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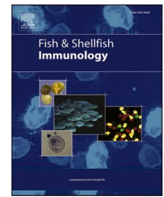

Full length article

Regulation of the molecular repertoires of oxidative stress response in the gills and olfactory organ of Atlantic salmon following infection and treatment of the parasite *Neoparameoba perurans*

Francisco Furtado [a,d], Mette W. Breiland [b], David Strand [c], Gerrit Timmerhaus [d], Danilo Carletto [d,e], Lars-Flemming Pedersen [f], Fernando Afonso [a], Carlo C. Lazado [d,*]

[a] CIISA, Faculty of Veterinary Medicine, University of Lisbon, 1300-477, Lisbon, Portugal
[b] Nofima, The Norwegian Institute of Food, Fisheries and Aquaculture Research, 9019, Tromsø, Norway
[c] Norwegian Veterinary Institute, 1433 Ås, Norway
[d] Nofima, The Norwegian Institute of Food, Fisheries and Aquaculture Research, 1433 Ås, Norway
[e] Department of Chemical, Biological, Pharmaceutical and Environmental Sciences, University of Messina Viale Ferdinando Stagno d'Alcontres, 31-98166 S, Agata-Messina, Italy
[f] Technical University of Denmark, DTU Aqua, Section for Aquaculture, The North Sea Research Centre, PO Box 101, 9850, Hirtshals, Denmark

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ABSTRACT

The present study investigated the involvement of key molecular regulators of oxidative stress in amoebic gill disease (AGD), a parasitic infestation in Atlantic salmon. In addition, the study evaluated how these molecular biomarkers responded when AGD-affected fish were exposed to a candidate chemotherapeutic peracetic acid (PAA). Atlantic salmon were experimentally infected with the parasite *Neoparameoba perurans*, the causative agent of AGD, by bath exposure and after 2 weeks, the fish were treated with three commercial PAA products (i.e., Perfectoxid, AquaDes and ADDIAqua) at a dose of 5 ppm. Two exposure durations were evaluated – 30 min and 60 min. Sampling was performed 24 h and 2 weeks after PAA treatment (equivalent to 2- and 4-weeks post infection). At each sampling point, the following parameters were evaluated: gross gill pathology, gill parasitic load, plasma reactive oxygen species (ROS) and total antioxidant capacity (TAC), histopathology and gene expression profiling of genes with key involvement in oxidative stress in the gills and olfactory organ. AGD did not result in systemic oxidative stress as ROS and TAC levels remained unchanged. There were no clear patterns of AGD-mediated regulation of the oxidative stress biomarkers in both the gills and olfactory organ; significant changes in the expression were mostly related to time rather than infection status. However, the expression profiles of the oxidative stress biomarkers in AGD-affected salmon, following treatment with PAA, revealed that gills and olfactory organ responded differently – upregulation was prominent in the gills while downregulation was more frequent in the olfactory organ. The expression of *catalase*, *glutathione S-transferase* and *thioredoxin reductase 2* was significantly affected by the treatments, both in the gills and olfactory organ, and these alterations were influenced by the duration of exposure and PAA product type. Parasitic load in the gills did significantly increase after treatment regardless of the product and exposure duration; the parasite was undetectable in some fish treated with AquaDes for 30 mins. However, PAA treated groups for 30 min showed lower macroscopic gill scores than the infected-untreated fish. Histology disclosed the classic pathological findings such as multifocal hyperplasia and increased number of mucous cells in AGD-affected fish. Microscopic scoring of gill injuries showed that AGD-infected-PAA-treated fish had lower scores, however, an overall trend could not be established. The morphology and structural integrity of the olfactory organ were not significantly altered by parasitism or PAA treatment. Collectively, the results indicate that AGD did not affect the systemic and mucosal oxidative status of Atlantic salmon. However, such a striking profile was changed when AGD-affected fish were exposed to oxidative chemotherapeutics. Moreover, the gills and olfactory organ demonstrated distinct patterns of gene expression of oxidative stress biomarkers in AGD-infected-PAA-treated fish. Lastly, PAA treatment did not fully resolve the infection, but appeared not to worsen the mucosal health either.

* Corresponding author.
E-mail address: carlo.lazado@nofima.no (C.C. Lazado).

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1. Introduction

Reactive oxygen species (ROS) refers to an array of derivatives of molecular oxygen that play a crucial role in aerobic life [1]. They are formed as a natural by-product of the normal metabolism of oxygen and are fundamentally important for the physiology, as functional signalling entities [1,2]. In aquatic animals, the antioxidant system is composed of enzymatic and non-enzymatic, low and high molecular mass antioxidants, that ensure ROS are kept under a non-deleterious level [3]. The balance between the production of ROS and the systems required to mitigate ROS is necessary to prevent the onset of a physiological state with deletrious consequences. The low mass antioxidants are known compounds, that can be water-soluble and function normally as free radical scavengers, like glutathione in its reduced form and ascorbic acid (Vitamin C), or lipid-soluble, such as retinol (Vitamin A), carotenoids (β-carotene included) and α-tocopherol (Vitamin E), as well as metal-binding proteins, such as ferritin and ceruloplasmin [3,4]. The high molecular mass antioxidants, abundant inside the cells, are enzymes such as *catalase* (cat), *superoxide dismutase* (sod) and *nicotinamide adenine dinucleotide phosphate* (NADPH). Due to their high reactivity, ROS can disrupt normal cellular function, and if not neutralised or scavenged, can lead to oxidative stress. In fish, oxidative stress can be triggered by various factors during production such as feeding, water quality, chemotherapies and infection, among a few others. In particular, the influence of oxidative stress in disease pathophysiology is an overlooked area in fish, despite an established connection in humans [5,6].

The sea-caged production of Atlantic salmon has two major parasitic infestation problems – sea lice and amoebic gill disease (AGD). They both entail biological, economic and societal concerns with varying costs for the industry. Although sea lice (*Lepeophtheirus salmonis* and members of the *Caligus* genus) remain the major parasitic issue, the threats posed by AGD outbreaks highlight the need to develop measures for sustainable salmon farming. The causative agent, *Neoparamoeba perurans,* is a free-living and opportunistically parasitic amoeba species, that attaches to the gill lamellae [7,8]. AGD manifests clinically as lethargy, anorexia, congregation at the water surface and increased ventilation rate [9], which leads to respiratory distress that can result in mortality if left untreated [10,11]. AGD is characterised by increased mucus production and the formation of white mucoid spots and plaques on the gill surface [12], which are used to score the severity of infection in the farms. Microscopically, infected gills exhibit epithelial multifocal gill hyperplasia, hypertrophy, oedema, and interlamellar vesicle formation [13]. Recently, oxidative stress has been implicated in the late stage of AGD in farmed Atlantic salmon [14] indicating that this fundamental phenomenon is likely involved in its pathophysiology. However, the limited indicators employed in the study posed a challenge in quantifying the magnitude of oxidative stress triggered by parasitism.

Peracetic acid (CH_3CO_3H; thereafter referred as PAA) is a potent oxidative disinfectant with a wide spectrum of antimicrobial activity and is generally accepted as an eco-friendly disinfectant because of its rapid degradation into innocuous by-products [15]. By releasing highly reactive oxygen radicals upon its decay, PAA oxidises the sulfhydryl and sulfur bonds in proteins, enzymes and other metabolites. This will lead to the impairment of chemiosmotic function of the lipoprotein cytoplasmic membrane and transport [16,17]. Oxidative disinfectants are often considered exogenous triggers of oxidative stress, especially that increased production of ROS is a causal feature in the toxicity of many xenobiotics [5], including PAA [18]. We have shown in earlier studies that bath exposure of Atlantic salmon smolts to PAA could induce transient oxidative stress [18,19], which is a major indicator for its informed use in aquaculture. PAA is a good candidate chemotherapeutic against a number of bacterial and parasitic infections [15] – how the disease status of fish interferes with the responses to PAA is yet to be unravelled.

The present study investigated the impact of AGD on the molecular repertoire of oxidative stress in the mucosal organs (i.e., gills and olfactory organ) of Atlantic salmon. The most widely used commercial treatment against AGD is freshwater bathing, though largely effective, the whole process requires high logistics cost. Chemotherapeutics such as chlorine-based chloramine-T (N-Chloro 4-methyl benzenesulfonamide, sodium salt) and the oxygen-based hydrogen peroxide (H_2O_2) are also being used, although environmental risks and conflicting laboratory and field results are often an issue. Here we explored PAA as a potential treatment for AGD and further identified how the infection interfered with the oxidative stress responses to the chemotherapeutics.

2. Materials and methods

2.1. Ethical use of fish for research

All fish handling procedures complied with the Guidelines of the European Union (Directive 2010/63/EU). The study was approved by the Norwegian Food Safety Authority under FOTS ID 20/23121.

2.2. Experimental infection with Neoparamoeba perurans

The fish experiment was conducted at the Tromsø Aquaculture Research Station (HiT), Norway. Prior to the trial, a representative number of experimental fish were sent to an external service laboratory for whole package diagnostics to ensure that only healthy fish were used. There were two main tanks at the start of the trial - Tank 1: Uninfected; and Tank 2: Infected groups. Each tank was stocked with 420 smolts, at around 70 g starting weight. Fish were allowed to acclimatise under the following conditions for 2 weeks: water flow rate in the tanks was 6–7 L/min, water temperature at 14.5 ± 1 °C, oxygen at >85% saturation, salinity at 35 ppt, pH at 7.9, photoperiod set at 24 light:0 darkness and continuous feeding regime with a commercial diet (Skretting Nutra Olympic 3 mm, Averøy, Norway) administered through a belt feeder. These conditions were likewise adopted all throughout the trial.

After 2 weeks, AGD was induced in Tank 2. Briefly, the water outlet was closed and *Neoparamoeba perurans* culture (provided by Sigurd Hytterød, Norwegian Veterinary Institute) was added to the tank to achieve a concentration of 1500 parasites/L. The fish were exposed to the parasites for 1 h. During the exposure period, the level of oxygen was routinely monitored to ensure that DO level did not go below 85% saturation. After the exposure period, water was flushed out and replaced with clean water. For Tank 1, the fish were handled similarly but without the addition of the parasite.

2.3. Treatment of parasitised fish with peracetic acid bathing

Three different commercial peracetic acid-based disinfectants were used in the trial: AquaDes (AQUA PHARMA U.S., INC. Kirkland, Washington, USA), ADDIAqua (Lillborg AS, Oslo, Norway) and Perfectoxid (Aquatiq Chemistry, Lillehammer, Norway). To ensure exact dosing, the actual concentration of PAA and hydrogen peroxide (Product PAA (%) H_2O_2 (%): Perfectoxid = 5.43, 17.9; ADDIAqua = 5.78, 17.9; AquaDes = 6.58, 23.5) in the products was empirically determined according to Falsanisi et al., 2006 [20].

After 10 days, fish were checked for their gill scores [21] and distributed to 0.5 m^3 circular tanks at a density of 30 fish per tank, according to the following treatment outlay which indicates 8 treatment groups in total, namely, 1) uninfected, untreated group; 2) infected, untreated group; 3) infected, AquaDes-treated group for 30 min; 4) infected, AquaDes-treated group for 60 min; 5) infected, Perfectoxid -treated group for 30 min; 6) infected, Perfectoxid -treated group for 60 min; 7) infected, ADDIAqua-treated group for 30 min; 8) infected, ADDIAqua -treated group for 60 min. Each group had duplicate tanks. Treatment was performed 5 days after transfer to smaller tanks, which was equivalent to 2 weeks post-infection. PAA Treatment protocol was

as follows for Groups 3 to 8: The water outlet was closed, using a bucket, *circa* 5 L of water were taken from the tank, PAA was added in the bucket and thereafter the water-PAA mixture was poured into the tanks and distributed to 6 different locations. Mixing was facilitated by aeration. Each PAA-treated group was exposed to a corresponding PAA target concentration of 5 mg L^{-1} either for 30 or 60 min. DO and pH were maintained above 90% saturation and 7.8–7.9. After the exposure period, the water was flushed out and replaced with clean water. This protocol had been standardised so that no residual PAA is present in the system after water replacement [19].

2.4. Sample collection

There were two samplings – 24 h (equivalent to 2 weeks post-infection) and 2 weeks (equivalent to 4 weeks post-infection) after treatment. For each sampling point, five fish were randomly dip-netted from each tank and humanely euthanised, with an overdose of Benzoak Vet (ACD Pharmaceuticals AS, Leknes, Norway) through an immersion bath. After the gill scores were assessed by trained personnel, fish lengths and weights were recorded. Thereafter, blood was collected using a lithium heparinised vacutainer (BD, Plymouth, United Kingdom) from the caudal artery, centrifuged for 10 min at 5200 rpm (Heraeus Labofuge 200, Thermo Scientific, Massachusetts, USA) and plasma was separated and stored at −80 °C until analysis. Gill swabs (Sarstedt, Germany) were taken from the left side of the gills and stored in ATL buffer (Qiagen, Hilden, Germany) for detection of the parasite by qPCR. The second gill arch was dissected and divided into two, where one fraction (non-lesion) was placed in RNAlater (Ambion©, Connecticut, USA), kept at room temperature for 12 h to allow proper penetration and afterwards stored at −80 °C until further use, while the other fraction was stored in 10% neutral buffered formalin (BiopSafe®, Stenløse, Denmark) for histological use. For the olfactory organ, the left rosette was collected for RNA, while the right rosette was separated for histology, both fractions were handled similarly to the gills.

2.5. Plasma analysis

Total antioxidant capacity (TAC) in the plasma was analysed by a colorimetric assay kit (Sigma-Aldrich, Missouri, USA) previously used in salmon [22]. The determination of reactive oxygen species (ROS) levels was done by using a fluorometric assay kit OxiSelect™ *In Vitro* ROS/RNS (Cell biolabs, Inc., San Diego, California, USA). All the samples were analysed in duplicates.

2.6. RNA isolation, cDNA synthesis and real-time quantitative PCR

Total RNA was isolated from the gills and olfactory organs using Agencourt RNAdvance™ Tissue Total RNA Purification Kit (Beckman Coulter Inc., California USA) in Biomek 4000 Benchtop Workstation (Beckman Coulter, Inc., Indianapolis, USA). NanoDrop 8000 spectrophotometer (Thermo Scientific, USA) was used to determine the RNA concentration and quality. Complementary DNA (cDNA) was synthesized through reverse transcription of 500 ng total RNA in a 20 μL reaction using Taqman™ Reverse Transcription Kit (Applied Biosystems, Massachusetts, USA). The PCR reaction was carried out in a Veriti™ 96-Well Thermal Cycler (Applied Biosystems, California, USA) with the following thermocycling parameters: 25 °C for 10 min, 37 °C for 30 min and 95 °C for 5 min.

QuantStudio™ 5 Real-Time PCR System (Applied 22 Biosystems, USA) was used to perform reverse transcription real-time quantitative polymerase chain reaction (RT-qPCR) for the quantification of selected transcripts detailed in Table 1. Each reaction mixture contained 4 μL 1:10 diluted cDNA, 5 μL of PowerUp™ SYBR™ Green Master Mix (Applied Biosystems, USA) and 0.5 μL 10 μM of each forward/reverse primer (Invitrogen, USA). Thermocycling parameters were as follows: 2 mins of pre-incubation at 95 °C, amplification with 40 cycles for 1 s at 95 °C and 30 s at 60 °C, and a melt curve stage of 15 s at 95 °C, 1 min at 60 °C and 15 s at 95 °C. All samples were run in duplicates. A standard curve with five times 2-fold dilution series was prepared from pooled cDNA to calculate the amplification efficiency. Expression was normalised using the geometric mean of two reference genes: *beta actin* (*actb*) and *elongation factor 1 alpha* (*ef1α*) for the gills, while *actb* and *hypoxanthine-guanine phosphoribosyltransferase* (*hprt1*) were used for the olfactory organ.

2.7. Real-time quantitative PCR detection of N. perurans

DNA was extracted from the gill swabs using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany). The samples were analysed with a *N. perurans* specific qPCR assay in order to detect *N. perurans,* the parasite and estimate DNA copies. The analyses were performed on the CFX96 Touch System (Biorad, California, USA) with 25 μl reactions consisting of 12.5 μl TaqPath qPCR Mastermix, 500 nM of each primer and 250 nM of probe (forward primer 5′-GTT CTT TCG GGA GCT GGG AG-3′, reverse primer 5′- CAT GAT TCA CCA TAT GTT AAA TTT CC-3′ and probe 5′-FAM/CTC CGA AAA/ZEN/GAA TGG CAT TGG CTT TTG A/3IABkFQ-3′), PCR grade water and 5 μl DNA sample. The thermocycling conditions were as follows: an initial denaturation at 95 °C for 20 s, followed by 50 cycles of denaturation at 95 °C for 3 s and annealing at 60 °C for 30 s. A 10-fold standard dilution using synthesized dsDNA (gBlocks™ gene fragment, Integrated DNA Technologies, Iowa, USA) of the qPCR target region with known DNA concentration was included in each qPCR run to estimate the DNA copies per reaction. Olfactory organ from a separate group of fish during each post-treatment sampling was collected and sent to an external diagnostics service laboratory for qPCR detection of the amoeba.

2.8. Histological assessments

The gill samples were sent to the Norwegian Veterinary Institute in Harstad, Norway, for processing and staining. The digitised Periodic Acid Schiff-Alcian Blue (AB-PAS) stained tissue sections were sent to Nofima for evaluation. The formalin-preserved olfactory organs were processed in-house in an automated tissue processor (TP1020, Leica Biosystems, Germany), embedded in paraffin (Leica EG1150H, Leica Biosystems, Nussloch, Germany), cut into 5 μm sections in a rotary microtome, and stained by an automated stainer (ST5010, Leica Biosystems, Nussloch, Germany) with AB-PAS and scanned with a digital slide scanner (Aperio CS2, Leica Biosystems, Illinois, USA).

For the analysis of the gills, 3 assessments were done for each of the samples. 1) *Overall damage scoring.* To perform this assessment a scoring system, ranging from 0 to 3, was used which based on the percentage of tissue injury in the gills per microscopic field at x100 magnification, as detailed in Supplementary Table 1. 2) *Quantification of non-specific lesions*. Six fields were randomly selected in the tissue section. In each field, 40 lamellae were selected giving a total amount of 240 investigated secondary lamellae per fish. Hyperplasia, lamellar fusion, epithelial lifting, lamellar clubbing, hypertrophy, necrosis, and aneurysms were documented. If none of the mentioned lesions were present, the lamellae were defined as "healthy". 3) *Morphometric assessment*. Three additional fields with 30 lamellae were selected and, in both filament and lamellae, mucous cells were quantified.

The olfactory organ was assessed based on a 0 to 3 scoring scheme which accounts for the degree of epithelial surface smoothness, loss of definition and structures, and signs of necrosis, according to the scoring scheme in Supplementary Table 2. In addition, measurements were carried out in 6 locations, for both epithelium and *lamina propria* thickness, in 3 randomly selected olfactory lamellae.

2.9. Statistical analysis

All statistical analyses were performed in SigmaPlot 14.0 (Systat

Table 1
Primers used in the study.

Gene name	Abbreviation	Sequences (5' → 3')	Reference
Glutathione peroxidase	*gpx*	F: GATTCGTTCCAAACTTCCTGCTA R: GCTCCCAGAACAGCCTGTTG	(Solberg et al., 2012)
Glutathione reductase	*gr*	F: CCAGTGATGGCTTTTTTGAACTT R: CCGGCCCCCACTATGAC	(Solberg et al., 2012)
Glutathione S-transferase	*gsta*	F: AGGGCACAAGTCTAAAGAAGTC R: GTCTCCGTGTTTGAAAGCAG	(Lazado & Voldvik 2020)
Manganese superoxide dismutase	*mnsod*	F: GTTTCTCTCCAGCCTGCTCTAAG R: CCGCTCTCCTTGTCGAAGC	(Solberg et al., 2012)
Copper/Zinc superoxide dismutase	*cu/znsod*	F: CCACGTCCATGCCTTTGG R: TCAGCTGCTGCAGTCACGTT	(Solberg et al., 2012)
Catalase	*cat*	F: GGGCAACTGGGACCTTACTG R: GCATGGCGTCCCTGATAAA	(Olsvik et al., 2011)
Thioredoxin-like	*txnl*	F: CTTCTTCAAAGGGCTGTCGG R: GCATTTGATTTCACAGTGTTGGG	This study
Peroxiredoxin 3	*prdx3*	F: TTTAAAGCTACAGCTGTCCAC R: GACAAACAAACGTGAAATCGAG	This study
Thioredoxin reductase 1	*txnrd1*	F: GTGAACGACGAGGAACAGAC R: GTAGTCACACTTGAGCGAGG	This study
Thioredoxin reductase 2	*txnrd2*	F: TGATCTCGTCGTTATTGGTGGT R: TAGTACCTTTAAGTGACGGCTCC	This study
Sulfiredoxin 1	*srxn1*	F: GAAGTTATCATACGACCAATCCC R: GTCTGTAGATTCCTGTATTGTACC	This study
Oxidation resistance 1	*oxr1*	F: GACCTTCCTCTTCACCTTCTG R: CCAAACTCACCACTTCCACC	This study
Thioredoxin-interacting protein-like	*txnip*	F: GAGAGTCTCGGCTATGAAAGTG R: CATCATGATCAGCTGGATGGT	This study
Elongation factor alpha 1	*ef1a*	F: GAATCGGCTATGCCTGGTGAC R: GGATGATGACCTGAGCGGTG	This study
β-actin	*actb*	F: CCAAAGCCAACAGGGAGAA R: AGGGACAACACTGCCTGGAT	This study
Hypoxanthine phosphoribosyltransferase 1	*hprt1*	F: CCGCCTCAAGAGCTACTGTAAT R: GTCTGGAACCTCAAACCCTATG	(de la Serrana & Johnston, 2013)

Software Inc, Berkshire, UK). Before performing ANOVA, the data set was subjected to Shapiro-Wilk test for normality check and Brown-Forsythe test, for equal variance requirement. Data was Log10 transformed when one of the ANOVA requirements was not fulfilled. The data was subjected to two-way ANOVA to compare differences between treatments and sampling points, as well as the interactions of these factors. Pairwise comparison was performed by Holm-Sidak method. The level of statistical significance was set at $P < 0.050$ in all analyses. For the heatmaps of gene expressions, qPCR results were imported into R (Version 4.0.2, https://www.r-project.org/). Mean expression values were calculated for each gene and treatment/time point group. One heatmap were calculated each for gill and olfactory organ expression, by using the heatmap.2 function (gplots package, no scaling) and a complete linkage algorithm for clustering (both dimensions). Mean expression values were coloured from blue to yellow.

3. Results

3.1. Production performance

There were no significant changes in the weight and length of the experimental fish. Daily monitoring of the feeding behaviour revealed no deviations. Except for the two dead fish found a day before the last sampling in AGD-affected-PAA-treated fish (one from AquaDes-30 and one from ADDIAqua-60), no significant mortality was recorded.

3.2. Level of ROS and TAC in plasma

There was a significant temporal difference in the plasma ROS level in all groups, where an increase was observed at 2 weeks post PAA treatment (Fig. 1A). There were no significant inter-treatment differences at 24 h post PAA treatment. However, significant inter-treatment differences were identified at 2 weeks post PAA treatment - the level was significantly higher in the groups exposed to AquaDes and Perfectoxid for 30 min compared with all treatment groups except in the infected-untreated and Perfectoxid-60 min groups.

The plasmatic total antioxidant capacity (TAC) did not exhibit significant inter-treatment differences both at 24 h and 2 weeks post PAA treatment (Fig. 1B). However, in the groups exposed to Perfectoxid for 30 min and ADDIAqua for 60 min, significant temporal difference was identified and characterised by a higher level at 2 weeks post PAA treatment.

3.3. Influence of AGD on the expression of genes related to oxidative stress in the gills and olfactory organ

We first evaluated how AGD affected the expression of genes relevant to oxidative stress in the gills (Fig. 2) and olfactory organ (Fig. 3) by comparing the uninfected-untreated (control) and infected-untreated (infected) groups at 2 weeks and 4 weeks after infection (this is equivalent to 24 h and 2 weeks post-treatment). There were significant temporal differences in the expression of *glutathione S-transferase* (*gsta*), *thioredoxin-like* (*txnl*) and *oxidation resistance 1* (*oxr*) in the gills – the expression was significantly higher at 4 weeks after infection in *gsta* and *oxr*, while an opposite trend was identified for *txnl* (Fig. 2). Only the expression of *gsta* showed significant inter-treatment differences where the expression was significantly lower in AGD-affected fish in both time points (Fig. 2A). In the olfactory organ, a number of genes displayed significant temporal changes including *catalase* (*cat*), *thioredoxin reductase 1* (*txnrd1*), *txnrd2*, *txnl*, *peroxiredoxin 3* (*prdx3*), *oxr* and *thioredoxin-interacting protein-like* (*txnip*) (Fig. 3A and B). For *cat*, *oxr* and *txnip*, both the control and AGD-affected fish displayed significantly higher transcript level at 4 weeks than at 2 weeks after infection. On the other hand, both groups displayed lower *txnrd1* transcript level at 4 weeks after infection. Only the AGD-affected group exhibited significant increase in *txnrd2* and *txnl* expression at 4 weeks after infection. For *prdx3*, the expression in the control group significantly decreased at 4 weeks after infection. Only the expression of *txnl* showed significant inter-treatment

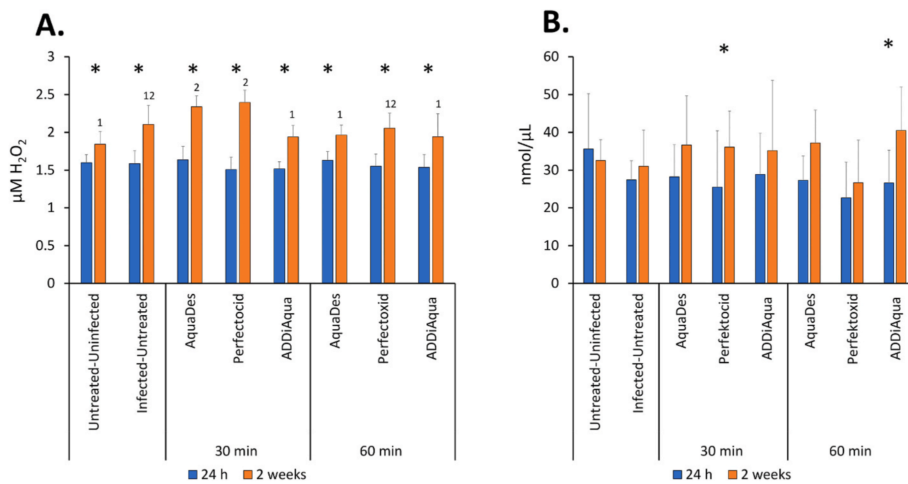

Fig. 1. Plasmatic levels of reactive oxygen species (ROS, expressed as H_2O_2) and total antioxidant activity (TAC) 24 h and 2 weeks after treatment with PAA. Values are means ± SD of 10 individual fish per treatment group at a particular timepoint. Asterisk (*) indicates significant difference between the two sampling points. Different letters denote significant difference among treatment groups at 2 weeks post treatment. No significant inter-treatment differences were identified at 24 h after treatment.

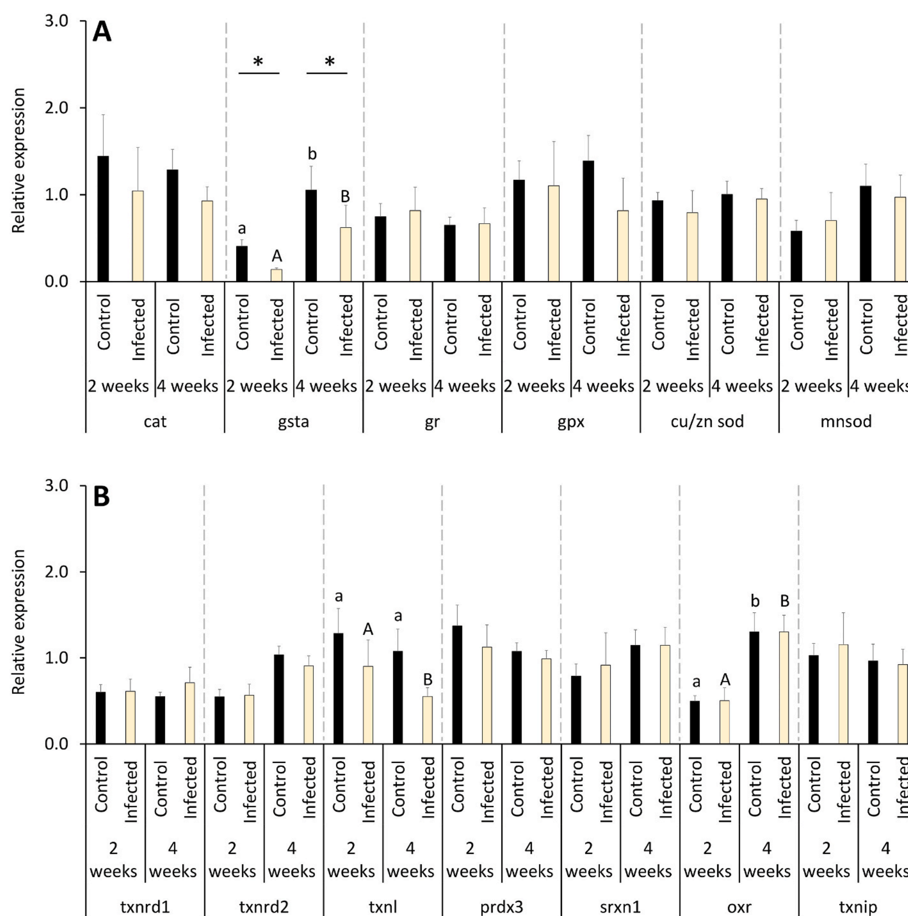

Fig. 2. Changes in the expression of oxidative stress genes in the gills of AGD-affected salmon. Values are means ± SD of 10 individual fish per treatment group at a particular timepoint. Asterisk (*) indicates significant difference between control (uninfected) and infected group at a particular sampling point. Different letters (lowercase: control; uppercase: infected) denote significant temporal difference within a treatment group.

difference, where the expression in the AGD-affected fish was significantly lower than the control at 2 weeks after infection (Fig. 3B).

3.4. Effects of oxidant treatment on the expression of genes related to oxidative stress in the gills and olfactory organ of AGD-affected salmon

There were significant treatment-related changes in the expression of oxidative stress genes in the gills and olfactory organ of AGD-affected salmon treated with different PAA products, either for 30 min or 60 min (Fig. 4, Supplementary Fig. 1). The changes in the expression of individual genes are provided in Supplementary Fig. 1. There was a distinct pattern in the expression of oxidative stress genes in the two organs – the majority of the biomarkers were upregulated in the gills, while downregulation was clearly exhibited in the olfactory organ.

For the gills, there were two major clusters of genes, depending on how they responded to the treatments. In particular, *gsta* formed a single

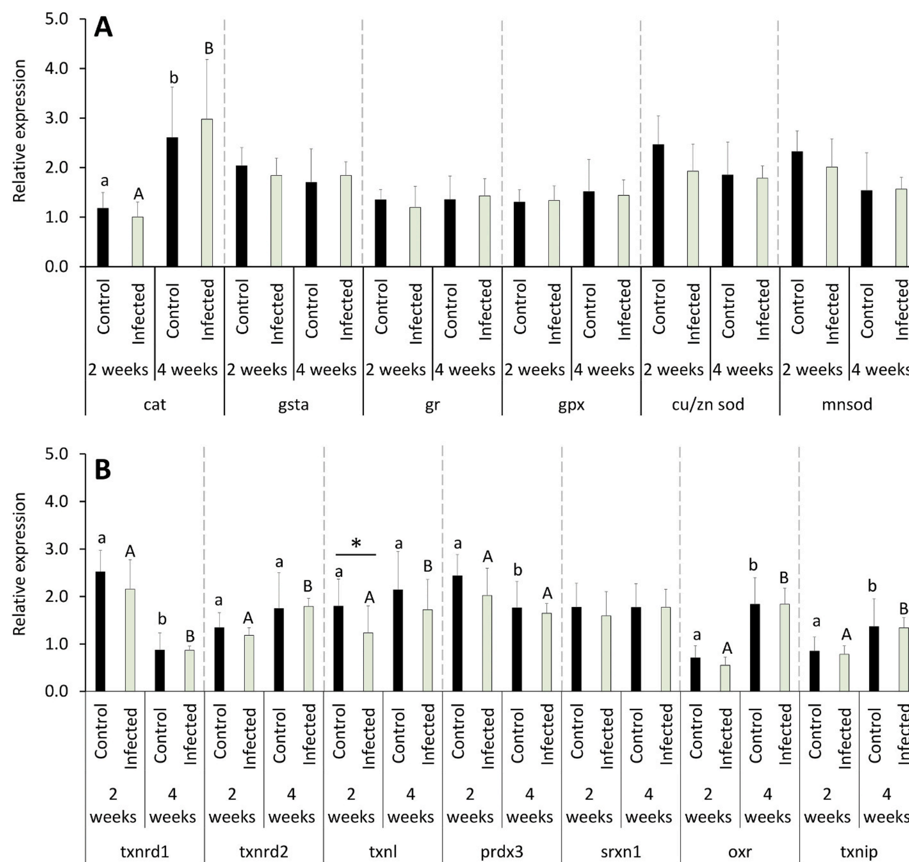

Fig. 3. Changes in the expression of oxidative stress genes in the olfactory organ of AGD-affected salmon. Values are means ± SD of 10 individual fish per treatment group at a particular timepoint. Asterisk (*) indicates significant difference between control (uninfected) and infected group at a particular sampling point. Different letters (lowercase: control; uppercase: infected) denote significant temporal difference within a treatment group.

cluster while of the rest of the oxidative stress genes were in a separate cluster (Fig. 4A). Looking into the changes in relation to time, two clusters were identified per timepoint. Twenty-four hours after treatment, the expression of the biomarkers was similar in AquaDes-30 and Perfectoxid-60, which was predominantly characterised by an upregulation. The other cluster, where the rest of the treatment groups formed, exhibited a downregulation. After 2 weeks, the expression patterns of the biomarkers in AquaDes-60 were different from the rest of the group, as indicated by a separated cluster which was marked by upregulation. The expression of *cat*, *gsta*, *glutathione peroxidase* (*gpx*), *copper/zinc superoxide dismutase (cu/znsod)*, *txnrd1* and *txnrd2* in the gills were significantly affected by the treatments. For *cat*, these changes were

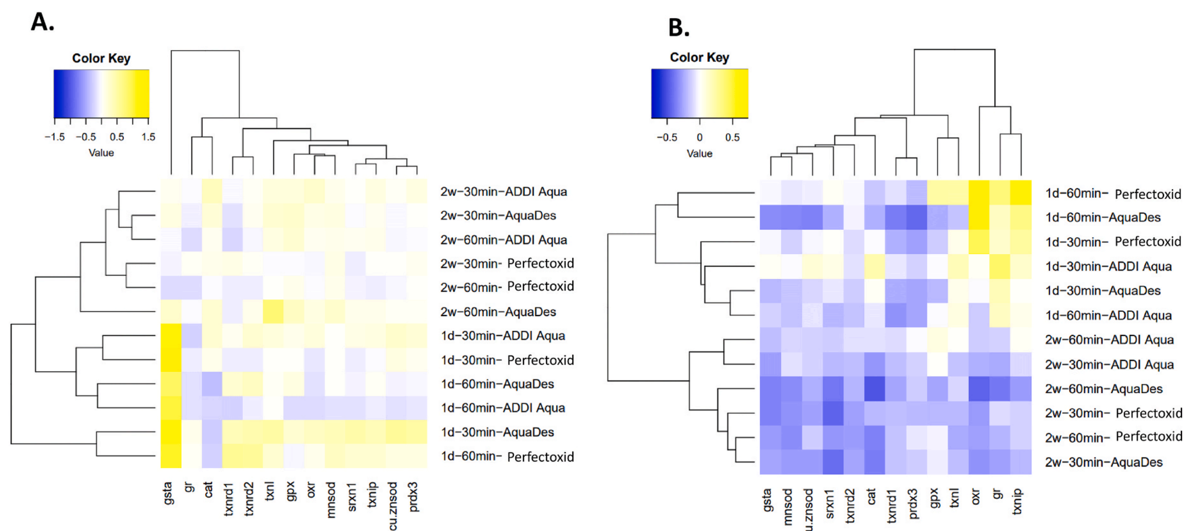

Fig. 4. Changes in the oxidative stress genes in the (A) gills and (B) olfactory organ of AGD-affected Atlantic salmon. Colours represent mean expression values from relative downregulation (blue) to upregulation (yellow). Dendrograms represent the clustering (complete linkage) of genes (columns) and treatment/time point groups (rows). N = 10 individual fish.

dependent on the exposure duration and PAA product while for *cu/znsod*, these alterations were dependent on exposure duration. For *gpx, txnrd1* and *txnrd2*, the changes in the expression were dependent on the product type and not on the exposure duration (Supplementary Fig. 1).

The majority of the oxidative stress biomarkers exhibited downregulation in the olfactory organ of AGD-affected salmon and treated with PAA particularly at 2 weeks after treatment. There were two major clusters in relation to treatment-related effects – a cluster formed by *oxr*, *glutathione reductase* (*gr*) and *txnip*, where upregulation was demonstrated at 24 h after treatment and downregulation 2 weeks thereafter, and another cluster formed by the rest of the genes, which was typified by downregulation in both timepoints (Fig. 4B). Two distinct major clusters were identified at each timepoint. Perfectoxid-60 and AquaDes-60 formed a separate cluster from the rest of the treatment groups at 24 h post treatment. Two of the ADDIAqua groups formed a separate cluster from the other treatment groups at 2-week post treatment. The changes of the individual genes in the olfactory organ demonstrated a clearer pattern of the effects of treatment when compared with the gills (Supplementary Fig. 1). The expression of *cat*, *gsta*, *gr*, *txnrd2*, *txnl*, *prdx3*, *sulfiredoxin 1* (*srxn1*), *oxr* and *txnip* was significantly affected by the treatments, however, these changes were neither dependent on the duration of the exposure nor product type, except for *txnip*. Moreover, the expression of *cu/znsod* and *manganese superoxide dismutase* (*mnsod*) showed to be significantly affected by the product type.

3.5. Gross pathological gill scores and parasitic load

Gills scores were taken 24 h and 2 weeks after treatment (Fig. 5A). At the beginning (24 h), all groups had a gill score between 1 and 2. From the 70 fish evaluated, regardless of the treatment a day earlier, 30 fish demonstrated gill score higher than 2, which accounted for around 42.8% of the population. Two weeks after treatment, all groups had an average gill score between 2 and 3. The group treated with AquaDes for 30 min had the lowest gill score among the groups. All groups treated with PAA for 30 min demonstrated lower gill score than the infected-untreated group.

The parasitic load in the gills is shown in Fig. 5B. There were no significant differences in the parasitic load across different treatments and timepoints. It is important to note that in AquaDes-30, only 6/10 fish in both time points had a detectable quantification cycle (Cq) value.

3.6. Histopathological changes in relation to infection and treatment

3.6.1. Changes in the gills

AGD affected fish showed the classic lesions including hyperplasia and fusion, which were more frequently found at 24 h than at 2 weeks post treatment (Fig. 6A, Fig. 7). Although it appears that fish treated with PAA for 60 min demonstrated higher frequency of hyperplasia, when compared with the infected-untreated group and PAA-treated groups for 30 min, no significant difference was identified with AquaDes-30. From the descriptive assessment of the histopathological lesions in the gills using an injury score, all groups affected by AGD, regardless of whether they had been treated or not, exhibited higher scores (which indicates higher degree of alterations), compared with the untreated-uninfected group at 24 h after treatment. At 2 weeks post treatment, some PAA-treated groups including AquaDes-30, AquaDes-60 and Perfectoxid-60 showed no significant difference to the uninfected-untreated group. ADDIAqua-30 and -60 were still significantly higher than the uninfected-untreated group, but significantly lower than the infected-untreated group. The infected-untreated group showed a significantly higher score than the untreated-uninfected group. In addition, all groups treated with PAA for 60 min showed a lower microscopic gill score at 2 weeks post-treatment than at 24 h after treatment.

The mucous cells in the gills showed no significant inter-treatment differences at 24 h after treatment (Fig. 6B). At 2 weeks after

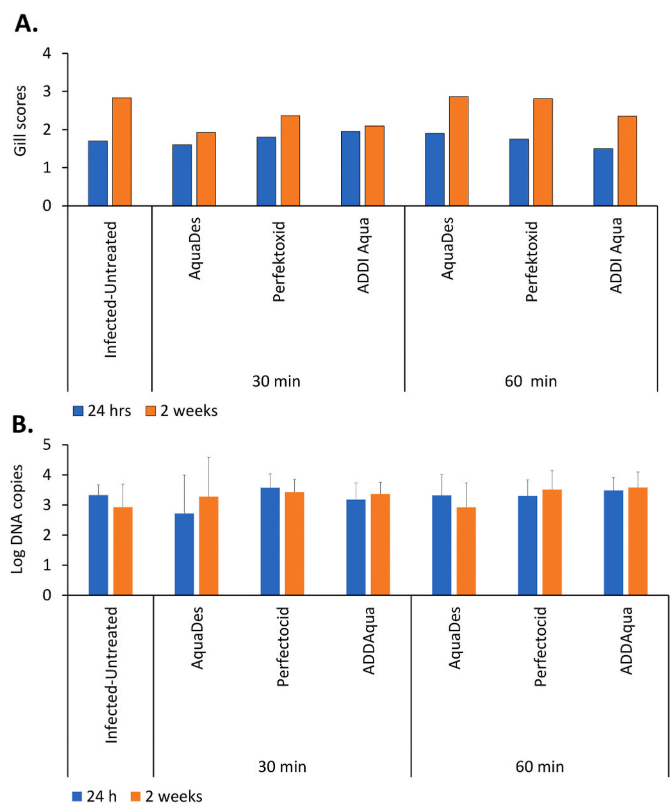

Fig. 5. Level of AGD infection assessed by (A) gross gill score and (B) qPCR of *N. perurans* from gill swabs. N = 10 individual fish. For the parasitic load in AquDes-30 group in both time points, only 6 out of the 10 analysed fish resulted in detectable Cq value.

treatment, mucous cells in AGD-affected groups, untreated and treated (except in ADDIAqua-30 and AquaDes60), showed significantly higher number than the uninfected-untreated group.

3.6.2. Changes in the olfactory organs

There were no significant temporal and inter-treatment changes in the thicknesses of the epithelium and lamina propria of the lamella of the olfactory organs (Fig. 8).

The width of the mucosal tip of the olfactory lamella did not significantly differ 24 h after treatment (Fig. 8). However, at 2 weeks post treatment, the width of the mucosal tip from the group treated with ADDIAqua-60 was significantly narrower, compared with the group that was not infected nor treated (Fig. 8). For the group treated with Perfectoxid for 60 min, the olfactory mucosal tip was significantly wider at 2 weeks than at 24 h post treatment. The injury scoring revealed no significant difference between timepoints as well as among treatment groups. (Fig. 8), though cases of degeneration could be observed sporadically (Fig. 9).

4. Discussion

Parasitic infestation and chemotherapeutics are known to have a strong influence on the oxidative stress status of an organism. In this study, we demonstrated that AGD did not trigger oxidative stress in Atlantic salmon smolts, as indicated by unaffected systemic and mucosal oxidative stress biomarkers following infection. However, when infected fish were treated with an oxidant, key regulators of oxidative stress response were significantly affected and the consequences were influenced by the PAA products, duration of exposure and, quite pronouncedly, by sampling point.

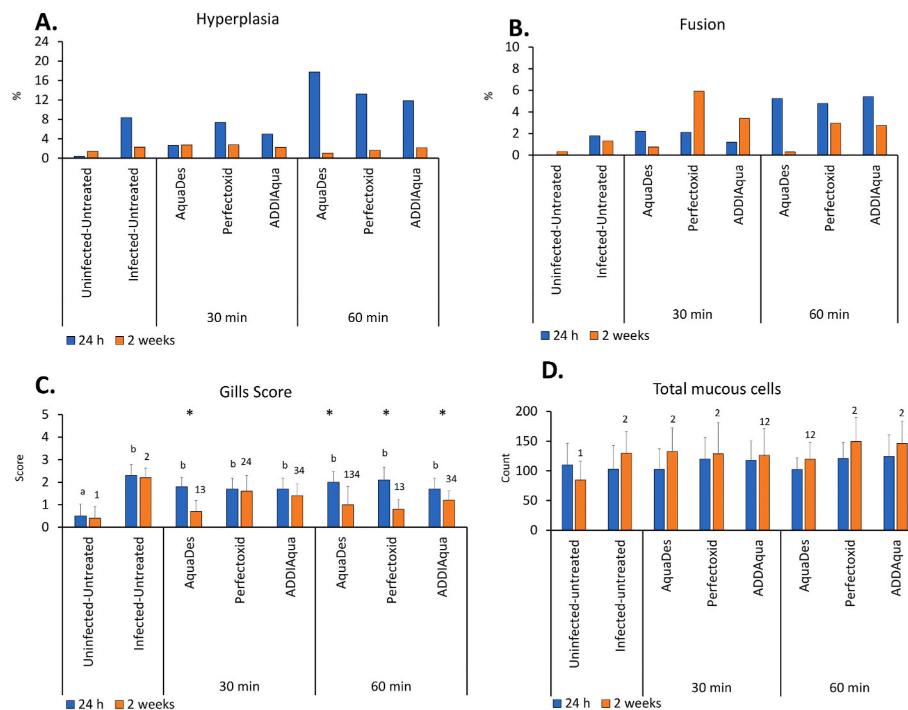

Fig. 6. Microscopic evaluation of Atlantic salmon gills. Frequency of (A) hyperplasia and (B) fusion had been evaluated. (C) Microscopic gill score assessed through an injury score system is likewise indicated. N = 10 individual fish.

4.1. AGD does not trigger systemic and mucosal oxidative stress in Atlantic salmon

Oxidative stress occurs when the balance between reactive oxygen species (ROS) and antioxidant enzymes is altered, either by lack of ROS excretion, by inefficient radical scavenging or by depletion of antioxidants [3]. During parasitic infection, the host generates toxic oxidants as an immune response and the balance of oxidants-antioxidants must be ensured to prevent untoward consequences [23]. It has been reported that Atlantic salmon presenting a gill score 2 showed a decreased antioxidant potential and this might be associated with exhaustion of antioxidant defences triggered by infection, such as by inflammatory response to the parasite. An inhibitory mechanism of the enzymatic production of antioxidants intrinsic to the parasite has been hypothesised earlier [14]. In the present study, we have shown that AGD-affected Atlantic salmon with gill score 1/2 did not exhibit an altered oxidative stress status either in plasma or on mucosal surfaces. Glutathione S-transferases (GSTs) are versatile enzymes that can affect parasite survival and parasite-host interaction [24]. We identified that *gsta* was the only gene which had its expression in the gills significantly downregulated in AGD-affected group. On the other hand, *txnl* was the only gene in the olfactory organ affected by AGD. This striking contrast from an earlier study [14] can be explained by the two different infection environments – natural versus laboratory controlled trial. Environmental parameters are strong modulators of oxidative genes, which might have influenced the responses in the earlier study.

When PAA degrades in water, oxygen radicals are formed and could induce a transient state of oxidative stress in fish [22]. We have documented in a series of studies that exposing salmon to PAA presented a state of oxidative stress which was reflected both at systemic and mucosal levels, and these changes were dependent on dose, exposure duration, frequency and stress status of the fish [18,19,22]. Though moderate AGD alone did not trigger significant change in plasmatic ROS level, exposing AGD-affected fish to PAA resulted in plasmatic ROS imbalance particularly at 2 weeks after treatment. In addition, this change was dependent on the type of PAA product (i.e., AquaDes and Perfectoxid) but not on exposure duration. We further found that TAC was not significantly altered in AGD-affected-PAA-treated fish which indicates that the infection might have interfered with the ability of fish to counteract, via antioxidants, the systemic ROS imbalance triggered by PAA. Elevation of plasma TAC was earlier found to be an important physiological counteraction of fish to PAA [22,25].

4.2. Expression of key oxidative stress genes in the gills following treatment of AGD-affected salmon with PAA demonstrates an acute response profile

Gills are the main target organ of AGD [26], while gills and the olfactory organ have been shown to be sensitive to PAA [18] in Atlantic salmon. Here we demonstrated that although the oxidative stress biomarkers in both organs showed minimal response to AGD alone, they demonstrated distinct expression profiles when exposed to PAA products suggesting that the response to the oxidant is organ specific. This further illustrates that the oxidant treatment, and not the infection, had a substantial impact on the oxidative stress markers in these two mucosal organs.

It is evident that the changes in the oxidative stress biomarkers in the gills were stronger at 24 h after treatment than 2 weeks after. This implies that the PAA-mediated regulation of the molecular repertoire of oxidative stress in the gills was likely transient and did not persist. Moreover, it was identified that treatment-related effects were more apparent 24 h after treatment. For instance, most of the oxidative stress genes were upregulated in the groups AquaDes-30 and Perfectoxid-60, while their counterparts in ADDiAqua-60 and AquaDes-60 were predominantly downregulated at 24 h after treatment. The *glutathione S-transferases* (GST) are a multigenic family of enzymes involved in the detoxification of xenobiotics [27], and have been shown to be vital in the protective mechanism against PAA-induced oxidative stress in the mucosal organs of salmon [18,28]. *Gsta* was one of the genes in the gills that demonstrated a strong response in AGD-affected salmon exposed to PAA. Such a response profile was clearly identified 24 h after treatment and was not dependent on either PAA product or duration of exposure, suggesting that *gsta* is likely a crucial detoxifying molecule against PAA in salmon gills. It is worth noting that *gsta* was significantly

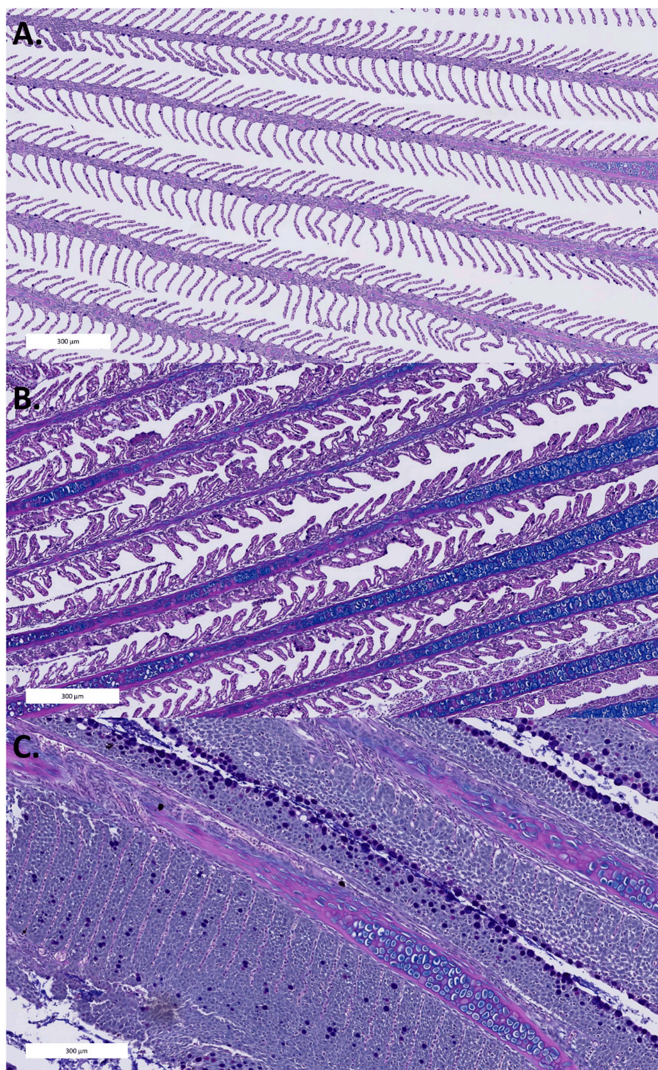

Fig. 7. Representative histological sections of A) healthy and B–C) AGD-affected, PAA treated Atlantic salmon gills. Note the cases of epithelial lifting/oedema, hyperplasia and lamellar fusion. Purple coloured cells are mucous cells.

downregulated in the gills of AGD affected salmon, thus, implying that infection did not interfere with its function following PAA treatment. Catalase (*cat*) is an inducible enzyme that protects the biological system against reactive oxygen species [29] by the neutralisation of hydrogen peroxide through decomposition [30]. Treatment of AGD-affected fish with PAA showed downregulation of *cat* expression in the gills in four out of six treatment groups at 24 h after treatment. This downregulation demonstrates that infection might have intervened with the acute *cat*-mediated response of salmon gills to PAA.

An important cellular system against oxidative stress is the thioredoxin system [31]. Thioredoxins are key component molecules of this central intracellular redox system. They are ubiquitously found in every cell type and function as an important regulator in ROS accumulation [32,33]. The prominent upregulation of the two *txnrd* genes in the gills of AGD-affected fish following oxidant treatment (i.e., AquaDes-30 and Perfectoxid-60) indicates their involvement in resolving the effects of the exposure to a chemical stressor and parasitic infection. This study provides new insight into the functions of thioredoxins in fish, which are not well explored, especially its dual role in immunity and radical neutralisation in mucosal organs.

4.3. Gene downregulation characterises the responses of the olfactory organ of AGD-affected fish to PAA treatment

The parasite was not detected in the olfactory organ, so the changes observed following infection and treatment were likely 1) a secondary response to AGD, and/or 2) they were mostly triggered by PAA and not by infection status. Unlike in the gills, where the changes in the oxidative stress genes showed a clear transient response, the olfactory organ displayed somewhat a persistent effect, as downregulation became more prominent at 2 weeks post-treatment. This was further exemplified by the magnitude of change, especially for most of the downregulated genes, becoming more marked at 2 weeks post treatment. The responses of the olfactory organs revealed that they were not heavily affected by AGD alone, however, exposure to oxidant could pronouncedly affect the molecular repertoire of oxidative stress. This provides another compelling support to earlier evidence that the olfactory organ of Atlantic salmon is sensitive to oxidative chemical stressors [28]. AquaDes-60 showed a prominent downregulation in the olfactory organs as shown by 10/13 genes at 24 h after treatment. At this timepoint, it appeared that AquaDes was a PAA product that could substantially alter the oxidative stress genes in the olfactory organ. This is the only instance where the effects were clearly established to be dependent on PAA product and exposure duration. In most treatment groups, the expression of *oxr*, *gr* and *txnip* in the olfactory organs at 2 weeks post treatment demonstrated downregulation. Particularly for *gr*, such a distinct response profile was identified in all treatment groups. Glutathione reductase maintains the supply of reduced glutathione, a major thiol in many cell types; the reduced form of glutathione plays key roles in the cellular control of reactive oxygen species [34]. We have earlier shown that PAA treatment altered the expression of *gr* in salmon, both using *in vitro* and *in vivo* models [19,22]. Therefore, the significant upregulation of *gr* following treatment is likely related to its role in ensuring the redox homeostasis, especially the glutathione system in the olfactory mucosa following exposure to an oxidant. Interestingly, *gr* downregulation was identified 2 weeks post-treatment in all treatment groups which could suggest either a form of recovery after a heightened state immediately after treatment or interference of *gr* functions as a persistent impact from the treatment. Oxidation resistance 1 (*Oxr1*) is a gene that is only found in eukaryotes and its function ranges from antioxidation to immune defence, ageing and cell cycle [35]. The expression profile of *oxr1* showed a similar pattern as in *gr* – upregulation at 24 h after treatment and downregulation at 2 weeks thereafter. *Oxr1* is one of the least studied oxidative stress genes in fish [35], hence, the present data provided evidence of its potential role in protecting the mucosa against an oxidative chemical stressor especially when the organ is parasitised with an amoeba. Interestingly, this duality of response was markedly demonstrated by the group AquaDes-60, therefore linking the role of this specific oxidative stress gene response to this particular PAA product. Thioredoxin-interacting protein has an important role in redox homeostasis, especially in increasing the production of reactive oxygen species (ROS), and oxidative stress [36]. Even though we did not document a significant increase in plasmatic ROS, 24 h after treatment, the increase in transcription of *txnip* indicates that it might have participated in triggering mucosal oxidative stress following the treatment, though such a striking change was not persistent.

Together with *cat*, *sulfiredoxin* showed a strong downregulation profile in the olfactory organ in all treatment groups except in ADDIAqua-60 at 2 weeks after treatment. Sulfiredoxin-1, an enzyme encoded by the *srxn1* gene, belongs to the family of oxidoreductases and catalyses the reduction of cysteine sulfinic acid, of hyperoxidised peroxiredoxins, and has a part in antioxidant defence [37]. The pronounced downregulation of these two genes points to a possibility that, though infection did not have direct impact, exposure to an oxidative stressor posed a constraint in their known roles in antioxidant defence.

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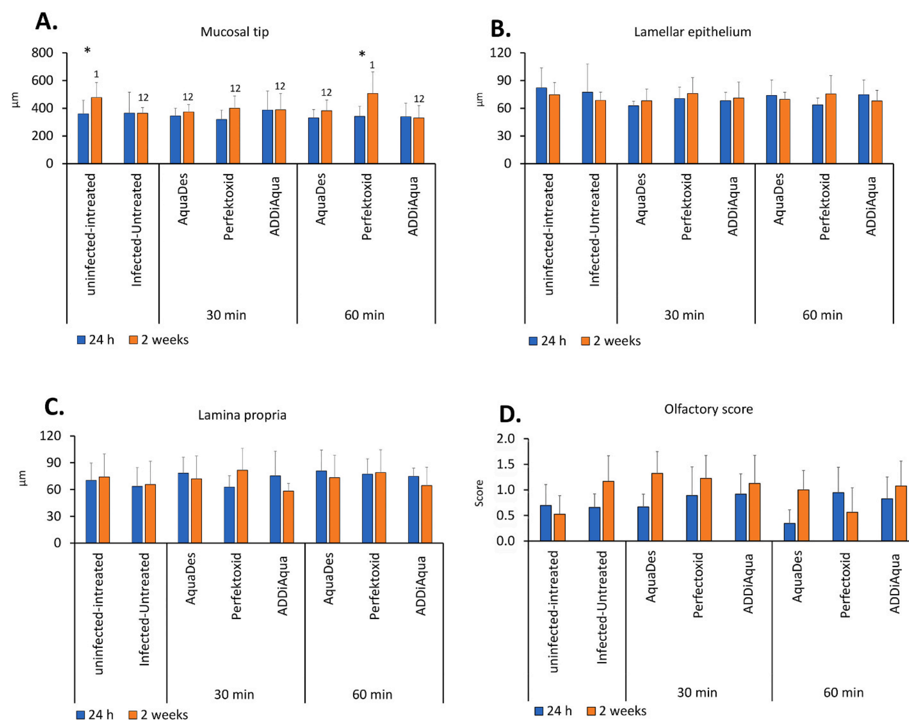

Fig. 8. Microscopic evaluation of Atlantic salmon olfactory organ. Measurements were taken from (a) mucosal tip, (B) lamellar epithelium; and (C) lamina propia. The overall health status of the olfactory organ was assessed by a scoring scheme, where a higher score denotes more damage. N = 10 individual fish.

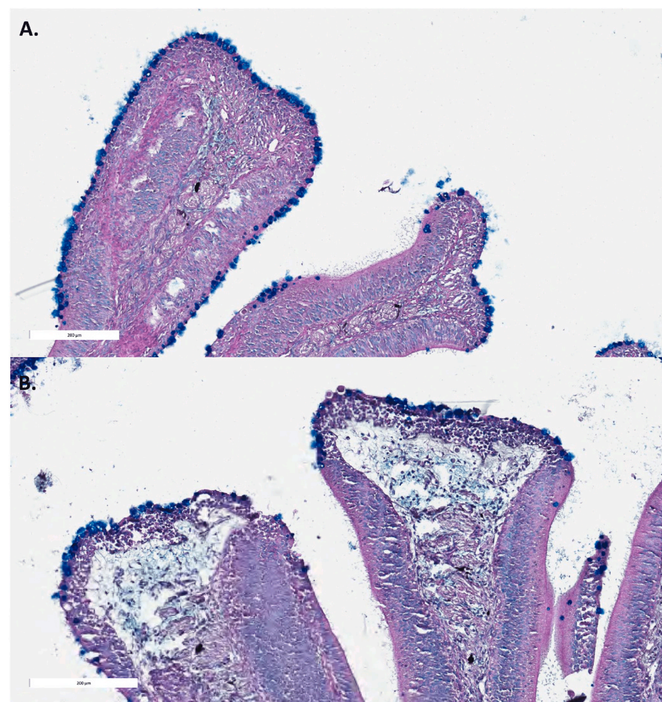

Fig. 9. Representative histological sections of the olfactory lamella from A) healthy and B) AGD-affected, PAA treated Atlantic salmon.

4.4. PAA treatments neither resolve nor aggravate the histological effects of parasitism in the gills

N. perurans is a free-living organism in the environment, contacts and adheres mainly to the gills of Atlantic salmon [38]. Scoring of the gross pathology of the gills is the most common on-site assessment of the severity of AGD infection. Gross gill score was 1–2 at the start and increased to nearly 3 after 2 weeks in fish that were not treated with PAA. It was apparent that fish treated with PAA for 30 min regardless of the product type showed relatively lower macroscopic gill scores than fish treated with PAA for 60 min and infected-untreated group. However, the inter-treatment resolution was not very clear which was further corroborated by the PCR quantification of amoeba. It is noteworthy to emphasise that some fish treated with AquaDes for 30 mins had undetectabe level of the parasite.

Then we investigated the microstructural changes in the gills of AGD affected fish following treatment. After colonising the organ, the parasite induces gill epithelial proliferation, causing, as main histological changes, hyperplasia and then fusion of the lamellae. Histological evaluation of the gills revealed that hyperplasia and fusion were observed in the group of infected animals, corresponding to the classic lesions observed in AGD [26,39]. Interestingly, in the infected-untreated group, these lesions were more frequently observed at the first sampling point than at 2 weeks after treatment, which seems to indicate that the severity of AGD somehow did not worsen, which supports the gross pathology and qPCR data.

With AGD-affected fish exposed to PAA treatments, there was a decrease in hyperplasia observed from 24 h to 2 weeks after treatment. Since this decreasing trend was very similar to the one observed in the infected-untreated group, PAA did not appear to potentiate tissue hyperplasia. The fusion of lamella, between the two sampling points, also decreased similarly to the infected-untreated group, except for two groups, Perfectoxid-30 and ADDIAqua-30, the same two groups where the decrease in macroscopic gill score was very modest. In the other treated groups, the reduction in microscopic gill score was quite considerable between the sampling days, pointing to the possibility that the PAA might have influenced the progression of the disease but not to an extent of reducing the parasite load. However, this is not generally true with some fish from the AquaDes-treated group.

Both the gross and microscopic scores, supported by the PCR quantification of the amoeba in the gills did not fully demonstrate that AGD did not progress within the 2-week timeframe. However, it remains to be

verified whether this non-progression was related to slower disease progression in the infection model (as shown by the infected-untreated group) or PAA might have interfered with this progression. Nonetheless, PAA did not worsen the histological lesions in the gills.

Gills infected with *N. perurans* parasite are often characterised with an increase in mucus production, likely due to hyperplasia and hypertrophy of mucus-producing cells [39,40]. In this study we observed that 24 h after treatment, inter-treatment differences in the number of gill mucus cells were found. However, after 2 weeks, the number of mucus cells in the gills of AGD-affected fish and those treated with PAA displayed higher counts than in the uninfected-untreated group, hence displaying the typical mucosal response to AGD. In addition, such a response to PAA of increase in mucus cell number in the gills corroborates an earlier documentation of such consequences in smolts exposed to PAA [41]. Gills exposed to an irritant stimulus increase the number of mucus cells as a protective response [42,43], and perhaps such a classic response might be working here. In addition, we also found an increased number of acidic mucous cells, which may represent an involvement in the defence mechanism, as a greater proportion of this cell type is closely linked to an increase in mucus viscosity, something that is associated with greater protection of the epithelium against damage [44].

4.5. Infection and treatment do not affect the structural integrity of the olfactory organ

The olfactory epithelium consists of a multi-lamellar olfactory rosette with sensory neurons, which are extremely sensitive to contaminants in the water [45,46]. In salmonids, the olfactory organ has a distinct immune function, characterised by the abundance of myeloid and lymphoid cells in the olfactory rosette which are important regulators of innate and adaptive immune responses [47]. AGD did not affect the structural integrity of the olfactory organ, neither did the treatment with PAA of AGD-affected fish. We did not visually find the amoeba in the olfactory organ, which was confirmed by qPCR, hence, the results demonstrate that the olfactory organ is likely not a target of the amoeba despite mounting an oxidative stress response to PAA in AGD-affected fish. A previous study revealed that some histological features (i.e. mucosal tip expansion, mucous cell hyperplasia) of the olfactory lamella are altered by recurrent PAA treatment [18]. We did not document such a consequence here. This apparent difference is likely attributed to the frequency of exposure – in the current study, we exposed salmon once to PAA while the previous study reported a periodic exposure. Though there were significant changes observed in the width of the olfactory mucosal tip, such as in Perfectoxid-60 and ADDIAqua-60, 2 weeks post-treatment relative to other treatment groups, these alterations did not reveal a clear profile to derive a possible implication.

5. Conclusion

In summary, the study demonstrated that the molecular repertoire of oxidative stress in the gills and olfactory organ of Atlantic salmon was not heavily affected by AGD, at least at the disease severity described in this study. However, such a profile was changed when AGD-affected fish were exposed to different PAA products either for 30 or 60 min. The two mucosal organs displayed distinct patterns of expression of oxidative stress biomarkers where transient upregulation was observed in the gills while a persistent downregulation was characterised in the olfactory organs. This striking response profile was not heavily influenced by either PAA product or duration of treatment. In future studies, the protein levels of these gene markers should be determined to further substantiate their biological functions in resolving parasitic infection and oxidative stress in salmon. Disease resolution was not fully established as assessed by gross pathology, histopathology and qPCR analysis of parasitic load.Nonetheless, PAA treatment of AGD-affected fish did not show to aggravate the lesions related to infection. Standardisation of exposure protocol is the next step in evaluating the chemotherapeutic potential of PAA against AGD. The non-detection of the parasite in AquaDes-treated group for 30 mins indicates that the treatment protocol is promising.

Author contributions

C.C.L., M.W.B., and L.F.P. conceived the research idea. C.C.L., M.W. B. and D.S. designed the trial. C.C.L., M.W.B., D.S., and F.F. performed the fish trial. L.F.P. analysed the PAA products. C.C.L., D.S., D.C., M.W. B., and F.F. collected the samples. F.F., D.C., and D.S. performed the lab analyses. C.C.L. and F.A. supervised F.F. C.C.L., G.T., and F.F. handled and processed the data. F.F., F.A., and C.C.L. interpreted the data. F.F. wrote the first draft of the manuscript. All authors contributed to the writing and review of the final version of the manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2022.09.040.

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