, which is energy demanding. The two PAA-exposed groups exhibited opposing general profiles in this gene cluster after the re-exposure – upregulation for the 0.6 mg/L and downregulation for the 2.4 mg/L group at both sampling points. Genes that were remarkably upregulated in the 0.6 mg/L group have key roles in cell protein folding and modification, cellular cytoskeleton, and tissue epithelium and glycan and, moreover, the magnitude of change increased at 2 weeks after re-exposure indicating that the impact may persist for some time. On the other hand, the changes in the 2.4 mg/L group were minimal in contrast to the 0.6 mg/L group following re-exposure. Whether this profile suggests tolerance or desensitisation to higher dose remains an open question.

Mucosal transcriptomic profiling of the skin identified 671 DEGs – around 12.5 % higher than the gills (Fig. 3). Moreover, the overall changes in the cutaneous expression in the PAA-exposed groups were considerably larger in magnitude compared with the gills. The full list of DEGs is provided in Supplementary File 2. In terms of surface area to water contact ratio, the gills are larger than the skin (Koppang et al., 2015). However, the results described here revealed that contact surface ratio did not entirely dictate the mucosal responses to PAA as the skin was identified as more responsive than the gills to the oxidant, at least in the concentration tested in the present trial. Though we could not fully ascertain as to why such a striking difference was observed, we speculate that this may be related to the prevalent cases of scale loss (Fig. 1) in both groups; it reduced the physical barrier thus rendering the outer layer of the epithelial surface a greater chance to come in contact with the oxidant. The DEGs can be further classified into 6 groups, with Clusters 1 (325 DEGs) and 3 (305) comprising the two most represented groups. In Cluster 1, the four annotation groups (cell: 24.9 %; immune: 24.3 %; metabolism: 19.7 %; tissue: 31.1 %) were almost equally represented. Both PAA-exposed groups showed downregulation at 48 h

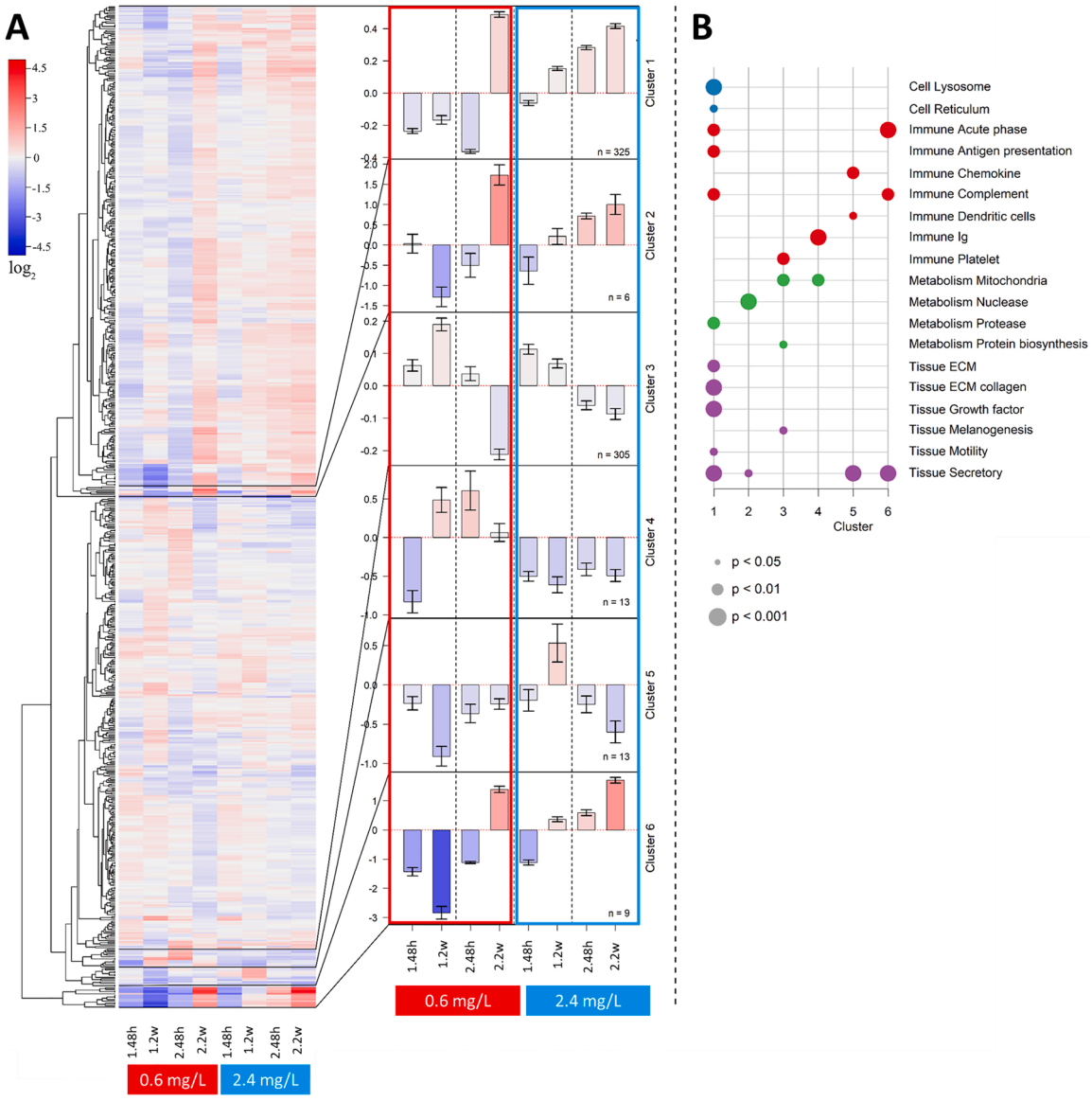

Fig. 3. Skin transcriptome of Atlantic salmon exposed to therapeutic doses of peracetic acid. Analysis and representation for DEGs of skin samples were conducted in the same way as for the gills. For further information, see the caption of Fig. 2.

after first exposure, with 0.6 mg/L group showing a relatively higher magnitude of response compared with the 2.4 mg/L group in this gene cluster. Some of the genes that were remarkably downregulated in both treatment groups are involved in immune lectin, lipid metabolism, protease metabolism and extracellular matrix. At the same time point after the re-exposure, an opposite trend was observed between groups in this cluster - downregulation was prominent in the 0.6 mg/L group while upregulation dominated the 2.4 mg/L group. The majority of the genes with marked downregulation in the 0.6 mg/L group were involved in acute immune response, antigen processing, complement, cytokines and immune effectors, which may likely suggest a transient immunosuppression. On the other hand, a significant portion of highly upregulated genes in the 2.4 mg/L group at this time point are involved in acute phase immune response, immune effector, immune lectin, and tissue secretion. Despite the contrasting profiles, the responses indicate interference of PAA on mucosal immunity. Both treatment groups displayed upregulation pattern in this gene cluster 2 weeks after the re-exposure where majority of substantially affected genes were crucial for immunity and metabolism. It is also interesting to observe that the magnitude of change was higher at this timepoint than other timepoints within this gene cluster, which may be indicative of a cumulative effect or a chronic-latent response. The genes that were markedly affected in both treatments groups include those with a role in protein folding, acute phase immune response, immune lectin, protease metabolism, tissue extracellular matrix and tissue growth factor. The overall pattern with which the four groups of annotated genes were equally represented in the DEGs indicates that the cutaneous molecular repertoire was under tight regulatory control to maintain homeostasis in the skin following exposure to the oxidant.

Cluster 3 is the second well-represented group in the DEGs in the skin (Fig. 3A). Around 40 % of the DEGs were annotated under metabolism while the rest of the genes fell within cell (~ 29 %), tissue (~ 20 %) and immune (~ 11.5 %) (Fig. 3B). This provides additional support for the foregoing observations that PAA exposure confers strong metabolic pressure on the mucosa, as this profile was also prominent in Cluster 4 in the gill transcriptome. Following the first exposure, Cluster 3 displayed upregulated expression regardless of the PAA dose and sampling timepoint. Some of the genes that were substantially upregulated at 48 h after first exposure in both groups have functions in cellular redox balance, mitochondrial metabolism, RNA metabolism and sugar metabolism.

Specifically, the upregulation of *glutathione transferase Ω-1* and *phosphoglycerate kinase* may be implicated to the control of the redox balance in the skin during the early phase post-exposure to the oxidant, since both genes are known to participate in this vital adaptive process when radicals are in abundance (Kim et al., 2017; Tsukamoto et al., 2013). On the other hand, genes the are considerably upregulated in both treatment groups 2 weeks after the first exposure are involved in lectin-mediated immunity and xenobiotic metabolism. Interestingly, the magnitude of change at this timepoint was larger in the 0.6 mg/L group compared with the 2.4 mg/L group. Genes with an important role in proteolytic metabolism were remarkably affected in the 0.6 mg/L group. The significantly elevated expression of proteolysis-related genes at this timepoint indicates the potential involvement of heightened protein turnover as a recovery mechanism to oxidant treatment, which influences tissue structure and integrity during the early period after exposure.

At 48 h after re-exposure, the genes in Cluster 3 were mostly upregulated in the 0.6 mg/L group and downregulated in the 2.4 mg/L group. It appeared that cutaneous metabolic processes were remarkably affected in the 0.6 mg/L group as most upregulated genes were involved in RNA and mitochondrial metabolism. Downregulation was the hallmark response in the 2.4 mg/L group for this gene group at 48 h after the re-exposure. The trend of the impact, however, was not quite pronounced compared with the 0.6 mg/L group. These profiles indicate that re-exposure may trigger a slight cutaneous metabolic disarray. Both groups exhibited downregulation profiles at 2 weeks after re-exposure, where the magnitude of response was more marked in the 0.6 mg/L than in the 2.4 mg/L group. The majority of the genes that were substantially downregulated in both groups are important in cell apoptosis, cell transcription, cell ubiquitination, immune regulation and mitochondrial metabolism. The downregulation in these genes may perhaps a form of compensation to counterbalance the substantial upregulation during first exposure.

Overall, the impact on the mucosal transcriptome of the lower PAA dose (i.e., 0.6 mg/L) was more remarkable than the high dose (i.e., 2.4 mg/L). The mucosa of the 0.6 mg/L-exposed group appears to exhibit substantial transcriptional changes, relatively robust responses based on the known function of identified DEGs and consistent response patterns. In overly represented gene clusters and in both mucosal tissues, a more marked response could be triggered 2 weeks after exposure, suggesting that there are numerous biological processes, particularly those involved in metabolism, that were activated for mucosal recovery and compensation. A cumulative effect is likely at play since dramatic changes in several gene clusters were often observed 2 weeks after the re-exposure, although, a chronic response is also possible for such a distinct profile.

The core PAA-mucosal response genes in salmon are represented with 22 DEGs that are common in both skin and gill (Table 1). It is apparent that genes involved in immunity were not strikingly represented, at only around 13.6 % of the total DEGs in the core group. PAA triggered strong immunological responses in the mucosa, as shown by the individual transcriptome profiles where several immune-related genes were affected. However, it is likely that immune regulatory mechanisms in response to PAA in the two mucosal tissues may differ because the number of shared DEGs is low. This highlights the possibility that the crosstalk between mucosal immunity and oxidant-induced modulation is complex and both tissues may have different fundamental underlying pathways. Mucins are important glycoprotein components of the mucus, an emblematic biological fluid covering mucosal surfaces. *Mucin-2*, in particular, is a common mucin in the intestinal mucosa, and only marginally expressed in the skin and gills of both naïve and stressed salmon (Sveen et al., 2017). Its regulation in response to PAA in both tissues reveals its essential role in maintaining mucosal glycopolymeric integrity and provides further insight into its functions beyond its well-established role in the gastrointestinal tract. *Mucin-2* expression was highest 2 weeks after the re-exposure in both mucosal tissues suggesting that it is important in the recovery of glycopolymeric

Table 1
List of differentially expressed genes that are identified both in the gills and skin of PAA-exposed salmon. An arrow indicates upregulation (↑) or downregulation (↓) relative to 0 mg/L. Each time point is represented with 2 arrows: the first arrow denotes the transcription in the gills while the second arrow was the expression in the skin.

Annotation	Gene Name	0.6 mg/L				2.4 mg/L			
		First exposure		Re-exposure		First exposure		Re-exposure	
		48 h gills: skin	2 w gills: skin	48 h gills: skin	2 w gills: skin	48 h gills: skin	2 w gills: skin	48 h gills: skin	2 w gills: skin
Cell Apoptosis	*Baculoviral IAP repeat-containing 2*	↑↓	↓↓	↓↓	↑↑	↑↓	↑↓	↓↓	↓↓
Cell Folding	*Serpin H1 (Hsp47)*	↑↓	↓↓	↓↓	↑↑	↓↓	↓↓	↓↑	↑↑
Cell GTP signaling	*Olfactomedin-4*	↑↓	↓↓	↓↓	↑↑	↓↑	↓↑	↓↑	↑↑
Cell GTP signaling	*Rho GTPase-activating protein 33*	↑↓	↓↑	↓↓	↑↑	↓↓	↓↓	↑↑	↑↑
Cell Myofiber	*Titin a - Ident 98*	↑↑	↓↑	↓↓	↑↓	↑↑	↑↑	↑↓	↓↓
Cell Reticulum	*Protein transport protein Sec31A*	↓↓	↓↑	↓↑	↓↑	↓↑	↑↓	↓↓	↑↓
Cell Reticulum	*Reticulocalbin 3_ EF-hand calcium binding domain*	↓↓	↑↑	↑↓	↑↑	↓↓	↓↑	↑↑	↑↑
Immune Lectin	*Rhamnose-binding lectin WCL1 [Salvelinus leucomaenis]*	↑↑	↑↓	↑↓	↓↑	↑↑	↑↑	↑↑	↓↑
Immune macrophage	*ATP binding cassette G1*	↑↑	↑↑	↓↓	↓↓	↑↑	↑↑	↑↓	↑↓
Immune T cell	*Rho GTPase-activating protein*	↓↓	↓↓	↑↓	↑↑	↓↑	↑↑	↓↑	↑↑
Metabolism Ion	*ATPase_ Na+/K + transporting_ beta 2b polypeptide*	↓↓	↑↑	↓↓	↓↑	↓↓	↑↑	↑↑	↑↑
Metabolism Lipid	*Lactosylceramide 1_3-N-acetyl-beta-D-glucosaminyltransferase A*	↑↑	↓↑	↓↑	↓↓	↓↑	↑↓	↑↓	↓↓
Metabolism Mitochondria	*NADH-ubiquinone oxidoreductase chain 5*	↑↑	↓↑	↓↑	↓↓	↓↓	↓↓	↑↓	↓↓
Metabolism Protease	*Carboxypeptidase E*	↑↓	↓↑	↑↓	↓↑	↑↓	↑↑	↑↑	↓↑
Metabolism Steroid, bile	*17 beta hydroxysteroid dehydrogenase 4 [Salmo trutta fario]*	↑↓	↓↑	↓↑	↑↓	↓↑	↓↓	↑↑	↓↓
Metabolism Sugar	*Glucosamine (UDP-N-acetyl)-2-epimerase/N-acetylmannosamine kinase*	↑↑	↓↑	↓↑	↓↓	↓↑	↓↓	↓↓	↓↓
Tissue Adhesion	*Cadherin*	↑↑	↓↑	↓↑	↓↓	↑↑	↑↑	↑↓	↓↓
Tissue bone cartilage	*TNF receptor member 11B*	↓↓	↓↓	↑↓	↑↑	↓↓	↑↑	↓↑	↑↑
Tissue ECM	*Novel protein similar to vertebrate leprecan-like 1 (LEPREL1)*	↓↓	↑↑	↑↓	↓↑	↓↓	↓↑	↑↑	↓↑
Tissue ECM collagen	*Collagen I alpha 2 chain*	↓↓	↑↑	↓↓	↑↑	↓↓	↓↑	↓↑	↓↑
Tissue ECM mucus	*Mucin-2*	↓↓	↓↓	↑↓	↑↑	↓↓	↑↑	↓↑	↑↑
Tissue ECM mucus	*Spondin 1a*	↓↓	↑↑	↑↓	↓↑	↓↓	↑↑	↑↑	↑↑

barrier mucosa after the treatments. *Spondin 1a* is also an epithelial mucus protein component (Lang et al., 2016) identified as significantly regulated by both treatments in both tissues. The shared regulatory profile between *mucin-2* and *spondin 1a* suggests that they are probably the main molecular drivers of mucus biophysical barrier features in the PAA response. The genes identified here (Table 1) have not been described as involved in the antioxidant response in salmon, thus, the list provides potential markers for the mucosal oxidant response that should be verified and characterised in future studies.

3.4. Presence of oxidant in the water does not trigger substantial disarray in the circulating metabolome, though metabolite-specific responses suggest key role in oxidative stress

In a previously published paper, we demonstrated that the total plasma antioxidant capacity of salmon was significantly increased at varying degrees by PAA treatment (Soleng et al., 2019). We then asked whether an increased level of environmental ROS (in this case, PAA) would also elicit global systemic responses, such as in small molecule substrates, intermediates, and products of metabolism. A total of 39 compounds (with authentic standards) were identified in the plasma (Table 2). The overall profile indicates that the exposure of salmon to 0.6 and 2.4 mg/L PAA either for 5 or 30 min did not significantly alter the plasma metabolomes. This was supported by the PCA (Fig. 4A) and the loading (Fig. 4B) plots, which show an absence of significant groupings in the metabolomes in relation to the treatment group and sampling point. This indicates that the effects of PAA exposure were minimal and further suggests that the treatment did not trigger global metabolomic disturbances in salmon. Both treatments did not show clear distinction in their metabolomes unlike in their transcriptomic responses. This underscores the sensitivity of the mucosa to mount varying responses to different environmental pressures. As the first line of defence and the point of contact, the mucosa mustered a robust response to counteract physiological PAA demands without leading to significant internal changes.

We further scrutinised the individual metabolites to identify metabolite-specific responses to PAA treatments. There were 14 differentially modulated metabolites, independent of PAA dose, duration and sampling time (Fig. 4C). Four of these were only affected at the low dose (0.6 mg/L) including valine, leucine, hexose and lysine. On the other hand, six metabolites were exclusively affected at the high dose (2.4 mg/L) and these were α-ketoglutaric acid, taurine, arginine, cytosine, pyridoxal and pyroglutamic acid. Five metabolites were identified as differentially modulated by the two PAA treatment groups including methionine, citrulline, histidine, tryptophan and trans-4-hydroxyproline. Looking at the loading plot (Fig. 4B), methionine and tryptophan behaved similarly while the other three compounds formed a separate cluster. All of these five metabolites have known functions in the mobilisation of the antioxidant response.

Methionine (Fig. 4D) is likely a key molecule in the response of salmon to low doses of PAA, elevated 48 h after each treatment.

Table 2
List of identified metabolites in the plasma and their $\log_2$ ratio relative to the 0 mg/L group. Values that are underlined and in bold are metabolites significantly affected by PAA treatment. Positive values indicate increases while negative values denote decreases relative to 0 mg/L.

Metabolite name	0.6 mg/L				2.4 mg/L			
	First exposure		Re-exposure		First exposure		Re-exposure	
	48 h	2 w	48 h	2 w	48 h	2 w	48 h	2 w
Lactic acid	−0.40	−0.19	−0.03	−0.09	−0.21	−0.20	−0.26	−0.12
Valine	**0.56**	0.12	0.18	−0.31	−0.36	0.18	0.16	−0.24
Leucine	**0.82**	0.01	0.19	−0.29	−0.15	0.31	0.02	−0.30
Isoleucine	0.58	−0.02	0.27	−0.12	−0.23	0.19	−0.09	−0.02
Hypoxanthine	−0.52	−1.06	0.29	−0.23	0.41	−0.30	−0.38	−0.66
a-Ketoglutaric acid	−0.01	−0.05	−0.35	−0.66	−0.59	0.43	**−0.46**	−0.14
Methionine	**0.80**	−0.34	0.48	0.32	**0.73**	0.34	−0.11	0.00
Tyrosine	0.25	−0.06	0.20	−0.33	−0.27	0.22	−0.25	−0.07
Taurine	0.50	−0.30	0.06	−0.23	**0.74**	−0.17	−0.27	−0.27
Citrulline	0.25	0.47	0.14	**−0.63**	−0.29	**0.50**	0.46	−0.26
Hexose	−0.04	−0.32	−0.26	**0.56**	−0.21	−0.32	−0.38	0.07
Inosine	−0.22	−0.70	0.03	−0.03	0.09	−0.02	−0.60	−0.23
?3-hydroxybuturylcarnitine (C4)	−0.42	0.09	0.49	0.12	−0.08	1.18	−0.05	0.09
?Succinylcarnitine (C4-DC)	−0.15	−0.08	0.33	0.14	0.27	0.53	0.27	0.45
4-aminobenzoic acid	0.13	−0.08	0.57	0.37	0.14	−0.05	0.27	0.35
Acetylcarnitine (C2)	−0.08	−0.19	0.53	−0.02	0.09	0.94	0.14	0.06
Adenine	−1.00	−0.54	−0.30	0.34	0.79	0.62	−0.02	0.37
Arginine	0.16	−0.12	−0.21	−0.22	−0.30	−0.12	−0.95	**−0.91**
Carnitine	0.08	−0.13	0.31	−0.18	0.14	−0.11	−0.03	−0.20
Choline phosphate (PCHO)	−0.50	−0.35	0.01	0.23	0.30	−0.01	−0.01	0.25
Creatine	0.21	0.18	0.38	0.35	0.55	0.48	−0.04	−0.01
Cyanocobalamin	−0.21	−0.36	0.15	0.54	0.33	−0.08	0.20	0.29
Cytidine	0.02	−0.41	−0.11	−0.29	−0.07	−0.34	−0.04	−0.44
Cytosine	−0.41	0.28	0.11	0.14	−0.41	0.50	**0.94**	0.09
Glutamic acid	−0.43	−0.23	0.03	0.35	0.13	−0.20	−0.13	0.19
Guanosine	−0.26	−0.45	0.01	0.02	−0.17	0.06	−0.19	−0.13
Histidine	**0.39**	0.49	−0.02	−0.63	−0.13	0.22	0.16	**−0.73**
Tryptophan	**0.70**	0.09	−0.04	−0.40	0.12	0.14	−0.01	**−0.34**
Lysine	**1.10**	−0.39	−0.06	−0.45	0.06	0.54	−0.18	−0.43
Nicotine amide	0.28	−0.20	0.07	0.23	0.36	0.10	0.06	0.13
Phenylalanine	0.15	−0.18	−0.05	−0.14	−0.11	−0.12	−0.15	−0.11
Propionylcarnitine (C3)	0.00	−0.02	0.46	−0.12	0.17	0.56	−0.03	0.12
Pyridoxal	0.24	0.24	0.16	−0.73	−0.46	0.37	0.17	**−0.94**
Pyroglutamic acid	−0.06	0.09	0.12	0.08	0.02	0.04	−0.17	**0.33**
Serine	0.02	0.13	0.10	0.21	0.19	0.88	−0.02	0.12
Sorbitol/Mannitol	−0.41	−0.08	−0.02	0.19	−0.43	−0.27	0.15	0.14
Threonine	−0.01	−0.06	0.04	0.47	0.18	0.90	−0.15	−0.11
Trans-4-hydroxyproline	0.33	0.77	0.10	−0.19	**0.43**	0.54	**0.63**	−0.03
Uridine	−0.49	−0.79	0.13	0.19	−0.36	−0.54	−0.16	−0.30

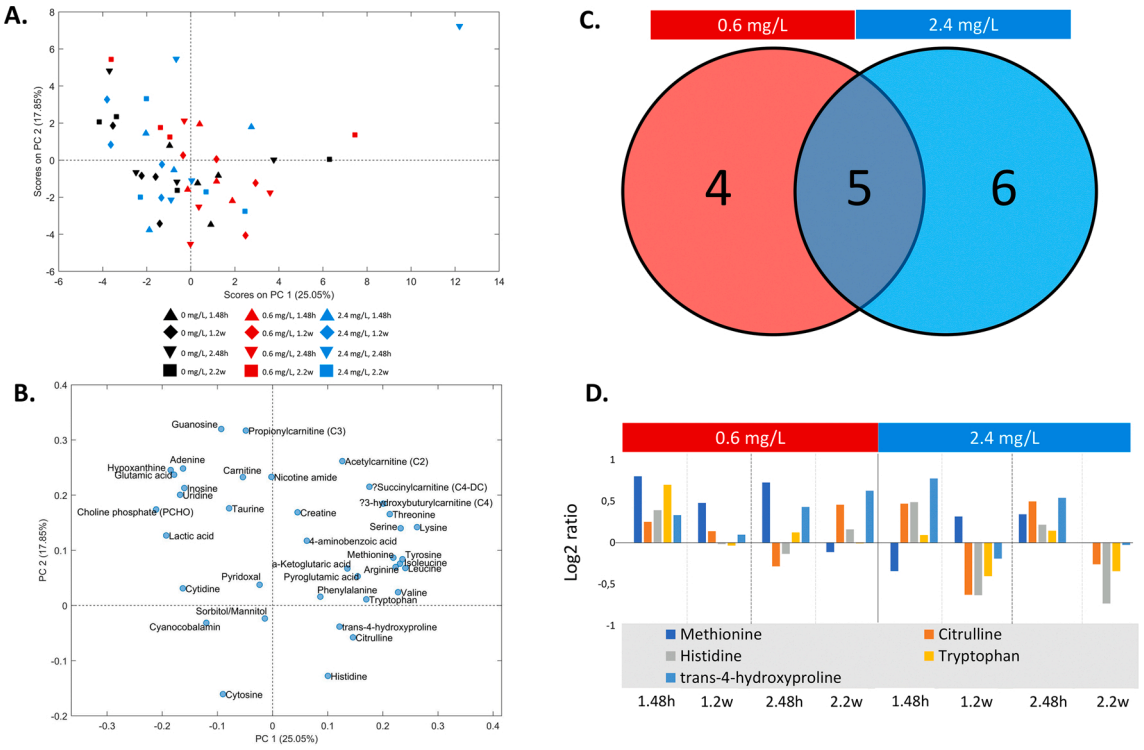

Fig. 4. Plasma metabolome of Atlantic salmon exposed to therapeutic doses of peracetic acid. **A)** Score plot from the PCA model calculated from the relative concentrations of the variables in the identified compounds. Data are auto-scaled. **B)** Loading plot from the PCA model calculated from the relative concentrations of the variables in the identified compounds. Data are auto-scaled. **C)** Venn diagram showing metabolites that were differentially modulated by PAA. **D)** Changes in the 5 metabolites that were differentially modulated in both treatment groups at least one timepoint. Values are expressed relative to the concentration in the 0 mg/L group at the same timepoint. Notations: 1.48 h = 48 h after initial exposure; 1.2 w = 2 weeks after first exposure; 2.48 h = 48 h after re-exposure; 2.2 w = 2 weeks after re-exposure.

Methionine is an aliphatic, sulphur-containing, essential amino acid (Martínez et al., 2017) that has been identified as a crucial regulator of the oxidative state in fish (Coutinho et al., 2017). Methionine residues may act as catalytic antioxidants, methionine sulfoxide reductases in most cells, catalysing a thioredoxin-dependent reduction of methionine sulfoxide, which is formed when methionine react with ROS, back to methionine (Luo and Levine, 2009). The cyclic interconversion of the methionine residues in proteins between their oxidised and reduced forms may, therefore, be regarded as an efficient ROS-scavenging mechanism (Levine et al., 1999; Wu et al., 2012). It could be possible that the substantial increase in methionine at 48 h in both treatment occasions in the 0.6 mg/L group is an important early phase protective mechanism against increased environmental ROS and the trigger does not depend on the dose.

For the 2.4 mg/L group, citrulline and histidine are the two molecules that were identified as early phase responders with their level were substantially increased at 48 h after each exposure (Fig. 4D). It has been demonstrated in a mammalian model that L-citrulline can enhance nitric oxide bioavailability and concomitantly reduce oxidative stress, especially in the vascular epithelium (Dawoud and Malinski, 2020). On the other hand, the role of histidine in the mobilisation of the response to oxidative stress has been documented in fish, mainly through dietary interventions (Jiang et al., 2016; Yang et al., 2019). Previous knowledge of the potential involvement of these molecules during oxidative stress leads us to hypothesise that their increased circulatory level is likely a counter response to the pressures associated with increased levels of environmental radicals that affected the internal redox balance (Soleng et al., 2019). However, it remains unknown whether they act by scavenging or neutralising increased ROS. The markedly low levels of these molecules 2 weeks post-exposures suggest a probable compensatory response that stabilises their levels in the plasma. It is interesting to evaluate this marked downregulation at 2 weeks post-treatment in the future, particular their role in inflammatory activity because both molecules have known anti-inflammatory functions (Darabi et al., 2019; Watanabe et al., 2008) and some immune genes involved in this process (e.g., cytokines) were downregulated as well at the mucosa.

Trans-4-hydroxyproline, an isomeric form of hydroxyproline, was significantly modulated both days and weeks after the initial and re-exposure in the 0.6 mg/L group (Fig. 4D). For the high dose, it was only markedly affected 48 h after treatments. This opposite response indicates that the trans-4-hydroxyproline-mediated response is not entirely dependent on the dose of PAA but may be dramatically influenced by how the precursors (i.e., proline) is affected by PAA and eventually the impact on the synthesis of the metabolite. Hydroxyproline is a structurally and physiologically important amino acid in animals. Its conversion into glycine enhances the production of glutathione, and the oxidation of hydroxyproline by hydroxyproline oxidase plays essential parts in cell antioxidative reactions, survival and homeostasis (Wu et al., 2017). One may speculate that the increase in the PAA-exposed groups may be important for antioxidant defence, nonetheless, it has a crucial role in collagen synthesis (Amit Kumar et al., 2016), which sheds light as to why PAA dramatically influenced the tissue extracellular matrix of the mucosa (Figs. 2 and 3).

The pattern of tryptophan response was similar in both treatment groups 48 h after exposure regardless of the duration, where a marked elevation was observed (Fig. 4D). This response was no longer present 2 weeks after exposure in the 0.6 mg/L group, however, the level was substantially reduced in the 2.4 mg/L group. The elevated level at the early phase after treatment can be attributed to the established antioxidant function of tryptophan. It is, together with some associated metabolites (e.g., melatonin, kynurenic acid, and xanthurenic acid) act as effective antioxidants, removing reactive oxygen, reactive nitrogen, and

active chlorine species and enhancing the organism's protection against free radical damage (Xu et al., 2018). Tryptophan deficiency is related to several physiological problems that have severe implications on fish growth (Hoseini et al., 2019). Though our current data cannot support the probability that the remarkable downregulation of tryptophan at 2 weeks post-treatments in the 2.4 mg/L group will lead to deficiency, it is logical to speculate that the decrease in plasma tryptophan level may not have been low enough to impair growth since both groups did not differ in weight after the trial.

3.5. Conclusions

Fish, in the wild and captivity, are constantly exposed to different environmental challenges that impact their health, fitness and survival. The use of strong oxidants, such as peroxides, are common in aquaculture to improve water quality, rearing conditions and treating diseases, but how the host fish respond to these compounds is often overlooked. The results of the present study provide the first comprehensive evidence for the global molecular responses that were mobilised in reaction to increased ROS levels in the rearing environment via the application of an oxidative chemotherapeutic PAA. Global transcriptomic profiles of the skin and gills of PAA-exposed fish identified the former as relatively more responsive to the tested treatment doses than the latter based on the magnitude of the transcriptional changes and the number of DEGs. The overall profiles further suggest that the mucosa could mount responses crucial to counteract the pressure of increased ROS levels. Moreover, plasma metabolome profiling showed that PAA treatments did not trigger metabolomic disturbances. Metabolite-specific responses, however, identified molecules with known functions during oxidative stress that were affected by PAA at both concentrations. The transcriptomic and metabolomic profiles corroborated the minimal impact of PAA on production performance parameters and external welfare indices. Therefore, PAA is a potential chemotherapeutic with minimal health and welfare impacts that salmon can physiologically adapt to. It is important to emphasise that the results presented here have to be considered as specific to the PAA product formulation and that we cannot rule out or guarantee that other product formulations (with different strengths, compositions and stabilisers) may lead to different responses, physiologically and behaviourally.

Author contributions

C.C.L. and L.F.P. conceived of the research idea. C.C.L. and L.F.P. designed the trials. C.C.L., L.F.P., G.T., M.W.B. and M.S. conducted the experiments and collected the samples. C.C.L., K.H.K., and M.S. performed the analyses in the laboratory. C.C.L, K.H.K., M.S. and G.T. analysed the data. C.C.L. and G.T. prepared the figures. All authors contributed to the writing of the draft and reviewed the final version of the manuscript.

Declaration of Competing Interest

The authors declare no competing interests. Mention of trade names in this manuscript does not imply any recommendation or endorsement by Nofima or DTU Aqua.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.aquatox.2020.105625.

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