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Nofima
Norwegian Institute of Food, Fisheries and Aquaculture Research

Peracetic acid as a potential treatment for amoebic gill disease (PERAGILL)

Final report

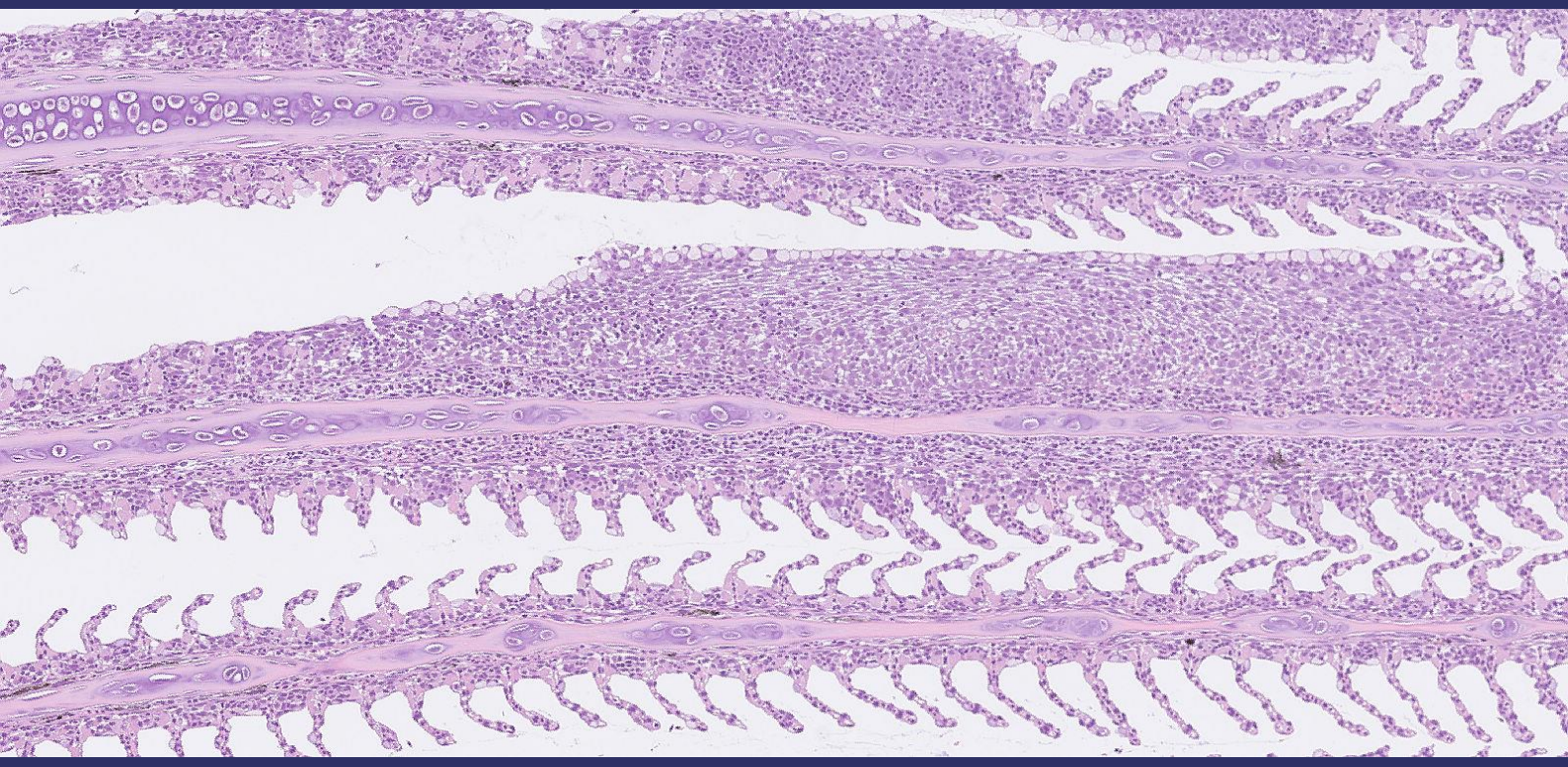

Illustration: M.Gjessing

Carlo C. Lazado, Lars-Flemming Pedersen, David Strand, Mette W. Breiland, Lisbeth Rørmark and Karin Pittman

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Norwegian Institute of Food, Fisheries and Aquaculture Research

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Report

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Title:

Peracetic acid as a potential treatment for amoebic gill disease (AGD) in Atlantic salmon

Tittel:

Pereddiksyre som en potensiell behandling av amøbegjellesykdom (AGD) i laks

Author(s)/project manager:

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Department:

Fish Health

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Keywords:

Peracetic acid, amoebic gill disease, oxidative stress

Summary/Recommendation:

The project investigated the potential of peracetic acid (PAA) as a treatment for amoebic gill disease (AGD) in Atlantic salmon. A series of studies were performed to 1) investigate the decay of PAA and the factors that influence this inherent chemical behaviour, 2) document the health and welfare consequences of using PAA in salmon smolts, and 3) evaluate the amoebicidal activity of PAA and its efficacy to treat AGD. Temperature, salinity, light and UV affected the decay dynamics of PAA. Salmon smolts could tolerate PAA doses of 0.6 to 10 ppm, however, behavioural and physiological responses were significantly influenced by duration and frequency of exposure, as well as with PAA commercial products. Stress and disease status of fish were documented to influence the responses of salmon to PAA. The application of PAA was identified to be a mild stressor and could induce both transient and systemic oxidative systems. Different physiological adaptive responses were mounted by the fish to counteract these impacts. PAA exhibited amoebicidal activity in vitro. Exposure of AGD-affected salmon to PAA using different treatment protocols resulted in an equivocal disease resolution. Though treatment could reduce parasite load, gross and microscopic pathologies persisted after the treatments. New insights about the pathophysiology of AGD had been generated particularly on the role of oxidative stress and circulating metabolites. Additional treatment optimisation is required for PAA as a treatment for AGD. Nonetheless, the project provided significant advancements in the chemistry and physiology of PAA in fish that will be crucial in developing the evidence-driven application of this disinfectant in aquaculture.

Sammendrag på norsk:

Prosjektet undersøkte potensialet til Pereddiksyre (PAA) som behandling av amøbegjellesykdom (AGD) hos atlantisk laks. Flere studier ble gjennomført for å 1) undersøke nedbrytningstid til PAA og faktorene som påvirker denne iboende kjemiske egenskapen, 2) dokumentere konsekvenser på helse og velferd hos laks ved bruk av PAA, og 3) evaluere effekt av PAA på amøben og dens effekt på behandling av sykdommen. Temperatur, salinitet, lys og UV påvirket dynamikken og nedbrytning av PAA. Laksesmolt kunne tolerere PAA doser fra 0,6 til 10 ppm, men både atferdsmessige og fysiologiske responser ble signifikant påvirket av varighet og frekvens av PAA eksponeringen. Hvilket kommersielt PAA produkt som ble testet påvirket også resultatene. Stress og sykdomsstatus til fisken ble dokumentert å være avgjørende for fiskens respons mot PAA. Tilsetning av PAA ble identifisert å være som en mild stressor for fisken, som kunne indusere både temporære og systemiske oksidative systemer. Ulike fysiologiske adaptive responser ble avdekket hos fisken for å motvirke disse ytre påvirkningene. Bruk av PAA in vitro viste seg å hemme amøbens aktivitet. Når laks smittet med amøber ble eksponert for PAA (ulike behandlingsprotokoller), gav dette tvetydige sykdomsbilder. Behandlingen kunne føre til redusert parasittbelastning, samtidig som amøbeobservasjoner og mikroskopisk patologi vedvarte etter behandling. Ny innsikt om patofysiologien til AGD har blitt generert i prosjektet, spesielt i forhold til oksidativt stress og sirkulære metabolitter. Det vil være behov for ytterligere optimalisering i forhold til behandling av AGD med PAA. Men prosjektet har ført til betydelige fremskritt angående kjemi og fysiologi i forbindelse med PAA behandling på fisk, som vil være viktig i videre utvikling og bruk (erfaringsbasert) av dette desinfeksjonsmiddelet i akvakulturnæringen.

Preface

PERAGILL received funding from the Norwegian Seafood, Research Fund under grant agreement 901472. The project was carried out from January 2018 to April 2022.

The project is a collaboration between Nofima, Norwegian Veterinary Institute, Technical University of Denmark, Quantidoc AS and Lilleborg AS.

An earlier version of this report appeared in:

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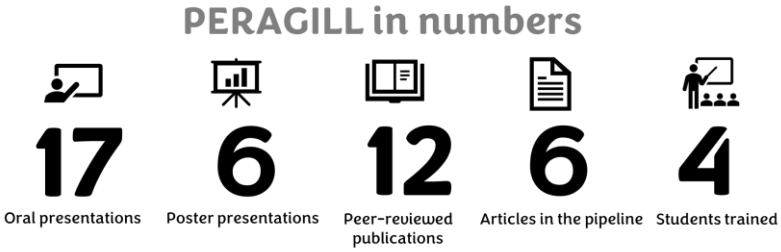
This project would have not been possible without the help of research and technical personnel from the different collaborating partners.

Innhold

1 Summary

The project investigated the potential of peracetic acid (PAA) as a treatment for amoebic gill disease (AGD) in Atlantic salmon. A series of studies were performed to 1) investigate the decay of PAA and the factors that influence this inherent chemical behaviour, 2) document the health and welfare consequences of using PAA in salmon smolts, and 3) evaluate the amoebicidal activity of PAA and its efficacy to treat AGD. Temperature, salinity, light and UV affected the decay dynamics of PAA. Salmon smolts could tolerate PAA doses of 0.6 to 10 ppm, however, behavioural and physiological responses were significantly influenced by duration and frequency of exposure, as well as with PAA commercial products. Stress and disease status of fish were documented to influence the responses of salmon to PAA. The application of PAA was identified to be a mild stressor and could induce both transient and systemic oxidative systems. Different physiological adaptive responses were mounted by the fish to counteract these impacts. PAA exhibited amoebicidal activity in vitro. Exposure of AGD-affected salmon to PAA using different treatment protocols resulted in an equivocal disease resolution. Though treatment could reduce parasite load, gross and microscopic pathologies persisted after the treatments. New insights about the pathophysiology of AGD had been generated particularly on the role of oxidative stress and circulating metabolites. Additional treatment optimisation is required for PAA as a treatment for AGD. Nonetheless, the project provided significant advancements in the chemistry and physiology of PAA in fish that will be crucial in developing the evidence-driven application of this disinfectant in aquaculture.



1.1 Summary in Norwegian

Prosjektet undersøkte potensialet til Pereddiksyre (PAA) som behandling av amøbegjellesykdom (AGD) hos atlantisk laks. Flere studier ble gjennomført for å 1) undersøke nedbrytningstid til PAA og faktorene som påvirker denne iboende kjemiske egenskapen, 2) dokumentere konsekvenser på helse og velferd hos laks ved bruk av PAA, og 3) evaluere effekt av PAA på amøben og dens effekt på behandling av sykdommen. Temperatur, salinitet, lys og UV påvirket dynamikken og nedbrytning av PAA. Laksesmolt kunne tolerere PAA doser fra 0,6 til 10 ppm, men både atferdsmessige og fysiologiske responser ble signifikant påvirket av varighet og frekvens av PAA eksponeringen. Hvilket kommersielt PAA produkt som ble testet påvirket også resultatene. Stress og sykdomsstatus til fisken ble dokumentert å være avgjørende for fiskens respons mot PAA. Tilsetning av PAA ble identifisert å være som en mild stressor for fisken, som kunne indusere både temporære og systemiske oksidative systemer. Ulike fysiologiske adaptive responser ble avdekket hos fisken for å motvirke disse ytre påvirkningene. Bruk av PAA *in vitro* viste seg å hemme amøbens aktivitet. Når laks smittet med amøber ble eksponert for PAA (ulike behandlingsprotokoller), gav dette tvetydige sykdomsbilder. Behandlingen kunne føre til redusert parasittbelastning, samtidig som amøbeobservasjoner og mikroskopisk patologi vedvarte etter behandling. Ny innsikt om patofysiologien til AGD har blitt generert i prosjektet, spesielt i forhold til oksidativt stress og sirkulære metabolitter. Det vil være behov for ytterligere optimalisering i forhold til behandling av AGD med PAA. Men prosjektet har ført til betydelige fremskritt angående kjemi og fysiologi i forbindelse med PAA behandling på fisk, som vil være viktig i videre utvikling og bruk (erfaringsbasert) av dette desinfeksjonsmiddelet i akvakulturnæringen.

2 Introduction

Infectious diseases remain a major bottleneck in Atlantic salmon aquaculture in Norway. There are two major parasitic infestations in the sea-caged production of Atlantic salmon – sea lice and amoebic gill disease (AGD), 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 plight in fostering sustainable salmon farming. Climatic changes pose one of the factors that may increase the prevalence of AGD and expand its geographic distribution. Hence, the industry must be kept abreast with sustainable treatment options to combat this emerging threat, as there is no universally effective prophylactic measure available at present. Developing effective and eco-friendly strategies should be based on holistic knowledge about the inherent features of the candidate therapies, the risks involved, and the factors that influence its effectiveness and consequences.

2.1 Amoebic gill disease

It was unequivocally identified that the free-living and opportunistically parasitic amoeba *Paramoeba perurans* (syn. *Neoparamoeba perurans*) is the causative agent of AGD [1] and the Koch's postulates were later fulfilled in 2012 [2]. Besides the Atlantic salmon, the facultative parasite has been identified to infect other salmonid species (*e.g.*, rainbow trout, *Oncorhynchus mykiss*; brown trout, *S. trutta*; chinook salmon, *O. tsawyschta*), Ballan wrasse (*Labrus berhylta*), sharp snout sea bream (*Diplodus puntazzo*), seabass (*Dicentrarchus labrax*), ayu (*Plecoglossus altivelis*) and blue warehouse (*Seriolla brama*) [3-6]. Fish affected with AGD shows lethargy, anorexia, the congregation at the water surface and increased ventilation rate [7], which will lead to respiratory distress that can result in mortality between 50-80 % if left untreated [8, 9]. AGD is characterised by increased mucus production in the gills. The branchial epithelial hyperplasia results in the characteristic hyperplastic plaque on the gills infiltrated with inflammatory immune cells. The white mucoid spots and plaques on the gill surface [10] are being used as a gross pathological indicator and their presence is often use to indicate the severity of infection in the farms. Microscopically, infected gills exhibit epithelial multifocal gill hyperplasia, hypertrophy, oedema, and interlamellar vesicle formation [11]. Acute cellular necrosis is a canonical pathological manifestation following AGD infection [12, 13].

Picture 1 Severity of infection is often assessed by gross gill scoring, as shown here. (Photo: Mette W. Breiland)

AGD was first described in Tasmania during the early 1980s and has since been confirmed in most salmon producing regions, including United States, Chile, Ireland, Spain, France, Japan, and Norway [9]. The first documented case of AGD in farmed salmon in Norway was in 2006 and the report suggested that the marine environment was a reservoir for the amoeba [9]. It was detected for the second time in 2012 with 5 positively diagnosed cases. An increment of about 1000 % was documented in 2013 with 56 cases and the number increased even more to 70 in 2014 [6]. Though AGD has been steady in the last years, the treats of climatic changes may alter the epidemiology of the disease in Norwegian localities. The rate of increase and the expansion of localities where outbreaks have been identified are clearly pointing to the imminent threat of AGD to Norwegian aquaculture. It is known that the two most important risk factors for outbreaks of AGD are high salinity and high seawater temperatures. The warming ocean and more frequent heatwaves in Norwegian waters (IPCC, 2019) are daunting factors that may trigger several AGD outbreaks in the near future. These interactions have already been documented in Scotland, and Australia (Benedicenti, et al., 2019; Foyle, et al., 2020), and preliminary evidence from our group is likewise pointing to such a relationship.

2.2 Treatments for AGD

No available vaccine for AGD at the moment, therefore therapeutic interventions are the most common options. Currently, the only effective commercial treatment against AGD is freshwater bathing which has been in place since the disease became established in Australia in the mid-1980s. The common practice in commercial scale involves transferring fish from their original pen to a tarpaulin liner filled with freshwater. There is a clean net cage under the tarpaulin. The freshwater is oxygenated at 120 – 200 % air saturation. Stocking density in the freshwater bath can be up to 40 kg m^{-3}. After the bathing period, which usually last 2-3 hours, the tarpaulin is removed by winch and the awaiting cage below collects the fish [14-16]. Ionic disturbances caused by the acute net efflux of Na^+ and Cl^- ions during freshwater transfer are relatively minor [15]. Though this method is effective in controlling AGD to a greater extent, the strategy entails significant infrastructure cost and is labor expensive. One important consideration and remains a major challenge is a requirement for a nearby freshwater source [17].

Chemotherapeutics are still used to treat fish diseases, however, several strategies have been in place to ensure safety. In many countries, the focus is on developing therapies that are more environment friendly [18, 19]. Chemotherapeutics have also been used to address AGD. Most common would be the application of strong disinfectants with a wide spectrum of antimicrobial activity. Unlike freshwater bathing, oxidative disinfectants often provide varying results and potential toxicity risk is a factor that must be thoroughly considered. Nonetheless, this chemotherapeutics offer alternative when freshwater bathing is a major issue. The two main oxidative disinfectants include the chlorine-based chloramine-T (N-Chloro 4-methyl benzenesulfonamide, sodium salt) and the oxygen-based hydrogen peroxide (H_2O_2). It was shown earlier that AGD-affected Atlantic salmon bathed in seawater with chloramine-T showed reduced amoeba density, with a comparable result with freshwater bathing [20]. Cl-T is used as a disinfectant to inactivate viruses and bacteria [21, 22] and often used for the treatment of gill diseases in the freshwater aquaculture industry [23]. The major issue with its use in the marine phase is that the toxicity of chloramine-T to salmon smolt is enhanced in seawater. Hydrogen peroxide is a strong oxidant and considered “green” as it breaks down into nontoxic, environmentally benign by-products [24]. This oxidative antimicrobial agent has a documented inhibitory property against fungal, bacterial and protozoan infections [25]. *In vitro* studies provided the potential of H_2O_2 against AGD, though *in vivo* trials indicated that its spectrum of efficacy was limited and required a higher concentration, with mortality problem as an associated issue [6, 17]. It often indicated that its use could only delay the progression of the disease and it could not fully resolve the infection [17, 26]. By manipulating the temperature and duration of exposure, it was demonstrated that H_2O_2 treatment in

seawater successfully ameliorated a clinically light case of AGD under laboratory conditions, though the results were largely not convincing following a 3-week recovery period [17].

2.3 Peracetic acid (PAA)

Peracetic acid (PAA), an organic peroxide, and available commercially as acidified mixture of acetic acid and hydrogen peroxide. PAA is very reactive therefore stabilizers are required for all PAA-based products for commercial use. It is commonly used as surface disinfectants and in wastewater treatments. Its popularity in aquaculture has increased dramatically in the last years, predominantly attributed to its wide spectrum of antimicrobial activity, rapid degradation, and residuals that pose little risks. The potent antimicrobial activity of PAA is based on the formation of highly reactive free radicals and the release of active oxygen atoms that eventually disrupt the chemiosmotic function of the lipoprotein cytoplasmic membrane and transport through the dislocation or rupture of cell walls [27, 28]. Because of its fat solubility, PAA has a stronger disinfection power than H_2O_2, though the combination of PAA and H_2O_2 has been demonstrated to exhibit synergistic property [29, 30].

In this project, we explored PAA as an alternative treatment for AGD. This concept was under the assumptions that:

- **FIRST**, PAA degrades completely within several hours after application and is degraded into harmless, neutral residuals (acetic acid and H_2O_2 and eventually to H_2O), facilitating ease of discharge [31], hence, addressing issues on environmental impact.

- **SECOND**, the effective concentration of PAA is less than 2 mg L^{-1} against various pathogens, in contrast to hydrogen peroxide that needs a much higher concentration (over 20 mg L^{-1}) to achieve successful disinfection.

- **THIRD**, PAA application has little impact on fish health (at least in the fish species tested prior to its application in salmon), and if there are stress-related changes following application, this can be easily addressed by manipulating the mode of application [32, 33].

- **FOURTH**, PAA products are potentially suitable disinfectants for recirculating integrated multi-trophic aquaculture systems because some non-target organisms (*e.g.*, microalgae) are not susceptible to the treatment.

- **FIFTH**, exposure to PAA at concentrations less than 1 mg L^{-1} had no severe impact on biofilter performance, hence, addressing issues on system disturbance and applicability in production system operating in RAS technology (*e.g.*, closed-containment system).

- **AND MOST IMPORTANTLY**, PAA exhibits anti-parasitic effects. It is a strong oxidising agent with a wide spectrum of antimicrobial activity and its oxidation potential is larger than that of chlorine, hypochlorite and hydrogen peroxide. The antimicrobial activity and anti-parasitic effects remain potent over a wide temperature range, including temperatures below 10 °C [34, 35]. PAA has a far more antimicrobial effect than H_2O_2 [28, 35, 36].

  The characteristics enumerated above underscore the potential of PAA as a treatment for AGD.

3 Objectives

The overarching objective of PERAGILL was to explore the potential of peracetic acid (PAA) as an alternative and sustainable treatment for amoebic gill disease, a gill health issue that is a threat to Norwegian Atlantic salmon aquaculture.

The project was divided into two stages (described in Section 4), with the specific objectives for each stage:

STAGE I:

1. To investigate the amoebicidal activity of PAA and identify factors influencing this feature
2. To assess the impact of PAA treatment on fish health and welfare
3. To determine the potential environmental risk of PAA treatment
4. To develop integrative assessment toolbox to evaluate treatment impacts and efficacy

STAGE II:

1. To identify additional factors that may affect PAA degradation and its associated residuals
2. To develop further the assessment toolbox related to salmon response to PAA and AGD
3. To evaluate the efficacy of PAA treatment against AGD-infected fish under different scenarios in the laboratory, as well as in the field.

4 Project execution

4.1 Work organisation

PERAGILL was organised into 4 Work Packages (WPs) and were conducted in a 2-step process. Stage I explored the chemistry, and health and welfare impacts of PAA on smolts. Stage II explored the chemotherapeutic potential of PAA for amoebic gill disease.

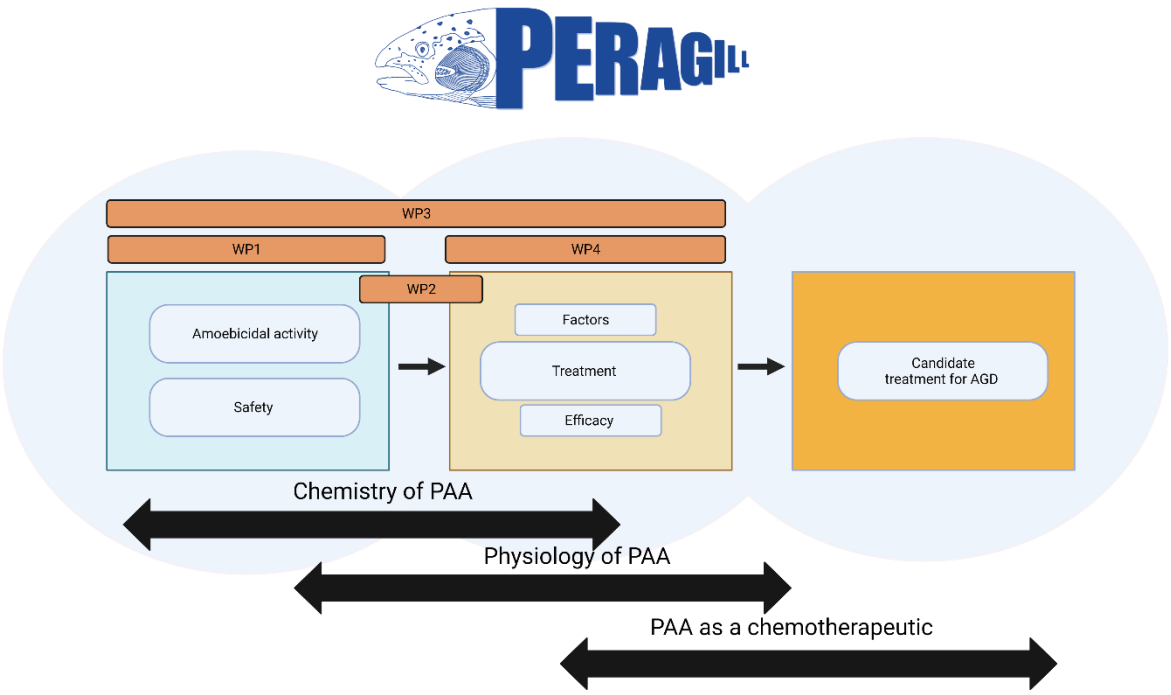


Figure 1 The PERAGILL project and the 4 Work Packages. These WPs were instrumental in establishing the different aspects that define the potential of PAA as a treatment for AGD.

4.2 Project organisation

PERAGILL was led by **Nofima**. **Carlo C. Lazado** of the Department of Fish Health served as the Project Leader. Other participants from Nofima with major involvement in the project were Gerrit Timmerhaus, Lill-Heidi Johansen and Mette Serine W. Breiland.

4.2.1 Academic and R&D Partners

Norwegian Veterinary Institute. NVI was represented by David Strand (Stage II) and Sigurd Hytterød (Stage I). Mona Gjessing and Saima Nasrin Mohammad were likewise involved in the analysis.

Technical University of Denmark. DTU Aqua was by led Lars-Flemming Pedersen and supported by Peter Vilhelm Skov.

4.2.2 Industry partners

Quantidoc AS. The company was represented by Karin Pittman.

Lilleborg AS. The company was represented by Lisbeth Rørmark.

4.3 Reference Group

The Reference group was composed of Linda Andersen (iLAB), Jon Åge Aune (Pharmaq) and Amund Litlabø (Aqua Pharma). Aune was only part of the Reference group in the first 8 months of the project. His position was taken over by Litlabø.

4.4 General approach

The experimental strategies included both *in vitro* and *in vivo* trials. *In vitro* studies adhered to the principles of 3Rs in research. To provide a holistic overview of the impacts of PAA treatment, we explored the effects on the causative agent of the disease, on the fish subjected to the treatment and on the rearing environment where the treatment was performed. This approach allowed us to dissect the extent of the potential of PAA as an alternative treatment. The effects of PAA treatment on infected and non-infected fish were studied from molecular, cellular, and organismal levels using up-to-date tools and techniques. This was important not only in providing a wider perspective on how the infected fish responded to the treatment, but also presented opportunities for marker identification and development of systematic protocols to assess treatment efficacy.

5 Findings, discussion, and conclusion

This section is presented in 3 main sections. Each section deals with the different aspect of PAA, all connected in building the foundation of PAA as a treatment for amoebic gill disease.

Section 1. The chemistry of PAA

In this section we discuss the main findings on the chemical behaviour of PAA. We also present the decay kinetics of PAA in different matrices, focusing on how factors such as temperature, salinity, light and UV influenced the inherent chemical characteristics.

Section 2. The physiology of PAA

In this section we present the health and welfare of naïve Atlantic salmon following PAA administration. An integrative approach was employed to document the physiological consequences of using PAA in Atlantic salmon smolts. In particular, the trials aimed to document the responses from the different levels of biological organisation – how genes were regulated and up to what extent external phenotypes were impacted by the treatment. The mechanism of action of PAA in Atlantic salmon is presented.

> **Trial 1.1.** Salmon were exposed to 0, 0.6 and 2.4 ppm PAA for 30 mins. After 2 weeks, the treated fish were exposed again to the same PAA doses and duration.
>
> **Trial 1.2.** The effects of crowding stress were studied in this trial. Prior to treatment with 4.8 pm PAA, fish were exposed to crowding stress for 1 h.
>
> **Trial 1.3.** Fish were exposed to 10 ppm PAA either for 15 mins or 30 mins. Exposure was performed 3 times over a 45-day period (FOTS ID 19321).

Section 3: PAA as chemotherapeutics for AGD

Here we focus on the amoebicidal activity of PAA against *N. perurans in vitro* and its impact on disease resolution *in vivo*. We also present the first high throughout metabolome data of AGD-affected Atlantic salmon.

> **Trial 3.1.** AGD-affected salmon were treated with either 5 ppm for 30 mins or 10 ppm for 15 mins (FOTS ID 23121).
>
> **Trial 3.2.** AGD-affected salmon were treated with 3 different PAA products at a target concentration of 5 ppm. Two exposure duration was tested - 30 or 60 mins. (FOTS ID 24455).

5.1 The chemistry of PAA

5.1.1 Decay of peracetic acid in seawater

The final version of the paper appeared on Aquaculture Environment Interaction.

Pedersen, L.F., Lazado, C.C. 2020. Decay of peracetic acid in seawater and implications for its chemotherapeutic potential in aquaculture. Aquaculture Environment Interactions. 12:153-165.

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Decay of peracetic acid in seawater and implications for its chemotherapeutic potential in aquaculture

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ABSTRACT

Peracetic acid (PAA) is a widely applied disinfectant in aquaculture. Knowledge on PAA decay in seawater (SW) is crucial for its potential successful implementation in SW aquaculture productions systems. Furthermore, residual decay of PAA is likely to have practical implications on developing effective treatment protocols by taking into account target efficacy and fish physiological responses to PAA in SW. We investigated the decay dynamics of PAA in SW under controlled conditions to assess the potential effect of temperature, salinity and light. We also applied PAA to 22 tanks with Atlantic salmon (*Salmo salar*) post-smolt in full-strength SW (33 ‰) over a realistic range of therapeutic concentrations (0.15-4.8 mg l^{-1}) to simulate relevant treatment scenarios. The study showed that PAA degrades rapidly in saltwater. The degradation follows exponential first-order decay with half-lives in the order of minutes to hours. Salinity and temperature significantly increased degradation of PAA, showing a four-fold faster degradation in full-strength seawater compared to freshwater. The decay of PAA was not significantly related to the nominal concentration of PAA in the concentration range tested. The other two active ingredients in PAA products, hydrogen peroxide (H_2O_2) and acetic acid were found to degrade at a much slower rate. H_2O_2 half-lives in SW were found to range from 15 to 70 h and minimal acetate degradation in clean SW was found. Finally, we compiled published data on PAA decay in relevant water matrices and discussed the potential environmental impacts, mitigation options and future research.

Introduction

Peracetic acid (PAA) or peroxyacetic acid, a biocidal peroxygen compound, constitutes the main active component in PAA containing trade products (cf. excellent reviews by Kitis, 2004 and Luukonen & Pehkonen, 2017). PAA is considered easily degradable and forms harmless residuals (i.e., acetate and eventually CO_2). PAA cannot persist in its pure form and is purchased solely as an acidified mixture of acetate and hydrogen peroxide:

$$CH_3COOH + H_2O_2 \leftrightarrow CH_3COOOH + H_2O \quad (1)$$

ACETIC ACID + HYDROGEN PEROXIDE **PERACETIC ACID + WATER**

The trade solutions can contain PAA from 5-40 w/w % (typically between 10-15 %) while hydrogen peroxide (H_2O_2) and acetate each range from 15-30 % (Kitis 2004, Muñio & Poyatos 2010, Liu et al. 2015).

PAA products have been used for more than 60 years (Greenspan & MacKellar, 1951), and a number of studies have documented high disinfection efficiency against viruses, bacteria, fungi, protozoa, spores and cysts (Baldry 1983, Baldry et al. 1991,Liberti & Notarnicola 1999, Kitis, 2004; Muñio & Poyatos 2010). PAA has a powerful biocidal and biostatic effects as it forms free radicals such as hydroxyl (cf. mode of action by Wessels & Ingmer (2013)), effective at low temperatures and requires markedly lower dosing (nominal concentrations) to achieve sufficient inhibition compared to H_2O_2 (Rajala-Mustonen et al. 1997,Finnegan et al. 2010, Flores et al. 2014). The decay of PAA is controlled by abiotic factors, primarily the presence of dissolved and organic particulate matter, transition metals, but also temperature, pH, mode of addition etc. (Yuan et al. 1997, Pedersen et al. 2013, Liu et al. 2014, Luukonen & Pekhonen 2017).

PAA forms free radicals in reaction with organic matter and transition metals, however considered unspecific in their mode of action which leaves PAA resistance quite unlikely (EU 528/2012). The cidal effect is a combination of direct oxidation of cell-membranes and destruction of sulfhydryl (-SH), disulphide (S-S) and double bonds (C-C) (Dröge 2002, Wessels & Ingmer, 2013), as well as protein and enzyme destruction (Block 1991, Kerkaert et al. 2011). The inhibition of catalases has been proposed as a mechanism of the synergistic effect of PAA and H_2O_2 (Flores et al. 2014).

PAA is widely used in various industries (Stampi et al. 2001, Gehr & Cochrane 2002, Wagner et al. 2002, Caretti & Lubello, 2003, Vinnerås et al. 2003). According to Luukonen & Pehkonen (2017), the annual global PAA consumption accounted for 29·103 MT for wastewater treatment and 55·103 MT for the food industry in 2013. PAA products are also used for ballast water disinfection (De Lafontaine et al. 2009) and implemented as water disinfectants in freshwater aquaculture (Meinelt et al. 2015). Though it is now being considered as a new sustainable and effective disinfectant in seawater production systems, there are still knowledge gaps especially on its decay and fate dynamics, including potential factors in SW matrices.

The management and control of parasitic infections are challenging to both land-based and open sea aquaculture systems (Rico et al. 2012, Buchmann, 2015, Shinn et al. 2015). The challenges associated with anti-parasitic drugs encompass not only delivering the right dose to ensure effective treatment, but also worker safety, fish welfare issues and concern over potential adverse environmental effects. Parasitic outbreaks such as from sea lice and gill amoeba remain a major concern in sea cage production of Atlantic salmon (Shinn & Bronn, 2012, Svåsand et al. 2017), posing substantial challenges both the economical, ethical and environmental sustainability. Hence, there are active initiatives in developing treatment protocols addressing these biological challenges.

From an environmental perspective, easily degradable chemicals which do not form toxic disinfection by-products or accumulate in aquatic organisms are preferred (Werschkun et al. 2014). Hydrogen peroxide fulfils these criteria and considered to be the ideal disinfectant as it is readily biodegradable and turn into oxygen and water upon decay (Jančula & Maršálek, 2011). H_2O_2 is currently applied as a chemotherapeutant against salmon sea lice (Overton et al. 2018). However, the handling and volumes used are not ideal and the treatment practice has recently been questioned due to treatment-related mortality and adverse environmental effects (Holan et al. 2017, Bechmann et al. 2019). Recently, the prevalence of amoebic gill disease (AGD), an infestation of gill amoeba (Paramoeba perurans) (Adams et al. 2012) has increased markedly in Norway with recorded cases at different geographic locations. Currently, freshwater and H_2O_2 bathing are the most commonly practiced treatments for AGD in

Norwegian salmon farming. However, none of the treatments appears to be 100 percent effective, and the treatment resolution between laboratory trials and field practice do not always correspond well. Nonetheless, treatment with freshwater is more gentle for salmonids and seems to have a better effect on the amoeba than treatment with H2O2 (Powell et al. 2015, Hjeltnes et al. 2019).

The anti-parasitic function of PAA against the causative agent of AGD is currently being investigated and comprehensive studies of PAA on fish physiology and stress response have recently been reported (Soleng et al. 2019). The development of new water disinfection routines and chemotherapeutants with water as a delivery matrix requires knowledge of the residual fate to ensure safe and effective treatment regimens and to evaluate the potential adverse environmental impact on the receiving water bodies. The present study aimed to investigate central aspects of the chemical behavior of PAA when exposed to seawater at realistic treatment concentrations. To address the objective, a series of controlled in situ test and pilot-scale trials with post-smolt salmon were conducted to identify the magnitude of PAA decay and the factors affecting decay kinetics. The results of the study further substantiate the potential of PAA as a chemotherapeutant for biological challenges faced by salmon at sea, such as AGD. The discussion on the implications of the results has been contextualized for salmon sea cage production, though the information provided may still be relevant to other farmed marine fish.

Materials and Methods

The studies were divided into small scale, controlled batch experiments in beakers (2.1) and pilot-scale trials with Atlantic salmon post-smolts in full-strength seawater mimicking aquaculture conditions (2.2). The trials that involved fish were performed in accordance with national and EU legislation (2010/63/EU) on animal experimentation.

In situ beaker trials

Temperature effect on PAA and H_2O_2 decay

The temperature effect on PAA and H_2O_2 degradation was investigated by controlled PAA and H_2O_2 spiking and subsequent analysis of either PAA or H_2O_2 residuals over time. Briefly, 34 ‰ seawater (piped water supply from Skagerrak) was incubated at four different temperatures (i.e., 5, 10, 15 and 20 °C) and divided into 1000 ml beakers with temperature control and with magnetic stirring (n=12). A nominal PAA concentration equivalent to 1.00 mg l^{-1} (fresh stock solution of 1000 mg/l made with Divosan Forte in Milli-Q water) PAA was added, and water samples were analyzed after t = 0, 10, 20, 30, 45, 60, 120, 180 and 240 min. These experiments were made as true triplicates.

Salinity effect on PAA and H_2O_2 decay

The salinity effect on PAA and H_2O_2 degradation was investigated in 1000 ml beaker with magnetic stirring at 20 °C. Water salinity matrices (i.e., 0, 5, 10, 15, 20, 25, 30, 33 ‰) were made by mixing seawater (33 ‰ piped water supply from Skagerrak) with non-chlorinated municipal tap water (< 0.1 ‰). A nominal PAA concentration equivalent to 1.00 mg l^{-1} was added to each beaker (true triplicates from all salinities), and water samples were analyzed after t = 0, 15, 30, 60, 120, 180 and 240 min.

Light effect on PAA decay

The effect of light on PAA degradation was investigated in a similar set up as described above. Three beakers were placed directly outside in the sunlight and three beakers were placed next to but shielded by aluminum foil. Ambient temperature changes were monitored during the trial. A nominal PAA

concentration equivalent to 1.00 mg l^{-1} was added to each beaker and water samples were analyzed after t = 0, 5, 15, 30, 60, 120, 180 and 240 min.

Decay of acetate and H_2O_2 at elevated nominal concentrations

H_2O_2 degradation was determined from two sets of experiments, i) by measuring residual H_2O_2 over a period of four hours (sampling at T = 0.25, 0.5, 1.0, 2,3 and 4 h.) at different salinities (see 2.1.2) and ii) by adding hydrogen peroxide at increasing nominal concentrations (i.e., 6.25, 12.5, 25, 50, 100 and 200 mgl^{-1} H_2O_2) into seawater and then measure subsequent residual H_2O_2 over a period of 4 h. Decay kinetics of acetate was similarly determined in *in situ* beaker experiments, by adding either pure acetate (C_0 = 10 mg l^{-1}) to full strength seawater and taking samples for residual analysis at T = 0, 1, 4 and 24 h after addition. These experiments were made at 20 °C and with the use of magnetic stirring.

Tank trials with seawater and Atlantic salmon smolts

The fish were obtained as smolts from a neighbouring commercial recirculating aquaculture system. After the transfer to full-strength seawater (33-34 ‰) in an experimental RAS, the fish were fed commercial diets with a daily ration equivalent to 1.5 % bodyweight. The fish were reared under stable and constant conditions for weeks prior to experiments, and no mortality was recorded during the period.

Fish density

The effect of fish density on PAA degradation was investigated in eight tanks each holding 300 L of full-strength seawater (33.5 ± 0.5 ‰, 15.2 ±0.4 °C). The tanks were stocked with three densities (approx. 8.2 kg/m^3, 16.5 kg/m^3 and 33 kg/m^3) in duplicates as well as two control tanks without fish. Just prior to and during the PAA exposure trial, water inlets to each tank were stopped to avoid any loss of PAA due to dilution. Pressurized air was delivered via air diffusers at the bottom of each tank to ensure sufficient oxygen and facilitate swift mixing of PAA. A quantity of 1.6 ml Divosan Forte equivalent to a nominal PAA concentration of 1.0 mg l^{-1} was added to a 500 ml beaker with tap-water and immediately distributed at several locations of each tank. Water samples were collected at fixed times after PAA spiking (t= 0, 10, 20,30,40,50 and 60 min) and immediately analyzed for PAA.

Nominal PAA concentration exposed to fish

PAA decay kinetics was investigated during simulated water disinfection trials in ten tanks with 300-L full-strength seawater as described earlier (Soleng et al. 2019). The study included two experimental trials where five nominal concentrations of PAA (C_0 = 0.15, 0.30, 0.60, 1.20 and 2.4 mg l^{-1} PAA) were added. In both trials, water samples were collected after t= 2, 4, 6, 15, 40 and 58 min and immediately analyzed for PAA residuals.

The first trial included transfer of 50 Atlantic salmon post-smolts (approx. 150 g each) to tanks in flow-through system (15.4 ± 0.5 °C, pH = 7.8-7.95, oxygen saturation 85-95 %). After 25 minutes following transfer, water exchange was stopped and 5 minutes later, PAA was added to each of the closed, static tanks. The fish were exposed to PAA for five minutes and were then swiftly netted and returned to recovery tanks while water sampling continued for another 55 minutes. The second trial was made two weeks after and was slightly modified with lower fish number (i.e., 20 post-smolts of approx. 160 g) and extended exposure period (30 minutes) before return to recovery tanks, otherwise as trial one. Three months later, a subsequent slightly modified tank trial was made with addition of PAA at a nominal concentration of 4.8 mg l^{-1}. The PAA quantity was added to two 500 l full strength seawater tanks holding post-smolts and sampled and analyzed as above (Soleng et al. 2019).

Chemical analysis

The concentration of PAA and H_2O_2 in the commercial PAA trade product (Divosan Forte®, Lilleborg AS, Norway) was analyzed by two consecutive autotitrations (0.1 M ceriumsulphate and sulfuric acid reaching transition at 960 mv to determine H_2O_2 concentration, followed by titration with 0.1 M sodium thiosulfate to determine PAA concentration) according to a manufacturer's protocol. Residual PAA in seawater samples was immediately analyzed by the DPD method according to Falsanisi et al. (2006) and Pedersen et al. (2013) by adding 250 µl N,N-diethyl-phenylene-diamine-sulphate salt (reagent 1) into 2.5 ml water sample. Thereafter, 250 µl potassium iodide buffer solution (reagent 2) was added, gently mixed and allowed to react for 30 seconds, before the color intensity was measured on a Hach Lange 2800D spectrophotometer at 550 nm. The absorbance values were used to calculate exact PAA concentration based on a standard curve made of adding aliquots of a 1000 mg l^{-1} PAA stock solution with Milli-Q to vials with seawater (33 ‰) and the absorbance was immediately measured to prevent in-vial decay.

Hydrogen peroxide was measured according to the method by Tanner & Wong (1998) and modified as described in Arvin & Pedersen (2015) based on standard curves made at the applied salinity. Acetate was measured by ion chromatography (Metrohm; Glostrup, DK), salinity was measured by use of a refractometer and oxygen concentration, pH and water temperature were measured with a HQ40 multimeter (Hach, Düsseldorf, Germany).

Data analysis of PAA and H_2O_2 kinetics

Both PAA and H_2O_2 degradation exhibited exponential decay (Newman, 1995) and the first-order decomposition reaction rate constant (k) was therefore calculated from the exponential decay equation:

$$C_t = C_0 \cdot e^{-kt} \quad (2)$$

where C_t is the concentration of PAA or H_2O_2 at time t (h), and C_0 represents the initial concentration, and k represents the decay rate constant in h^{-1}. The decay rate constant *k* was deducted from exponential regression analysis using concentration above 0.1 mg l^{-1} only and/or calculated as the regression coefficient of Ln transformed concentrations values versus time using the same set of data.

The half-life ($T_{½}$) was calculated as $T_{½} = \ln 2/k$ (3)

Statistics

The statistical analyses were performed in Sigmaplot 13.0 Statistical Software (Systat Software Inc., London, UK). The calculated reaction rate constants and half-lives were subjected to one-way ANOVA to test the effect of individual fixed factors. Pairwise multiple comparison procedure was made by Holm-Sidak method to test for differences between groups. All tests for statistical significance were set at $P < 0.05$.

Results

Effects of different factors on PAA decay

Temperature

The PAA decay in all temperature experiments followed exponential decay (all $R^2 > 0.997$, N=12) and showed minimal variation within replicates. The temperature had a highly significant positive effect on the decay of PAA in full-strength seawater, with decay rate constants at 0.087 ± 0.001 h^{-1} at 5 °C and 0.35 ± 0.012 h^{-1} at 20 °C (Figure 1). The corresponding half-lives were significantly inversely related to temperature ranging from 8.1 h at 5 °C to 1.9 h at 20 °C ($p < 0.001$), see Figure 2.

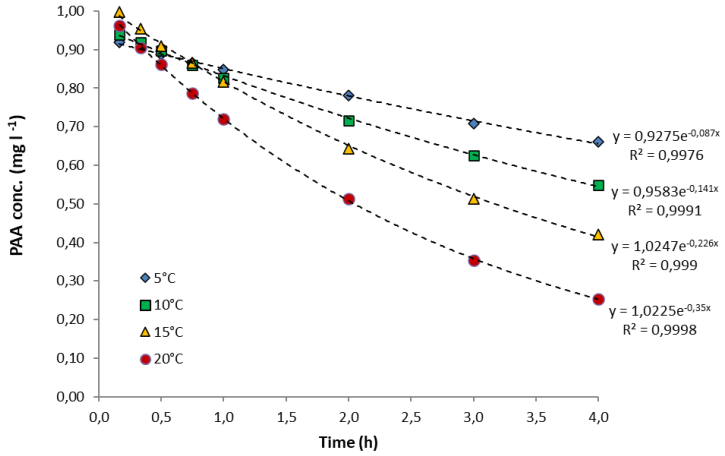

Figure 1. PAA decay in full-strength seawater at four different temperatures. Symbols reflect average PAA concentration (n=3) at four different temperatures with the respective 1 order exponential regression lines: Cx = C0.e-kx, where Cx is the PAA concentration at time x (h.), C0.e-kx, the nominal PAA concentration, x = time after addition (h.) and k is the decay rate constant (h-1).

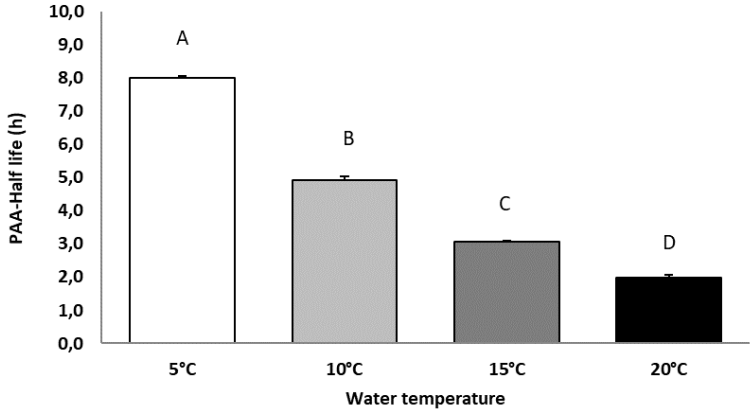

Figure 2. Calculated PAA half-lives (mean ± std.dev.) in full-strength seawater at four different temperatures, based on data from PAA decay with a nominal concentration of 1 mg l^{-1} PAA. Different letters denote significant difference ($p<0.01$).

Salinity

At all tested salinities, the PAA concentration was found to decline exponentially over time (N=24). Salinity significantly increased the decay of PAA. The decay rate constants increased linearly from 0.099 h^{-1} in freshwater to 0.390 h^{-1} in 33 ‰seawater (Fig. 3). The corresponding half-lives ranged from 6.7 h in freshwater to 1.7 h in full-strength seawater. The relationship between PAA decay rate constants (Y, in h^{-1}) and salinity (x, in ‰ or ppt) was highly significant; linear regression analysis: Y = 0.0094x + 0.1084 (R^2 = 0.9827, p< 0.01).

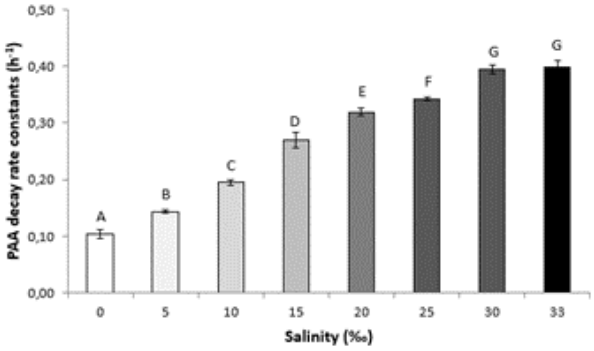

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Figure 3. Calculated first order PAA degradation rate constants (mean ± std. dev.) according to salinity, based on batch experiments performed at 20 °C with nominal PAA concentration at 1 mg l-1. All data are based on true triplicated experiments. Different letters denote significant difference (p< 0.05).

Light

The exposure to light increased the degradation of PAA in seawater compared to under dark conditions. Four hours after addition of PAA equivalent to 1.0 mg l^{-1}, residual PAA was measured to range from 0.275-0.29 mg l^{-1} and from 0.105-0.112 mg l^{-1} PAA under dark and light conditions, respectively. The decay rate constant under direct light was 0.58 ± 0.03 h^{-1} compared to 0.33± 0.01 h^{-1} in the dark. The corresponding half-lives were 1.2 ± 0.06 h and 2.1 ± 0.05 h, under light and dark conditions, respectively. The presence of light caused a temperature increase of 5.2 °C after four hours compared to the systems under darkness, see discussion.

Fish stocking density

Biomass increased the degradation of PAA to a minor degree, however not significantly (p=0.16) at the densities tested (Fig. 4). The decay rate constants from the tanks with 33 kg/m^3 were ca. 30 % higher compared to the tanks without fish (0.332 h^{-1}, vs. 0.249 h^{-1}) corresponding to half-lives of 2.1 and 2.8 h, respectively.

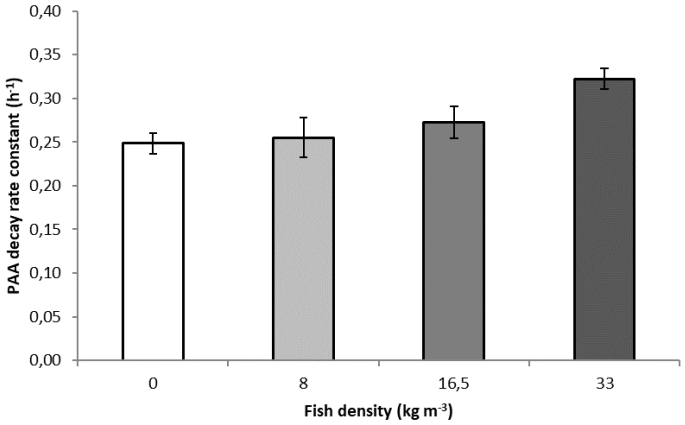

Figure 4. Calculated decay rate constants of PAA (mean ± std. dev.) when added to 300 l tanks (n=20) with full strength seawater and different biomasses. Each symbol represents a calculated PAA decay rate constant from an individual tank trial. The nominal concentration of PAA was 1.0 mg l-1, water temperature 15.2-15.8 °C.

Nominal PAA concentration during PAA treatment in tanks

PAA decreased exponentially in all 22 trials. At low PAA concentrations (0.15 and 0.30 mg l^{-1} PAA) and at 4.8 mg l^{-1}some variations were observed within the same treatments (Fig.5). The decay rates were generally higher in trial 2, however no significance effects of nominal PAA concentration were found. Over the entire nominal concentration range from 0.15 to 4.8 mg l^{-1} PAA, the decay value ranged from 0.17 h^{-1} to 0.39 h^{-1} with corresponding half-lives from 1.8 to 3.9 h.

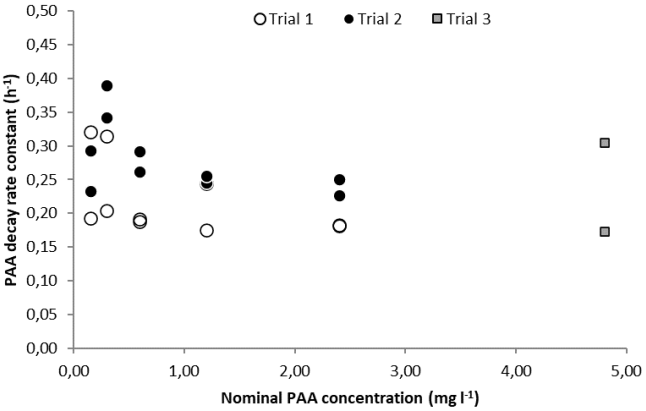

Figure 5. Calculated decay rate constants of PAA derived from trials with addition of PAA 300 liter seawater at 15 ºC in concentrations at C0= 0.15, 0.30, 0.60, 1.20 and 2.4 mgl-1 PAA (n=20). Each symbol represents a calculated PAA decay rate constant from an individual tank trial. Trial 1 included 25 kg/m -3 post-smolts exposure for 5 minutes, and trial 2 included 10 kg post-smolts exposed to PAA for 30 minutes before transfer. The squared grey symbols represent a subsequent trial where 4,8 mg l-1 PAA was added to two tanks with 500 liter seawater (15°C) in which 15 kg m-3 post-smolts were exposed to PAA for 30 minutes (n=2).

Degradation of hydrogen peroxide and acetate

H_2O_2 degradation rate constants calculated from the Divosan Forte addition to different salinities were ranged from 0.013 h^{-1} in freshwater to 0.05 h^{-1} at full strength seawater at 20 °C (Fig. 6a). There were some variations within and between salinities, and the degradation was generally positively correlated with salinity, however not statistically significant. The associated half-lives of H_2O_2 ranged from 15 to 69 h.

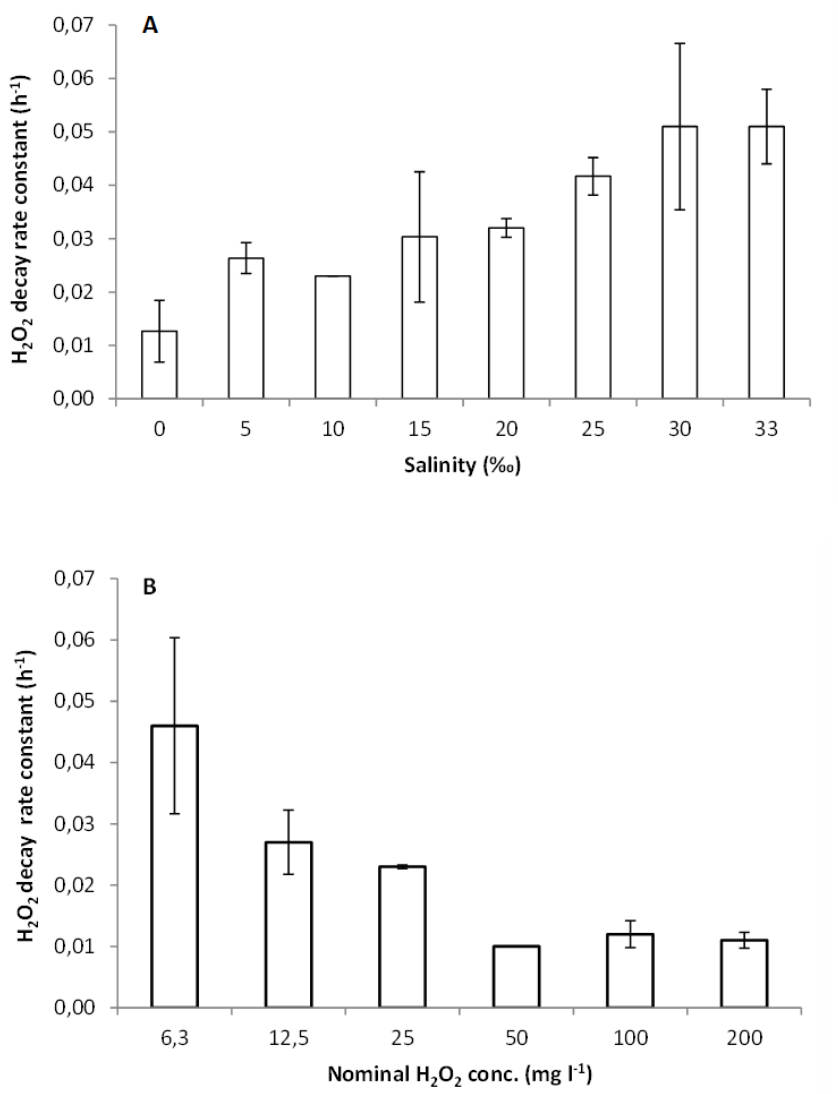

Figure 6. A) Calculated decay rate constants of H2O2 (mean ± std. dev.) derived from beaker experiments where Divosan Forte was added into water samples with different salinities at C0 of 1 mg/l PAA which corresponded to a C0 of 1.6 mg H2O2 l-1; B) Decay rate constants of H2O2 (mean ± std. dev.) derived from beaker experiments with different initial H2O2 concentration added to full-strength seawater. Values are based on duplicate experiments except C0 = 50 mg H2O2 l-1 where an outlier is removed. All trials performed at 22 °C.

Beaker trials with full-strength seawater spiked with H_2O_2 from 5 to 200 mg l^{-1} showed that the decay rates declined with increasing H_2O_2 start concentration (Fig. 6b). The decay rates ranged from 0.010 to 0.036 h^{-1} corresponding to half-lives up to three days.

The acetate concentration in the beakers with seawater was 11.0 ± 0.1 mg l^{-1} immediately after addition and 10.8 mg l^{-1} after four hours. The acetate concentration further dropped to 9.8 mg l^{-1} after 24 h. Degradation rate constants were not calculated due to a low number of analyses.

Table 1. Overview of studies about PAA decomposition in seawater

Water source	Trade product	Nominal conc.	Degradation kinetics	Half-lives	Remarks	Reference
10 and 30 ‰ seawater	Lspez E250; E400;	1 mg l^{-1}	Exponential decay	0.8 h 2 h > 5 h	Product specific degradation	Liu et al. 2014
10 and 30 ‰ NaCl solution	Lspez E250; E400;	1 mg l^{-1}	Linear decay	>> 5 h	Room temp.	Liu et al. 2104
Seawater	AQUA DES	5 mg l^{-1}	Exponential decay	1.3 h	Data according to Table 1A	Solvay, 2005.
Seawater	AQUA DES	50 & 100 mg l^{-1}	Exponential decay	1.9 and 5 h	Data according to Table 1A	Solvay, 2005.
Seawater	Peraclean	15-20 mg l^{-1}	Linear decay	3-5 d	Ice cold seawater 0.1-0.5 C	De Lafontaine et al. 2009
Seawater	Peraclean	20 mg l^{-1}	Exponential	1-2 days	6-7 ºC	De Lafontaine et al. 2009
Seawater (~20 ‰)	Sigma Aldrich	2 mg l^{-1}	Linear decay	2-3 h	Not 1 order	Chhetri et al. 2014
Seawater	PERASAN	1 mg l^{-1}	Exponential decay	0.5 h	H_2O_2 measured	Howarth, 2003
Seawater	PERASAN	20 mg l^{-1}	Exponential decay	0.3 h	H_2O_2 measured	Howarth, 2003
Seawater	Divosan Forte	1 mg l^{-1}	Exponential decay	1.7-8.1 h	5-20 °C	This study
Seawater	Divosan Forte	0.2-4.8 mg l^{-1}	Exponential decay	1.8-3.9 h	15 °C	This study
Seawater	Aqua Oxides	0.75 mg l^{-1}	Exponential decay	0.2 h	15°C; commercial RAS with high organic matter content	Unpubl. data

Note: Supplementary studies of PAA decomposition in other water matrices can be found in Luukonen & Pehkonen, 2017.

Discussion

Factors affecting PAA degradation

Generally, PAA degradation is considered a chemical oxidation process, in contrast to H_2O_2 where microbial related enzymatic activity is the main route of degradation (Arvin & Pedersen, 2015). This difference and the implication hereof are exemplified and discussed below.

Salinity

The *in situ* beaker trials conducted with different dilutions of seawater showed an apparent effect of salinity on the degradation of PAA. Compared to freshwater, seawater accelerated the PAA degradation by a four-fold with half-lives in seawater below 2 hours. As bioavailable organic matter (BOD_5 < 1 mg 0_2 l^{-1}), pH (7.8-7.95) and temperature (20 °C) were similar in all trials, ion content was the only difference. PAA degradation is accelerated by the presence of transition metals (Yuan et al.

1997). Liu et al. (2014) observed significant PAA degradation in 10 and 30 ‰ seawater but not in 10 and 30 ‰ saltwater made by NaCl only (Table 1). Howarth (2003) reported half-lives of PAA in seawater as short as 0.5 and 0.3 h when spiked with 1 and 20 mg $^{-1}$l PAA (Table 1). All previous studies have shown that the half-lives of PAA in saline water in the order of 1-5 hours, with the exception reported by de Lafontaine et al. (2009) showing a much lower decay; they demonstrated that 20 mg l^{-1} PAA at 0.1-0.5 °C took 3-5 days to degrade. The low half-lives values are a significant advantage from an environmental point of view, as the active compound will only remain for a limited period of time and reduce the likelihood of spreading outside the area of operation (i.e., sea cage production).

The chemical reactions of PAA in saline waters include formation of secondary oxidants during reaction with halide ions (Shah et al. 2015). The inorganic ion composition affects the initial PAA consumption and decay PAA and addition of transition metals, i.e. Fe_2Cl or $KMnO_4$ or addition of reducing compounds such as sodiumthiosulphate may be potential mitigation options to accelerate PAA decay (Henao et al 2018b).

Temperature

The disinfection efficacy of PAA is positively correlated with temperature (Stampi et al. 2001), however scarce information is available about temperature impact on residual PAA. In this study, we demonstrated that temperature had a pronounced effect on the degradation of PAA, with an estimated 9.5 % increase in the decay rate constant per degree Celcius. This corresponds to a four-fold increase in the temperature range tested (5 to 20 ^{o}C).

Linear regression on the decay rate constants (Y, in h^{-1})) *vs* temperature (X, in ^{o}C) showed Y = 0.017X (R^2=0.954). By applying this equation for very low-temperature conditions, i.e., 0.5 ^{o}C as in the case reported by de Lafontaine et al. (2009) the decay rate constant is registered at 0.009 h^{-1}, corresponding to a significantly low half-life of more than 3 days as reported by the authors. The temperature effect found in this study is more markedly compared to previous studies (Pedersen et al 2013), of temperature effects in freshwater matrices with substantial organic matter content. In that study, the effect of organic matter on PAA degradation was exceedingly predominant and thereby masked the sole effect of temperature on PAA decay.

Temperature fluctuations are common in open sea farming and these environmental alterations may influence the kinetics of degradation when PAA is applied in sea cages or well boats. Knowledge of the effects of temperature on the degradation of PAA may provide important information that must be taken into account in its application in the field and may serve as a correction factor in using PAA at different time of the year and under varying environmental thermal conditions.

Light

The effect of light on PAA degradation demonstrated in the present study indicate potential involvement of photodegradation or photocatalysis. The presence of light (i.e., from sunlight) caused an approx. 20 % increase in the degradation of PAA after 4 hours compared to under dark conditions, with the model corrected for temperature effect. This effect may not have any particular consequences for the treatment efficacy of PAA or impacts on the environment but indicate a potential additional decomposition pathway (Howard et al. 1991, Zhao et al. 2008). Higashi et al. (2005) noted that PAA decomposed when exposed to UV irradiation at 182 nm. After 30 seconds of irradiation, they found that all PAA (0.02 wt% solution) decomposed within a short period of three minutes. There is an increasing consciousness on the risk of discharges from the use of chemotherapeutants in sea cage aquaculture hence, ways on how to increase the rate of decay in SW must be explored. The effects of light on the

degradation of PAA in SW provide implications on how to manage the discharge after treatment in sea cages, potentially by applying UV irradiation.

Fish density and organic matter

As PAA reacts with organic matter – suspended or by direct contact with surfaces – the bare presence of fish is expected to influence the degradation of PAA. In the current study, we found a positive correlation between PAA decay and fish density, however with some variation and with a modest impact. In freshwater, Pedersen et al. (2009) found that the half-life of PAA dropped by around 40 % at 35 kg/m^3 compared to tanks without fish, which was more pronounced than the reduction of 23 % observed in the present study with seawater under similar laboratory conditions. The information of fish density effects on PAA decay is useful and relevant in treatment water planning, emphasizing the adjustments needed to take biomass into account both from treatment and environmental perspectives.

The main factor affecting PAA consumption is the presence of organic matter (Koivunen & Heinonen-Tanski 2005,, Munio & Poyatos 2010, Henao et al. 2018a). The presence of large amounts of organic matter may lead to an instantaneous consumption of PAA (Pedersen et al. 2013, Henao et al. 2018b). This initial oxidative demand can be substantial and the initial PAA consumption can be used as a parameter for a modified kinetic model (Haas & Finch 2001) and described by Chhetri et al (2018) and Henao et al (2018b). None to limited initial PAA consumption was observed in the present study and a simple first order decay model was applied.

In open net-pen operation, organic matter content is expected to be low and of minor importance, whereas in land-based RAS with high feed loading and elevated organic matter content can be the main driving factor affecting PAA degradation. For example, PAA degradation was measured in a commercial brackish water RAS by spiking 5 ml Aqua Oxides/m^3 water (C_0 ~ 0.75 mg l^{-1}PAA); here the degradation rate was found to be 4.26 h^{-1} corresponding to a half-life of PAA below 10 min (*unpublished data*). From a practical point of view, contact with organic matter and/or biofilm on colonized surfaces could be a technical solution to further facilitate rapid PAA degradation and thereby reduce the discharge of PAA. A potential solution could be application of a floating wood chip compartment where PAA enriched water could be directed hence facilitating rapid degradation when exposed to large surface areas (von Ahnen & Pedersen 2019). De Lafontaine et al. (2008) documented a significant effect of the presence of sediments on PAA degradation in seawater and this finding can potentially also be applied.

Concentration of PAA

The PAA concentrations applied in this study reflects a realistic concentration range where PAA has proven treatment efficacy against a number of pathogens (Pedersen et al. 2013, Soleng et al. 2019). Furthermore, since PAA is biocidal and very potent, the threshold level for being toxic to fish species begins at a relatively low concentration range below 5 mg l^{-1} PAA (Strauss et al. 2018).

Based on the tank trials with full strength seawater, the start concentration of PAA did not affect the PAA decay rate constants. A proportionally higher net removal of PAA was observed when PAA dosing increases, but the decay rates and half-lives are fairly consistent (Table 1), and an inherent consequence for the exponential decay kinetics.

The implication of PAA decay kinetics can be relevant when planning PAA water treatment regimes, recalling treatment efficacy is a combination of *de facto* PAA concentration and exposure time (Rach et al. 1997). In such situation dealing with easy degradable disinfectant, successful treatment requires knowledge of "unintended" PAA consumption and ideally including analytical verification on the location.

H_2O_2 and acetate degradation

While PAA degradation primarily is governed by chemical oxidation processes both H_2O_2 and acetate rely on microbial activity (Rojas-tirado et al. 2019). When applying PAA products, H_2O_2 and acetate are directly introduced and formed during PAA degradation, eventually to become O_2 and CO_2. H_2O_2 degradation is controlled by enzymatic activity (Anderson & Miller, 2001, Mishra & Imlay, 2012, Arvin & Pedersen, 2015), but photo-induced degradation and chemical oxidation have also been reported (Cooper et al. 1994). The concentration of H_2O_2 and hence the PAA: H_2O_2 ratio can differ substantially from product to product range-wide (Liu et al. 2015) however, common PAA and H_2O_2 concentrations are within the same order of magnitude. Recalling the low dose of PAA applied, the associated H_2O_2 is often in the order of a few mg H_2O_2 per liter. These H_2O_2 concentration levels itself are order of magnitudes below applied anti-parasitic treatment concentrations of H_2O_2 and hence non-toxic (Wessels & Ingmer, 2013).

Addition of Divosan Forte (nominal concentration of H_2O_2 equivalent to 1.6 mg l^{-1}) into water samples with different salinities showed that H_2O_2 degraded at low rates with a half-life of up to 3 days. The degradation of H_2O_2 increased with salinity, however to a far less extent than compared to PAA and more than one day was required to consume the small amount of H_2O_2. Addition of technical grade H_2O_2 (from 6.3 to 200 mg l^{-1} H_2O_2l) to full-strength seawater showed degradation rate constants from 0.01 to 0.05 h^{-1} (half-life from 15-70 h) with decreasing half-lives at increasing H_2O_2 concentrations. Substantial variation in H_2O_2degradation was found in both experiments. The experiments were performed in July and subsequent similar analysis of H_2O_2 degradation in seawater samples from October showed degradation rates of H_2O_2 below 0.001 h^{-1} at 5 and 50 mg l^{-1} H_2O_2 added (*unpubl. data*). In both cases, H_2O_2 degradation was tested in seawater samples (incubated to 22 °C) without isolating or quantifying the potential abundance of bacteria and microalgae and their effect on H_2O_2 degradation (Pedersen et al. 2019). Future studies are therefore needed to investigate to which degree H_2O_2 degradation rates are affected by light and/or seasonal abundance of microorganisms in seawater samples. A study by de Lafontaine and colleagues (2008) showed that presence of sediment increased H_2O_2 degradation initially, however after 1-2 days, no further H_2O_2 degradation occurred probably due to bacterial inactivation (Arvin & Pedersen 2015).

Acetate is an energy-rich and readily biodegradable carbon source for many bacteria and other microorganisms (Canelhas et al. 2017). Acetate is directly transferred to the water during application of peracetic acid, given the composition of all PAA products. The addition of 10 mg l^{-1} acetate to pre-filtered over 24 hr at 20 °C showed also a marginal decrease in acetate concentration of 1.1 mg l^{-1} after 24 hours corresponding to a decay rate of 0.004 h^{-1} and a half-life of approx. 7 days.

The cause of degradation H_2O_2 is primarily related to bacterial activity, and in this particular case, impeded by lack of nutrients and low bacterial activity present at the start. More research is needed to evaluate the degradation of acetate in relation to practical PAA application and to assess bacterial regrowth issues (Zhang et al. 2019) and the potential environmental implications (see section 4.3.). Peracetic acid including acetate abatement by bioaugmentation is an unexplored option so far, but could potentially be applied as a measure to neutralize residual compounds.

Environmental aspects and management perspectives using PAA

The rapid decay of PAA may complicate correct dosing but reduces the exposure risk to the surrounding environment and hence, favorable from an environmental point of view. The existing antiparasitic treatment with H_2O_2 requires a substantially higher concentrations (500-1000 fold), and with a much lower rate of decay it has recently been found to be environmentally inexpedient (i.e., effects on non-target organisms).

There are both differences and similarities between use of PAA for ballast water disinfection and aquaculture disinfection/treatment in open net pens at sea. In both cases, a certain treatment efficacy is required; for ballast water disinfection overdosing is not critical as such when compared to aquaculture disinfection where the dose to be used must not compromise fish health and welfare. It is inherent that in identifying the effective treatment dose these three arbitrary criteria must be taken into consideration: 1) low environmental risk (e.g., rapid degradation, do not pose challenge to non-target organisms) 2) no substantial impact to fish health and welfare 3) effective against the target causative agents.

The potential environmental impact is similar; ballast water operates at a far higher PAA concentration but deals with smaller water volumes (LaCarbona et al. 2010, Hess-Erga et al. 2019). In case of excess residuals of PAA during de-ballasting, different mitigating options are available such as addition of catalase or the reducing agent thiosulphate (Luukonen & Pehkonen 2017). Whether it is an issue for aquaculture operation at all, the rapid degradation and the low concentrations of PAA applied similar options could be applied. It remains to be investigated whether additional harmless salts/compounds containing transition metals (i.e., $KMnO_4$) can be applied or whether UV has any potential to accelerate degradation of PAA, especially in the field. The environmental impact of the H_2O_2 related to PAA application is considered negligible considering the diminishing small amount of H_2O_2 applied compared to commercial sea lice operation procedures (Adams et al. 2012).

PAA products are often highlighted as degradable via the rapidly biodegradable acetate. Acetate release is not critical from a toxicological point of view, but attention has been allocated toward potential bacterial regrowth (Kitis, 2004, Zhang et al. 2019). Sánchez-Ruiz et al. (1995) confirmed longevity of coliforms after PAA treatment compared to untreated controls, which was earlier stressed by Lefevre et al. 1992. More recently, Stehouwer et al. (2013) documented acetate consumption and regrowth associated to ballast water disinfection. Biofilm growth in freshwater tanks with continuous PAA addition have been observed (Liu et al. 2017) and even the mode of acetate addition has been found to affect the microbial communities (Canelhas et al. 2018). An important benefit of PAA compared to other chemicals is that PAA does not cause formation of toxic disinfection by-products. Henao et al. (2018c) recently made a comprehensive review of the potential ecotoxicological effects of PAA where disinfection by-products were compiled as well as a toxicity overview of various aquatic species. They concluded that PAA does not form genotoxic or persistent disinfection by-products (see Shah et al. 2015, for protective effect of H_2O_2 on secondary oxidants) that bioaccumulation of PAA is unlikely and that environmental impact of PAA disinfection will be minimal and transient, and that prolonged PAA exposure with PAA is toxic mainly against bacteria and algae (Henao et al. 2018c).

Conclusion

The degradation of PAA is rapid (half-lives in the order of 1-2 hours) and half-lives are shortened in saline water compared to freshwater. PAA degrades via acetate to CO_2 and water and does not form toxic by-products. Moreover, its degradation profile is affected by several factors including light, salinity, temperature, stocking density and nominal dose. PAA is far more environmentally advantageous to use than existing chemical treatments, especially targeting ectoparasitic infections in fish. Future research is needed to optimize the full-scale application of PAA products including measures to reduce PAA and acetate residuals.

The results presented and discussed are essential in the eventual use of PAA as a chemotherapeutant against diseases affecting salmon during the seawater stage of production. The factors identified here must be included in the risk analysis that will be conducted for the application of PAA in salmon at sea.

5.1.2 Methods to improve degradation of peracetic acid

(*This trial is expected to be developed into a peer-reviewed manuscript*)

During stage I, experiments from WP2 documented, that PAA is easy degradable in seawater with half-lives in the order of hours. PAA decomposition correlated positive to salinity and temperature in clean seawater. The degradation half-lives of PAA in seawater ranged from 2-8 hours inversely related to temperature (Pedersen & Lazado, 2020). The recommended PAA concentration used to treat AGD was ≤ 5 mg PAA/l. Accordingly, we tested potential practical solutions to increase the degradation of peracetic acid. The studies reflected scenarios of PAA treatment in seawater in closed confinement PAA treatments and PAA concentrations up to 5 mg PAA/l as found in stage I. Here we report results from batch experiments with chemical addition, UV and vacuum UV and include information from published studies.

The tests involved chemical enforced PAA degradation using iron salts (Fe^{2+} and Fe^{3+}) and sodium thiosulphate ($Na_2S_2O_3$). Iron(II-&III) salts ($FeSO_4 \cdot 7H_20$ and $FeCl_3 \cdot 6H_20$) were chosen, as iron is a common transition metal available in large quantities and with no/very limited environmental impacts when discharged. Sodium thiosulphate is a reducing agent commonly applied to neutralize chlorine and is also consider environmental safe.

Tests of UV irradiation (254 nm) and vacuum UV (185/254 nm) were also made with the purpose to assess the potential photo-oxidative capacity on PAA degradation. Photo oxidation is a chain process incorporating chemical reactions which are subsequent to the outcome of the primary event, absorption of a photon, which induces breakdown to free-radical products.

Materials and Methods

The tests of UV irradiation on PAA degradation were made in tanks with 100 liter water and included different UV lamps (8-11-18 & 37W). The trade PAA product Aqua Oxides was tested in clean seawater and freshwater as well as freshwater from intensive pilot scale RAS (Fig. 1). Each experiment included sampling and immediate analyses of PAA residuals after 5, 10, 15, 20, 30, 45, 60, 90 and 120 min. The degradation of PAA was calculated as exponential decay with the rate constant k (h^{-1}). The degradation half-life (h) was calculated as Ln2/*k*. Water temperature ranged from 14.5-16 °C; pH in tap water and seawater ranged from 7.9 to 8.5. Tests with single and combined effects of PAA and UV (2 ppm PAA; 2 ppm PAA+UV, UV and control) on microbial water quality included a two hrs exposure and subsequent measurements on the microbial dynamics.

Figure 1. Test of PAA degradation in clean freshwater and seawater (left) with and without UV. PAA addition with and without UV were tested in mature RAS water.

Test of vacuum UV (VUV) on PAA degradation was performed in a static 20 l bench scale setup with a 350 W quartz lamp, a circulation pump and a flowmeter (Fig. 2). Seawater was brought Ultra Aqua, Ålborg, Dk where the setup was installed.

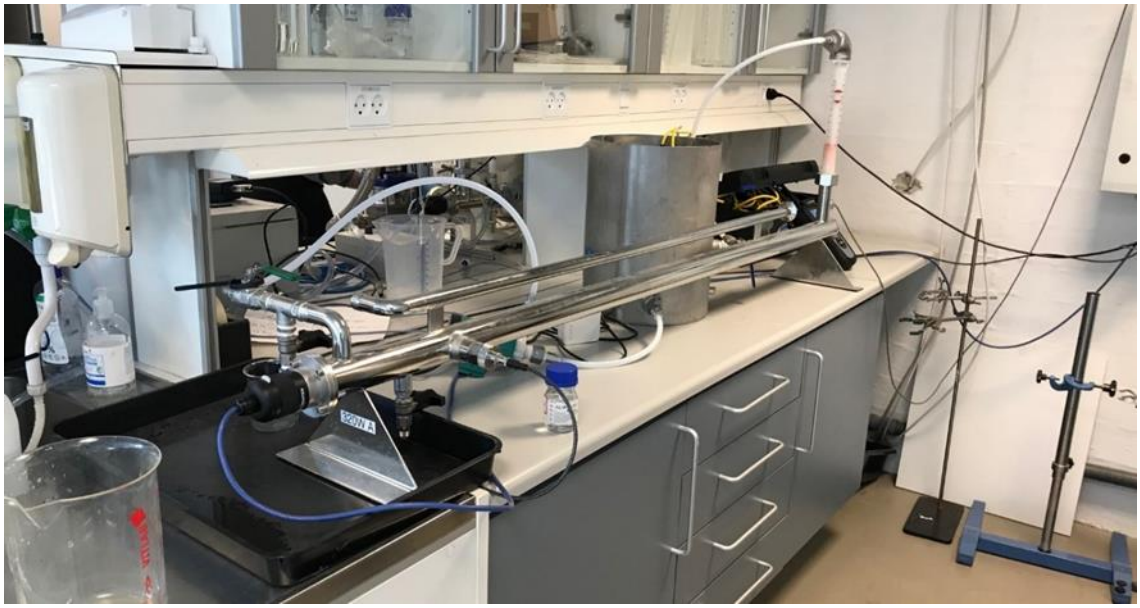

Figure 2. Experimental bench scale vacuum UV setup. Bench scale VUV setup. A 25 l reservoir connected to a circulations pump (440 l/h) providing water to the inlet of the VUV reaction chamber with synthetic quartz lamp and returns to the reservoir.

PAA (1000-ppm PAA stock solution made of Aqua Oxides) was added to the system at 1, 2 and 5 mg PAA/l with and without VUV irradiation.

The experiments with transition metals Fe(II) and Fe(III) and reducing chemical (sodium thiosulphate) included batch scale degradation tests in 1-liter Pyrex® glass beakers with seawater. Water samples were collected at regular intervals for immediate PAA analysis.

Results and Discussion

UV

The degradation rate of PAA was more than three times lower in freshwater compared to seawater when water circulated with the UV lamps off (Fig. 3). The UV irradiation increased the degradation rate of PAA in both fresh and seawater. In freshwater, UV irradiation decreased the half-live by 63 % compared to a reduction of 26 % in seawater.

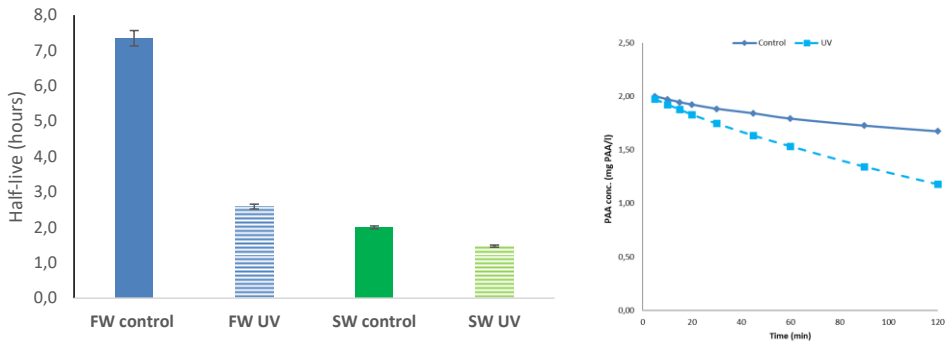

Figure 3. A) Calculated half-lives of PAA based on PAA degradation in 100 l tanks with seawater at 15 °C. The nominal concentration of 2 mg PAA/l and the UV used was 37 W. Control reflects circulation over the UV lamp turned off. B) Example of PAA measurements from two tanks with and without UV turned on.

The UV irradiation dose significantly affected the degradation of PAA in both freshwater and seawater (Fig. 4). In seawater, the half-life of PAA (C0 = 2 mg PAA/l) were reduced by 14 min/W compared to a reduction of 1.5 min /W in freshwater. The half-lives in seawater ranged from 1.5 to 2.4 hours.

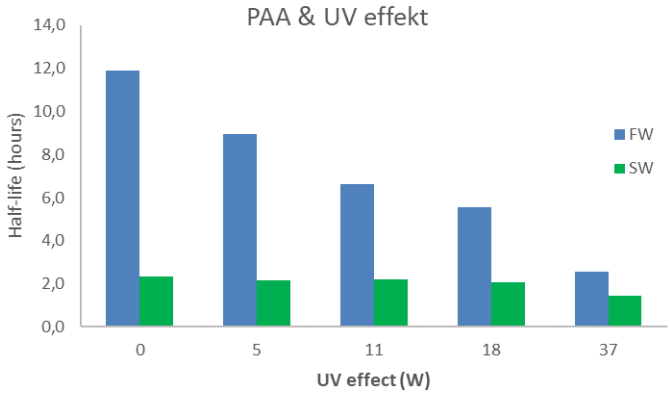

Figure 4. Degradation half-lives of PAA in 100 l tanks with seawater at 15 °C and different sizes of UV irradiation based on tank experiments.

The experiments with RAS water showed that PAA caused delayed microbial growth as indicated by increased mibrobial activity in water samples measured 48 hours after PAA addition. In the untreated group (control), the microbial activity decreased by approx. 50 % compared to the values before treatment (Fig. 5.). Similar in the UV treated water, whereas PAA addition – with and without UV irradiation led to an approx. 40 % increase compared to the start values. The bacterial activity increased 6-8 fold compared to the activity after 24 hours. This example illustrates that nutrient rich RAS water with a high bacterial load involves a substantial indirect PAA consumption – which leads to insufficient disinfection. The degradation of PAA and the accompanying acetate serves as an easily biodegradable C-source for the remaining bacteria in the water (Ricão Canelhas et al., 2017; Rojas-Tiroda et al., 2019). This will only occur in (closed) systems with long retention time in the order of days and at sub inhibitory PAA dosages.

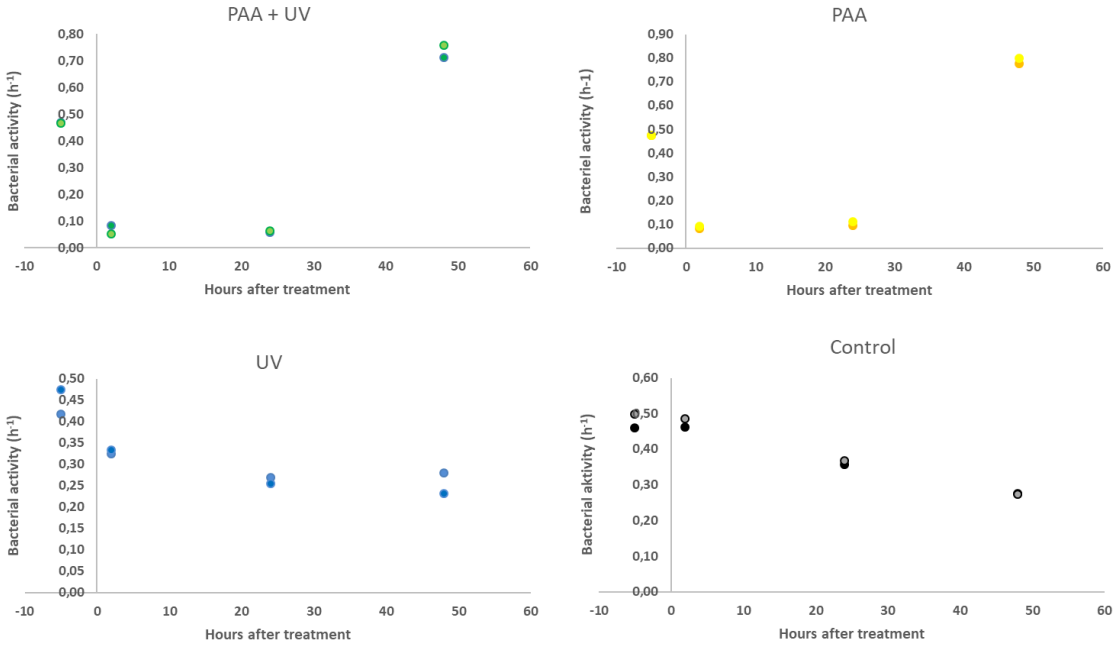

Figure 5. Bacterial activity, based on hydrogen peroxide assay, measured in RAS water with 4 different treatments. Peracetic acid (PAA) was added equivalent to 2 mg PAA/l, and UV (37W) exposure for 2 hours. Control indicates tanks with RAS water with PAA addition and with UV lamps off.

Vacuum UV

Degradation of PAA increased significantly when exposed to VUV (Fig. 6). The PAA concentration showed an exponential decay immediately after VUV activation. The rate constants were similar (0.116-0.133 min-1) across PAA concentrations from 1.0 to 5.0 mg PAA/l (Fig. 7, Table 1). The VUV associated half-lives of PAA degradation was 5.5 ± 0.3 min, which was 10-40 times faster than PAA degradation under control conditions. The VUV itself did not form any radicals interfering with the measurement applied. The UV lamp led to temperature increments up to 22 °C starting at 10 °C.

The results from the experiment included both photo oxidation (185 nm) and degradation from UVC (254 nm) and the current setup did not allow to calculate the interrelationship.

Table 1. Estimated 1. Order exponential decay rates r (min^{-1}) during VUV exposure and during control conditions (Off). The degradation half-lives (t½) are calculated as Ln(2)/r.

Nominal PAA concentration	Rate const. VUV on (min^{-1})	Half-life t½ (min)	Rate const. VUV off (min^{-1})	Half-life t½ (min)	Ratio (On:Off)
1.0 ppm	0,133	5,21	-	-	
1.0 ppm	0,118	5,87	0,012	58	9,8
2.0 ppm	0,128	5,42	0,003	231	42,7
5.0 ppm	0,116	5,98	0,007	99	16,6

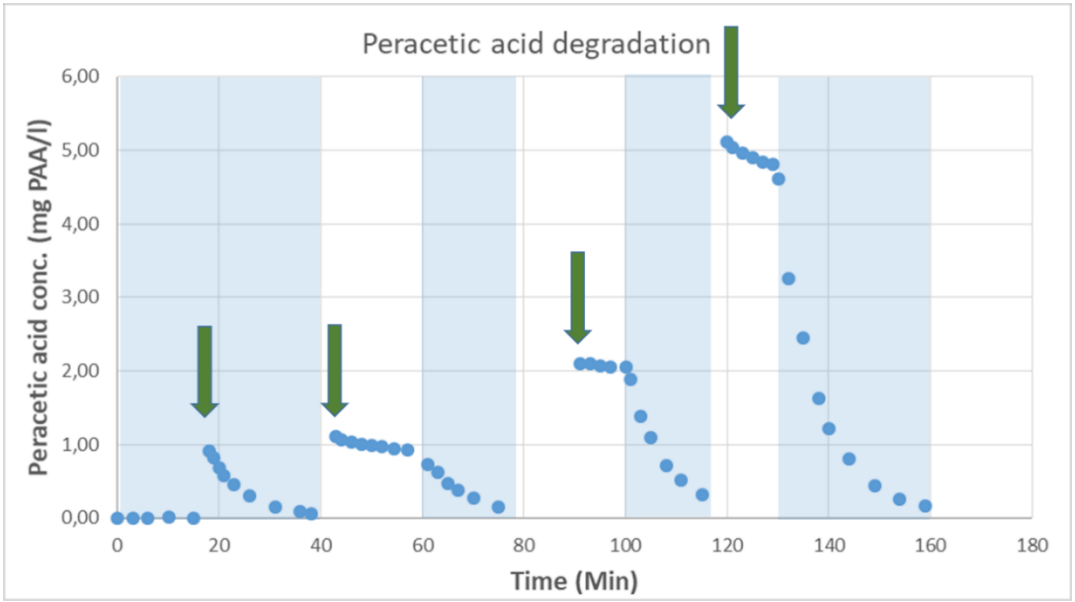

Figure 6. Results of peracetic acid (PAA) degradation in seawater (34 ppt) testes in a bench scale setup with vacuum UV irradiation. The green arrows indicate time of addition of PAA (stock solution based on Aqua Oxides). The blue shaded areas indicate periods with the VUV lamp turned on, while white areas refer to periods with circulation over the VUV in off state.

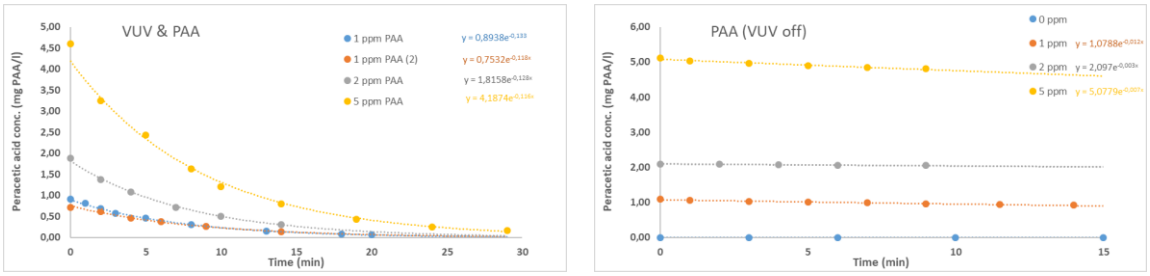

Figure 7. Degradation kinetics of peracetic acid in seawater in the presence of vacuum UV and with VUV turned off. Exponential regression was used to estimate removal rates of peracetic acid (All R2 > 0.99).

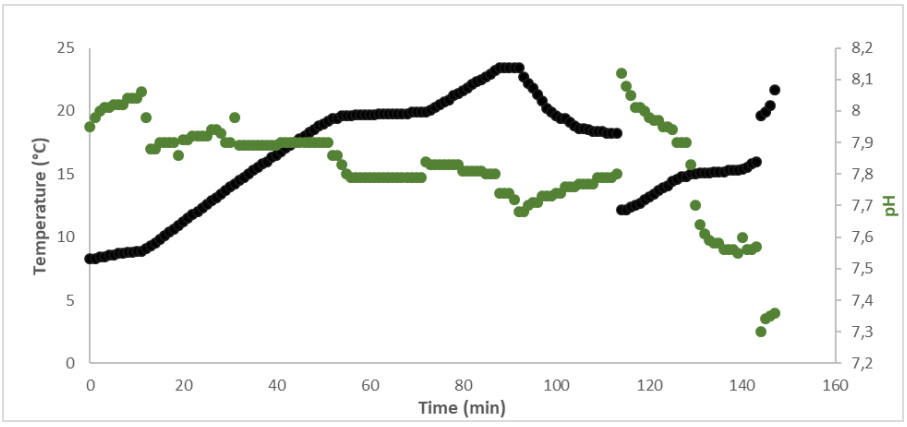

Figure 8. Recorded water temperature and pH during the experiments. New water was added after 115 min.

The degradation capacity and kinetics can potentially by applied in systems with high concentrated PAA and a low volume. This could be during terminal disinfection of a RAS where the combined effect og PAA and VUV have two advantages: i) a combined antimicrobial effect (advanced oxidation process

including photo - and chemical oxidation and radical formation) ii) an intrinsic degradation of PAA to neutralize the water before discharge.

Transition metals

Addition of Fe(III)-salts had a minor positive effect on the degradation of PAA compared to the degradation in seawater (Fig. 9). The degradation of PAA with ferric iron was twice as fast as compared to seawater control with a half-life at approx. 1.2 hrs.

The addition of Fe(II)-salts lead to a spontaneous consumption (Fig 9) directly related to the concentration of Fe^{2+}. The mechanisms are not further tested (Ao et al., 2021), but based on the results, a 1:1 PAA – Fe^{2+} ratio leads to a momentary reduction within 5 minutes.

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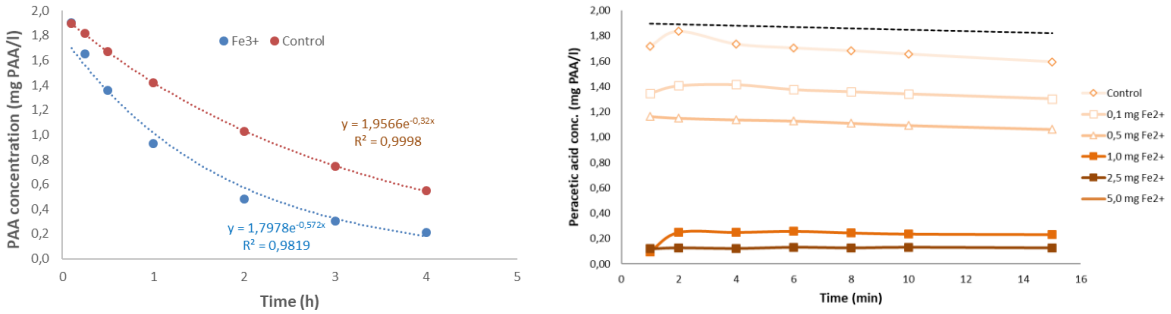

Figure 9. Degradation of PAA following addition of 10 ppm Fe^{3}+ (left) and Fe^{2+} (right) at different concentrations. PAA was added equivalent to 2 mg PAA/l in 20 °C seawater. The dotted black line represents decay in seawater.

Neutralization with a reducing agent

Batch experiments with 2 mg PAA/l in seawater at 20 °C proved complete PAA removal with 5 min after addition of sodium thiosulphate (Fig. 10). By adding 0.5 mg Na2S2O3/l to the PAA enriched seawater (ration 1:4), approx. 50 % of PAA was degraded. With a ration of approx. 1:1 more than 75 % of the PAA was degraded and completely neutralized after 10 min (Fig. 10). As a result, the respective half-lives ranged from 15 minutes to below 1 min.

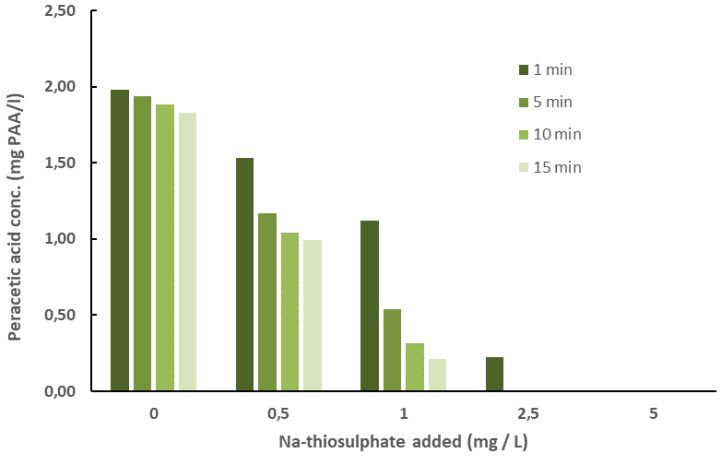

Figure 10. Degradation of PAA following addition of sodium thiosulphate at various concentrations. The batch experiments were performed I 1-liter Pyrex beakers with seawater at 20 °C.

Other options to increase PAA decay

Alternative methods to remove PAA can include mechanical and biological treatment processes. Pedersen et al. (2009) have previously reported removal rates of PAA in biofilter in the order of 10 mg PAA/m2/h. Recently, von Ahnen et al. (2021) documented wood chip bioreactors as an effective solution to remove aquaculture therapeutants. The tested concentrations of PAA (50-350 mg PAA/l) exceed by far applied PAA concentrations during AMG bath treatments but may reflect situations where PAA is applied to systems without presence of fish. Complete removal of PAA at 50 ppm was found within five hours of retention time, which emphasizing the large removal capacity of wood chips. An additional asset of the wood chip treatment unit is the associated microbial degradation of acetate to carbon dioxide and reduction of hydrogen peroxide to water and oxygen.

Hydrogen peroxide and acetate

Acetate and hydrogen peroxide are associated compounds in peracetic acid trade products. Acetate is an easily biodegradable organic compound and a central carbon source for heterotrophic bacteria. The abiotic degradation of acetate is minimal, however at the concentrations applied (< 5 ppm) acetate do not pose any environmental risks. In closed systems with long retention time, acetate has been found to support bacterial growth in the water phase and on surfaces as biofilm because of the bacterial utilization.

Hydrogen peroxide does not pose any environmental risks at the dosages applied when added as PAA products. With a dosage of 5 ppm PAA, H2O2 does not exceed 10 ppm, which is > 100-fold, lower than when applying H2O2 directly to treat against sea lice.

Degradation of acetate and hydrogen peroxide increases by leading PAA residuals to compartments where organic matter and bacteria are present in abundance. This could be sewage, sludge, wood chips, bioreactors or similar.

Conclusions

The potential environmental impact of peracetic acid use relates to the amount and concentration of PAA discharged to the environment (Henao et al., 2018). PAA dosage against AGD is low

(< 5 mg PAA/m^3) and PAA degrades relative fast in seawater (Table 1). The current knowledge makes it possible to assess and apply PAA in a safe way where excess PAA can be treated.

The recent findings show simple and advanced methods to remove PAA faster, and based on the specific PAA application, practical and sustainable solutions can be applied.

For example, if a RAS facility or a well boat has to be sterilized by use of a very high dose PAA – potentially *vacuum* UV could be and option. The combination will optimize the disinfection by advanced oxidation processes and the PAA is degraded at a high rate. UV and vacuum UV may enhance the antimicrobial effect when combined with PAA and could be a relevant combination to investigate further. Addition of a reducing agent as thiosulphate could also be a solution to neutralize PAA, involving only costs of the chemicals applied. Beside the physical and chemical treatment, mechanical and biological options (end-of pipe biofilter or wood chip reactor) could also be relevant in situations (i.e., land-based RAS) where a high volume of low concentrated PAA is discharged. Here, not only PAA but also residuals of acetate and hydrogen peroxide will be reduced.

Table 1. Overview of studies about PAA decomposition in seawater (Modified from Pedersen & Lazado, 2020).

Water source	Trade product	Nominal conc.	Degradation kinetics	Half-lives	Remarks	Reference
10 and 30‰ seawater	Lspez; E250 E400	1 mg l^{-1}	Exponential decay	0.8 h 2 h >5 h	Product specific degradation	Liu et al. (2014)
10 and 30‰ NaCl solution	Lspez E250; E400	1 mg l^{-1}	Linear decay	≫5 h	Room temp.	Liu et al. (2014)
Seawater	AQUA DES	5 mg l^{-1}	Exponential decay	1.3 h	Data according to Table 1	Massey (2005)
Seawater	AQUA DES	50 & 100 mg l^{-1}	Exponential decay	1.9 and 5 h	Data according to Table 1	Massey (2005)
Seawater	Peraclean	15–20 mg l^{-1}	Linear decay	3–5 d 0.1–0.5°C	Ice-cold seawater	De Lafontaine et al. (2009)
Seawater	Peraclean	20 mg l^{-1}	Exponential	1–2 d	6–7°C	De Lafontaine et al. (2009)
Seawater (~20‰)	Sigma Aldrich	2 mg l^{-1}	Linear decay	2–3 h	Zero-order	Chhetri et al. (2014)
Seawater	PERASAN	1 mg l^{-1}	Exponential decay	0.5 h	H_2O_2 measured	Howarth (2003)
Seawater	PERASAN	20 mg l^{-1}	Exponential decay	0.3 h	H_2O_2 measured	Howarth (2003)
Seawater	Divosan Forte	1 mg l^{-1}	Exponential decay	1.7–8.1 h	5–20°C	This study
Seawater	Divosan Forte	0.2–4.8 mg l^{-1}	Exponential decay	1.8–3.9 h	15°C	This study
Seawater	Aqua Oxides	0.75 mg l^{-1}	Exponential decay	0.2 h	15°C; commercial RAS with high organic matter content	L. F. Pedersen unpubl.
Seawater	**Aqua Oxides**	**1- 5 mg l^{-1}**	**Exponential decay**	**0.1 h**	**Vacuum UV 12-20°C**	**This report**
Seawater	**Aqua Oxides**	**2 mg l^{-1}**	**Momentary reduction**	**90 %**	**Addition of Fe^{2+} (0.1-5.0 ppm) 20°C**	**This report**
Seawater	**Aqua Oxides**	**2 mg l^{-1}**	**Exponential decay**	**< 0.05 h**	**Addition of sodium-thiosulphate (0,5 – 5 mg/l) $Na_2S_2O_3$ 20°C**	**This report**

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5.2 The physiology of PAA

5.2.1 Effects of repeated low dose PAA treatment

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Lazado, C.C., Timmerhaus, G., Soleng, M., Kirste, K.H., Breiland, M.B., Pedersen, L.F. 2020. Oxidant-induced modifications in the mucosal transcriptome and circulating metabolome of Atlantic salmon. *Aquatic Toxicology*. 227, 105625

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Full length article

Atlantic salmon (*Salmo salar*) mounts systemic and mucosal stress responses to peracetic acid

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(This is part of Malene Soleng's MSc thesis submitted to UiT-The Arctic University of Norway)

ABSTRACT

Peracetic acid (PAA), a strong organic peroxide, is considered a relatively sustainable disinfectant in aquaculture because of its broad effectivity against many pathogens at low concentrations and because it degrades spontaneously to harmless residues. The impacts of PAA on fish health must be determined before its use as either a routine disinfectant or chemotherapeutant. Here we investigated the systemic and mucosal stress responses of Atlantic salmon (*Salmo salar*) to PAA. In experiment 1, salmon were exposed to different nominal concentrations (0, 0.6, and 2.4 ppm) of PAA for 5 min, followed by a re-exposure to the same concentrations for 30 min 2 weeks later. Sampling was performed before exposure to PAA and at 2 h, 48 h, and 2 w after exposures. In experiment 2, fish were subjected to crowding stress prior to PAA exposure at 4.8 ppm for 30 min. The fish were sampled before exposure and 1 h, 4 h, and 2 w after. The two trials were performed in a recirculation system. Both systemic (i.e., plasma cortisol, glucose, lactate, total antioxidant capacity) and mucosal (i.e., expression of antioxidant coding genes in the skin and gills) stress indicators were affected by the treatments at varying levels, and it was apparent that the fish were able to mount a robust response to the physiological demands of PAA exposure. The cortisol levels increased in the early hours after exposure and returned to basal level afterwards. Prior exposure history to PAA did not markedly affect the levels of plasma lactate and glucose when fish were re-exposed to PAA. Crowding stress before PAA treatment, however, did alter some of the stress indicators (i.e., lactate, glucose and expression of antioxidant genes in the gills), suggesting that stress history serves as both a confounding and compounding factor on how stress responses to PAA are mobilised. Nonetheless, the changes were not substantial. Gene expression profile analyses revealed that the antioxidant system was more responsive to PAA in the gills than in the skin. The increased antioxidant capacity in the plasma, particularly at 2.4 ppm and higher, indicates that antioxidants were produced to neutralise the internal redox imbalance resulting from PAA exposure. In conclusion, the results show that salmon were able to mount a robust adaptive response to different

PAA doses and exposure times, and a combined exposure to stress and PAA. These results underscore the potential of PAA as a chemotherapeutant for salmon at PAA concentrations commonly applied to control parasitic infestations.

Introduction

Peracetic acid (PAA) is a highly reactive peroxygen compound and is recognised as a sustainable disinfectant in aquaculture [37, 38]. PAA is commercially available as an equilibrium mixture of acetic acid, hydrogen peroxide (H_2O_2), and water. The potential of PAA for improved biosecurity in aquaculture is underscored by its broad range of antipathogenic activity and rapid decay into neutral residuals (*i.e.*, carbon dioxide, oxygen and water) [31, 37-41]. The fat solubility of PAA significantly contributes to its potent antimicrobial activity [29], in which the main mode of action is oxidative disruption of cell membranes via hydroxyl radicals [28, 42]. These radicals interrupt the chemiosmotic function of the lipoprotein cytoplasmic membrane and transport [43, 44]. Intracellular PAA acts upon essential enzymes, oxidising them, resulting in the impairment of biochemical pathways, active transport across membranes, and intracellular solute levels [45]. It is also suggested that it can oxidise the sensitive sulfhydryl and sulfur bonds in proteins, enzymes, and other metabolites [28, 39]. These features account for why PAA is potent against a wide range of microorganisms, including *Ichthyophthirius multifiliis*, *Aeromonas salmonicida*, *Flavobacterium columnare*, *Yersinia ruckeri*, *Saprolegnia* spp., *Aphanomyces* spp., and infectious salmon anemia virus [39], where, in most cases, the effective dose is less than 2 mg L^{-1}[38].

Most of the studies on the application of PAA in aquaculture have focused on degradation kinetics, antimicrobial activity, impacts on water quality, and biofilter nitrification [31, 38, 41, 46]. Though toxicological data exist [39], the physiological responses of fish to PAA exposure are not well documented, and this might undermine its potential as a sustainable prophylaxis and chemotherapeutant in aquaculture. As a strong oxidant, PAA likely triggers physiological imbalances and hence might require fish to mount suitable countermeasures. The stress axis has been shown to mount an adaptive response to the presence of PAA, and this had been documented in common carp (*Cyprinus carpio*) and rainbow trout (*Oncorhynchus mykiss*) [37, 47, 48]. A hallmark response in these studies demonstrated that PAA-exposed fish exhibited an increase in the levels of plasma cortisol after initial exposure, but repeated exposures to PAA resulted in lower cortisol response. The lower cortisol response indicated that fish might have habituated to PAA, but desensitisation, physiological exhaustion, or PAA-mediated endocrine disruption might also explain the reduced corticosteroid response to repeated PAA treatment. Gesto and colleagues [37] demonstrated that the lower cortisol response after repeated PAA exposure was a true form of habituation since rainbow trout repeatedly exposed to PAA were able to execute a normal physiological stress response when prompted with a secondary stressor. As an oxidant, PAA produces hydroxyl radicals following its decay, and this likely results in an altered redox balance, hence triggering oxidative stress. The antioxidant system acts upon these excess reactive oxygen species (ROSD) or radicals, thereby protecting the fish from oxidative damage. How fish mobilise its antioxidants defences to PAA-induced oxidative stress is yet to be demonstrated.

In this study, we explored how the systemic (i.e., plasma) and mucosal (i.e., skin and gills) stress defences are mobilised to counteract the physiological pressures or stressors when Atlantic salmon are exposed to PAA. We measured the classical physiological stress indicators (i.e., cortisol, glucose and lactate) as well as the transcriptional changes of key antioxidant coding genes in mucosal tissues.

Materials and Methods

Ethical statement

All fish handling procedures complied with the Guidelines of the European Union (2010/63/UE), as well as with national legislation.

Experimental fish and husbandry conditions

Salmon smolts (Experiment 1: 150.3 ± 5.6 g, mean ± SE; Experiment 2: 131.3 ± 2.3 g) were purchased from Danish Salmon A/S (Hirtshals, Denmark). Experiment 1: Upon arrival at the recirculation aquaculture (RAS) facility of DTU Aqua (Hirtshals, DK), fish were sorted and moved to six 1-m2 holding tanks (water volume ≈ 600 L), with 60 fish in each tank. The RAS had a 40-µm drum filter, a submerged fixed bed biofilter, and a trickling filter with a makeup water exchange at approximately 0.4 m3/h, equivalent to a retention time of 1.5 days. Internal recirculation allowed more than two-times the tank exchange per hour. Fish were acclimated for 3 weeks under stable conditions, with daily monitoring of water quality parameters, which were kept within safe limits (Supplementary Table 1). The tanks had no direct light above them, and the photoperiod in the experimental hall was set at 16L:8D (06.00 – 22.00), similar to the natural photoperiod in April–May 2018 (57º35'N 09º57'E). Water temperature was at 15±1oC. The fish were fed (Biomar, EFICO Enviro, 4,5 mm) at a ratio of 1.0–1.5 % total biomass per day using a belt feeder. Feeding was gradually increased during the acclimation period and feeding behaviour of the fish in terms of uneaten feed pellets was registered by daily inspection of the swirl separator. Experiment 2: A second batch of smolts were transported to the aquaculture facility of DTU Aqua, sorted and moved to two 4-m2 holding tanks (water volume ≈ 1500 L) in a seawater flow-through system, with approximately 100 fish in each tank. The fish acclimated for 2 weeks under stable rearing conditions, with daily monitoring of water quality parameters (Supplementary Table 2). Water temperature was at 11±1oC. The photoperiod was set at 24L:0D and the dietary ration of 1–1.5 % total biomass (Biomar, EFICO Enviro, 4,5 mm) per day was provided using a belt feeder.

Peracetic acid exposure experiments

Peracetic acid (Divosan Forte™, PAA) was supplied by Lilleborg AS (Oslo, Norway). The disinfectant is a stabilised PAA solution (15 % v/v) which is non-foaming and completely free rinsing. The actual amount of PAA in the solution was verified by the DTU Aqua laboratory (Hirtshals, Denmark) to be at approximately ~18 % v/v. The solution was stored at 4 °C. During each exposure, the concentration of PAA in the water was experimentally verified [31] in real-time to ensure that the fish were exposed to the target concentration from start to termination of exposure.

Experiment 1: To represent the pre-exposure fish, on the day before the first exposure, two fish from each of the holding tanks were sampled, as described in detail in Section 2.4. Feeding was temporarily ceased 24 h prior to PAA exposure. Fish were netted from the holding tank, transferred to a transportation container, and immediately thereafter into a 300-L exposure tank. Each holding tank had its equivalent exposure tank, and water quality parameters were identical between these two tanks. The fish were allowed to settle for 10 min before the PAA solution was added to the tanks to achieve the following final concentrations: 0 (seawater), 0.6, and 2.4 ppm. Even PAA distribution was assured by vigorous aeration directly into the rearing tanks. The concentrations were pre-selected based on an earlier report on the toxicity of PAA for rainbow trout [39]. Each treatment group had two replicate tanks. During the exposure period, the water flow to the tanks was stopped, and the decay of PAA in the water matrix was followed. After 5 min, fish were immediately netted out of the tank and returned to their corresponding holding tank. Post-exposure samplings were carried out thereafter, as detailed in Section

2.4. Two days after the PAA exposure, feeding was resumed, similar to the protocol in Section 2.2. All husbandry conditions during post-exposure rearing were similar to pre-exposure conditions. Two weeks after the first exposure, the fish were re-exposed to the same concentration of PAA. The protocol used in the re-exposure experiment was identical with the approach employed in the initial exposure, with a slight modification on the duration of exposure. Instead of 5 min, fish were re-exposed to PAA at a similar concentration used in the first trial for 30 min. Fish were returned to their corresponding recovery tank, and post-exposure samplings were carried out thereafter. Post-exposure husbandry strategies, as described in Section 2.2, were followed.

Experiment 2: Fish were starved for 24 h prior to the exposure experiment. Before the experiment was carried out, four fish were collected from each holding tank to represent the pre-exposure fish. Fish were transferred to a closed-system 500 L exposure tank to achieve a density of 15 kg/m^3. The fish were allowed to settle for 15 min before a group was subjected to crowding stress for 1 h, by lowering the water volume to attain a density of 75 kg/m^3. Aeration was provided during crowding stress. Fifteen minutes after the water level returned to the initial level, one group of the stressed fish was exposed to 4.8 ppm PAA, double the highest concentration tested in experiment 1, while the other stressed group was exposed 0 ppm (seawater) for 30 min. Likewise, another group of fish was transferred to the same exposure tank but was not exposed to crowding stress. After allowing the fish to settle for 15 min, one group was exposed to 4.8 ppm PAA, and one group was exposed to 0 ppm (seawater) PAA for 30 min. After the exposure experiment, fish were transferred to their corresponding recovery tank, similar to what was used in experiment 1. Post-treatment husbandry protocols were followed, as detailed in Section 2.2. Each treatment group was represented with duplicate tanks.

Sample collection

For experiment 1, sampling was conducted at 2 h, 48 h and 2 w after exposure for each occasion. For experiment 2, sampling was carried out at 1 h, 4 h, and 2 w after PAA exposure. Five fish were taken from each replicate tank and were humanely euthanised with an overdose of 20 % benzocaine solution. After the length and weight were measured, blood was withdrawn from the caudal artery using a heparinised vacutainer, centrifuged at 1000 x g for 10 min at 4 °C, and plasma was collected and kept at –80 °C until analyses. The same sampling protocol was applied for fish that were collected before exposure. Tissue samples were collected for RNA isolation. A portion of the dorsal skin and the second gill arch was dissected and transferred to RNAlater (Ambion, USA). Tissue samples in RNAlater were left at room temperature overnight and thereafter kept at – 80 °C before RNA extraction.

Plasma stress indicators

Three commercially available assay kits were used to evaluate the level of plasma stress indicators (cortisol, glucose, and lactate). Plasma cortisol was analysed using an Enzyme-Linked Immunosorbent Assay (ELISA) kit (Neogen, USA), following the manufacturer's protocol. Plasma lactate was analysed using a Lactate Assay Kit (Sigma-Aldrich, USA). Plasma glucose was quantified using a Glucose Assay Kit (Abcam, USA). All samples were run in duplicates.

Total antioxidant capacity assay

Total antioxidant capacity in the plasma was colourimetrically quantified by a commercial kit, and the level was expressed relative to 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), a water-soluble analogue of vitamin E (Sigma-Aldrich).

Gene expression analysis

Total ribonucleic acid (RNA) was isolated from skin and gills by MagMAX TM-96 Total RNA Isolation Kit (Ambion). The RNA concentration and quality were determined using a NanoDrop 8000 spectrophotometer (Thermo Scientific, USA). Also, RNA quality was further assessed with an Agilent® 2100 Bioanalyzer™ RNA 6000 Nano kit (Agilent Technology Inc., Santa Clara, CA, USA). Total RNA was reverse transcribed to complementary DNA (cDNA) using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) with minor modifications. The 25-µL reaction was set up containing 15 µL (200 ng total) RNA template, 2.5 µL 10X RT Buffer, 1 µL 25X dNTP, 2.5 µL 10X RT random primers, 1.25 µL Multiscript Reverse Transcriptase, 1.75 µL nuclease-free H_2O, and 1 µL Oligo d(T) (Invitrogen, USA). The thermocycling parameters were as follows: 25 °C for 10 min, 37 °C for 120 min, 85 °C for 5 min, and 4 °C∞. Real-time quantitative polymerase chain reaction (qPCR) was performed using the QuantStudio 5 Real-Time PCR system (Applied Biosystems). Each reaction contained 10 µL Power SYBR Green PCR Master Mix (Applied Biosystems), 1.2 µL of each forward/reverse primer (5 µM), 0.6 µL nuclease-free H_2O, and 7 µL of 1:40 cDNA. Positive and non-template controls (NTC) were included in the assay. The following cycling parameters were used: 50 °C for 2 min, 95 °C for 10 min, 40 cycles of 95 °C for 15 sec, and 60 °C for 1 min. An eight-step standard curve of 2-fold dilution series was prepared from pooled cDNA to calculate the amplification efficiencies. Transcript level was expressed as relative expression following normalisation with the geometric mean of three reference genes (i.e., *β-actin*, *18S ribosomal RNA* and *elongation factor 1 alpha*). The primers used in the study are given in Table 1.

Table 1. Primers used in the present study

Gene name	Abbreviation	Sequence (5'-3')	Reference
glutathione peroxidase	*gpx*	F: GATTCGTTCCAAACTTCCTGCTA	[49]
		R: GCTCCCAGAACAGCCTGTTG	
glutathione reductase	*gr*	F: CCAGTGATGGCTTTTTGAACTT	[49]
		R: CCGGCCCCCACTATGAC	
manganese superoxide dismutase	*mnsod*	F: GTTTCTCTCCAGCCTGCTCTAAG	[49]
		R: CCGCTCTCCTTGTCGAAGC	
copper/zinc superoxide dismutase	*cu/znsod*	F: CCACGTCCATGCCTTTGG	[49]
		R: TCAGCTGCTGACAGTCACGTT	
β-actin	*β-actin*	F: CAGCCCTCCTTCCTCGGTAT	[50]
		R: CGTCACACTTCATGATGGAGTTG	
18s	*18S*	F: TGTGCCGCTAGAGGTGAAATT	[51]
		R: GCAAATGCTTTCGCTTTCG	
elongation factor 1 alpha	*ef1α*	F: CGCCAACATGGGCTGG	[50]
		R: TCACACCATTGGCGTTACCA	

Statistics

All statistical analyses were performed in Sigmaplot 14.0 Statistical Software (Systat Software Inc., London, UK). A Shapiro-Wilk test was used to evaluate the normal distribution and a Brown-Forsyth test to check for equal variance.

Data sets from experiment 1 were subjected to a two-way ANOVA to test for differences between groups over time. The Holm-Sidak test was used to identify pairwise differences. For experiment 2, a three-way ANOVA was used to test for time, treatments, and stress effects, as well as their interactions. To increase the fit to the model, backward elimination was used to remove insignificant factors from the ANOVA. A Holm-Sidak post-hoc test was applied when significant interactions were detected.

Kruskal-Wallis factor ANOVA and Dunn's post hoc test were used if the requirement for parametric statistics were not met. The transformation was applied where necessary, to meet the assumptions of the two- or three-way ANOVA. If the transformation was unsuccessful, the residuals were plotted for examination. If passed, an ANOVA test was performed. All tests for statistical significance were set at $P < 0.05$.

Results and Discussion

The development of any new chemoprophylactic or chemotherapeutic measures in aquaculture should consider the health and welfare consequences for the fish. PAA is widely considered as a sustainable disinfectant in fish farming because of its apparent advantages, yet little is known how fish respond to this strong oxidant. This is the first report to demonstrate the physiological coping strategies of salmon to oxidative stress induced by PAA. Our results highlight the adaptive responses of salmon to PAA and show that these responses can be altered by either re-exposure or stress history.

PAA alters the systemic antioxidant capacity

PAA is a potent oxidant, and its constituents and decay produce forms of hydroxyl radicals and other reactive oxygen species [28, 42]. Several studies have shown that the increase in the total antioxidant capacity (TAC) indicates loss of redox balance resulting from oxidative stress, thereby mobilising antioxidants to counteract the alterations [52, 53]. Exposing the fish to PAA for 5 min did not trigger significant changes in the TAC of plasma (Figure 1A). However, when the fish were re-exposed to PAA, but for a longer period, the plasma TAC significantly increased (Figure 1B). The overall response showed that PAA exposure resulted in increased TAC that lasted for 2 days. At 2 h after exposure, TAC increased by almost 35 % in the 2.4 ppm group compared with the 0 ppm group. This remained at a significantly elevated level at 48 h after exposure, though the rate of increase relative to the 0 ppm group decreased to about 10 %. There was a lag response in the 0.6 ppm group as TAC significantly increased, but only after 48 h, and at a similar rate of change as with 2.4 ppm when compared with the 0 ppm. No inter-treatment differences were observed at 2 w after the re-exposure, indicating that alterations of the antioxidant state were an acute response. Collectively, the increase in plasma TAC indicated that PAA might have triggered oxidative stress, hence the antioxidants were generated to attack the excess reactive oxygen radicals that might otherwise damage lipids, proteins, and DNA.

Crowding stress has been shown to influence the antioxidative state in fish [54]. The second experiment demonstrated that a stressful episode prior to exposure interfered with the systemic antioxidative response to PAA (Figure 1C). Fish that were not subjected to stress before PAA exposure showed a marked response, notably at 4 h after exposure. In particular, the TAC in fish exposed to PAA increased by 15 % compared with the control group, indicating that the fish were able to mobilise their systemic antioxidant repertoire against PAA-induced oxidative stress. Interestingly, the control and PAA-exposed groups that were subjected to crowding stress before PAA exposure displayed no significant differences. These results imply that a stressful episode before PAA exposure might be a confounding factor, and that it restricted the ability of fish to mount systemic antioxidative responses. Moreover, it appeared that regardless of post-stress treatments, crowding stress ensued a long-term effect on TAC. Both the control and the PAA-exposed groups of the group subjected to crowding stress had

significantly lower TAC at 2 w post-exposure compared with their counterparts in the no stress group at the same time point.

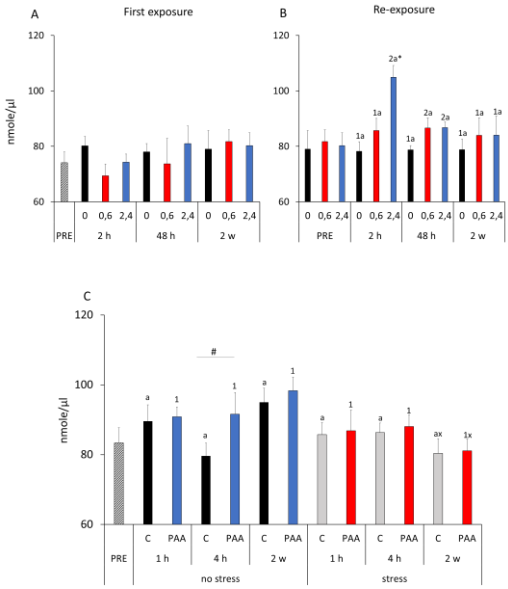

Figure 1. Changes in the level of total antioxidant capacity (TAC) in plasma of fish from Experiment 1 (A & B) and Experiment 2 (C). The level is expressed relative to Trolox standards. Values are mean ± SE of eight individual fish. Notations: For graphs A & B (Experiment 1): an asterisk (*) denotes significant difference between a treatment group and the pre-exposure group (PRE), different numbers signify significant differences between treatments within a sampling point, and different letters indicate significant differences within a treatment through time. For graph C (Experiment 2): different letters denote significant differences within control groups through time, while different numbers indicate differences within PAA exposed groups through time. The same notations are used for no stress and stress groups. x designates that the level of a particular group significantly differs between no stress and stress groups, whereas # indicates a significant difference between the control and PAA-exposed group at a particular time point.

Plasma stress indicators are activated following PAA exposure

Since the internal redox balance was altered, it was anticipated that stress indicators in the plasma would also change. These interactions might be because of three possible scenarios: 1) the altered redox balance elicits responses from other plasma stress defences to ensure that the organism adapts to the physiological demands of PAA; 2) increased antioxidant activity is a result of altered stress defences; and 3) a simultaneous well-coordinated response from both the classic participants of stress response and antioxidants defence is triggered by PAA. Though the present study could not conclusively identify the mechanisms involved, it is interesting to observe the changes in the plasma parameters during PAA exposure following crowding stress.

Plasma cortisol levels from experiments 1 and 2 followed the same pattern (Figure 2A) - a significant increase in the early hours after stress had been triggered, then followed by a decrease and return to the baseline values thereafter, which is the classical cortisol response to stress in fish [55, 56]. Moreover, this was in line with other studies on stress response in salmonids, including experiments on peroxide exposure [37, 57-59]. In the first exposure in experiment 1, all groups showed significantly elevated cortisol level 2 h after exposure, but inter-treatment differences were not observed. This suggests that the increase in cortisol response might be due to handling during the transfer of fish from the holding/recovery tank to the exposure tank, and not due to PAA. The elevated cortisol level of the 0.6 ppm group after 48 h was striking. Though it is quite difficult to provide a firm conclusion with the

current data, we can deduce that 0.6 ppm PAA combined with handling-related stress has a more acute impact than exposing the fish to 2.4 ppm. This marked difference was not observed when the fish were re-exposed to PAA.

A distinctive rise in cortisol level was observed in the 2.4 ppm group at 2 h post-re-exposure (Figure 2a). Nevertheless, the average plasma cortisol was lower compared with results from other stress studies on salmon [57, 59-62], indicating that the fish did experience a stressful episode, but its magnitude was not overly high. The previous history of PAA exposure appeared to magnify the cortisol response only in the 2.4 ppm group. Moreover, the results reveal that the fish recovered rapidly, as all groups had a cortisol level similar to the baseline at 48 h post-re-exposure. Cortisol values in experiment 2 were higher (~ 110 ng/mL) compared with the values identified in experiment 1, which indicates that the fish experienced a more intense compound stressor (i.e., handling, crowding and PAA) (Figure 2G). Interestingly, all groups – regardless of treatment (control, PAA, and/or stress) – had identical patterns in their average cortisol response, which was significantly elevated in the first 4 h after exposure. The similarities in the response of the two groups illustrate that potential interactions and additive effects did not alter the ability of fish to mount a cortisol response to a challenging condition.

PAA did not significantly alter the glucose level in either of the exposure occasions in experiment 1, though temporal variability was apparent (Figure 3B,E). This result reveals that in the tested PAA doses, prior exposure history did not pose a significant impact on glucose metabolism. In experiment 2, however, prior stress history and a higher PAA dose resulted in the differential activation of glucose metabolism (Figure 2H). At 1 h after exposure, the no stress-control displayed significantly elevated glucose level compared with the no stress-PAA group, though both groups were not significantly different from the baseline value. At 4 h after exposure, plasma glucose of both groups was significantly higher compared with the baseline value and the no stress-PAA displayed significantly elevated level compared with the no stress-control. Such an inter-treatment difference was still prominent 2 w post-exposure, though the glucose level of the no stress-control was similar with the baseline value. At this time point, the no stress-PAA group exhibited a glucose level a fold higher than the no stress-control. Prior stress history might interfere with glucose metabolism following PAA exposure (Figure 2H). No inter-treatment differences were documented in any of the time points, contrary to the profile of the group that was not subjected to a stressful episode prior to PAA exposure. The glucose level 4 h post-exposure was significantly lower for the stress group compared with the no stress group, regardless of the treatment. Moreover, this was still evident 2 w after exposure when comparing the stress-PAA and no stress-PAA groups. Glucose is mobilised following a stressful event to ensure energy is provided to overcome the physiological pressure of the situation [55]. The result in the stress group suggests that the fish might have already mobilised the stored glycogen during the crowding stress [63]. Thus, no adaptive changes were identified when subjected to another stressor. As the glycogen deposit in the liver is limited, no further glucose could be mobilised [64]. This illustrates that crowding stress possibly interferes with the glucose stress response to PAA. The higher glucose level 2 w post-exposure in no stress-PAA indicates a delayed and prolonged effect of the stressors, and that the elevated glucose levels might be due to a heightened state of gluconeogenesis to meet the metabolic demands of PAA and handling [63]. The long-term metabolic consequences of PAA exposure, therefore, deserves further investigation.

Lactate is known to increase as a response to a stressful condition [55]. Experiment 1 revealed that the tested PAA concentrations, exposure duration, and re-exposure did not significantly alter the plasma lactate level (Figure 2C,F). This result corroborates other stress parameters (i.e., cortisol and glucose) in this experiment and further illustrates that though PAA exposure at tested concentrations triggered stress (i.e., changes in plasma cortisol), the magnitude of the stress was not high. The lactate level in experiment 2 revealed more obvious dynamics (Figure 3.I). Plasma lactate of the no stress-PAA group

exhibited a significant rise relative to the baseline value 1 h after exposure. The level returned to the basal value thereafter. In addition, no significant difference was identified between no stress-control and no stress-PAA. In contrast to the no stress group, both sub-groups in the stress group had significantly elevated lactate levels 2 w after exposure. We could not ascertain whether crowding stress before exposure might contribute as a compounding factor in the lactate response to an additional stressor (i.e., PAA) since stress-control and stress-PAA displayed no significant difference. It is reported that lactate levels for smolt should not rise above 5 mmol/L (≈ 450 ng/µL) after a stressor [65], and no group had values above this level. The plasma lactate level was slightly higher than the levels found for PAA exposed rainbow trout [37] and on the same level as the control group in a hydrogen peroxide study in salmon [59].

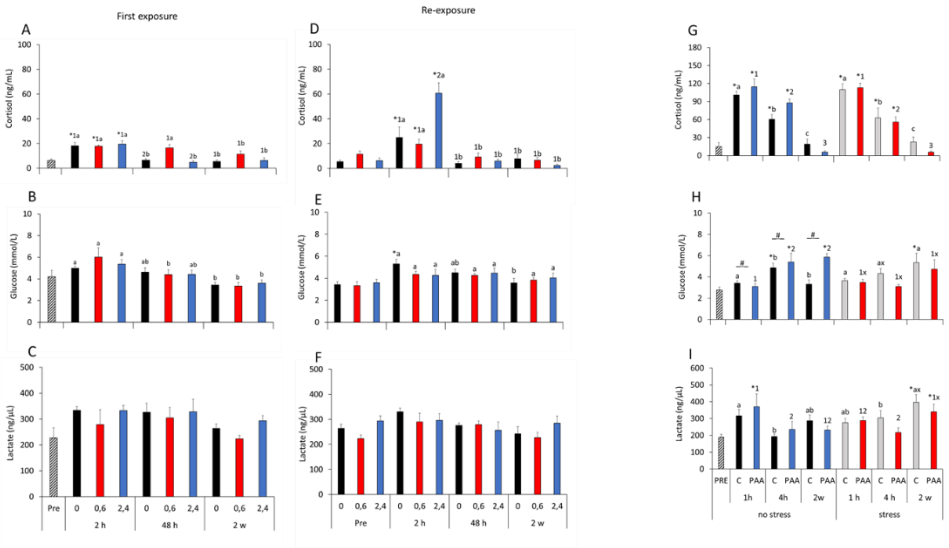

Figure 2. Changes in the level of plasma stress indicators (cortisol, glucose and lactate) of fish from Experiment 1 (A-F) and Experiment 2 (G-I). Values are mean ± SE of eight individual fish. Please refer to Figure 1 on statistical notations.

The antioxidant defences are more responsive to PAA in the gills than the skin, and prior stress imposes a potential confounding factor

Oxidative stress occurs when the balance between ROS and the antioxidant defence system is disturbed [66]. Excessive levels of ROS might have detrimental effects on lipid metabolism, protein synthesis, and DNA [67]; therefore, scavenging of the radical surplus must be performed effectively. H_2O_2 and perhaps PAA – since it degrades into H_2O_2 and O_2 – might induce oxidative stress and provoke a defence mechanism against ROS. We have shown that PAA exposure influenced the systemic antioxidant capacity, indicating that oxidative stress might have been triggered during exposure and that a robust humoral antioxidant defence was mounted (Figure 1). We then asked whether such an antioxidative defence could also be elicited from the mucosal surfaces since they are in contact with water to which PAA was added. The gills and skin are mucosal tissues that function as the first line of defence and are highly responsive to changes in the immediate environment [68], including the levels of ROS. In experiment 1, initial PAA exposure did not affect the expression of any of the antioxidant genes in both tissues, though time-dependent changes in some of the treatment groups were observed (Figure 3). Re-exposing the fish to the same PAA concentrations resulted in the differential modulation of antioxidant gene expression, particularly in the gills (Figure 3a,b,c,d). The expression of *gpx* in the gills of fish subjected to 0.6 and 2.4 ppm was significantly higher compared

with the 0 ppm group at 48 h, and the trend persisted until 2 w post-exposure (Figure 3a). Upregulated *gpx* expression was only observed in the skin 48 h after exposure (Figure 3e). *Gpx* was the only studied gene for which expression was modulated both in the skin and gills in experiment 1, implying a critical role for *gpx* in the antioxidant defence against increased ROS at mucosal surfaces, as noted in other fish studies [69, 70]. The gill expression of *mnsod* and *gr* was also modulated in the 2.4 ppm group after re-exposure, where the former displayed an earlier response while the latter exhibited a late response. Overall, the not so dramatic changes in the expression of the antioxidant genes in these mucosal tissues suggest that the tested PAA concentrations in experiment 1 were not able to elicit a strong mobilisation of the antioxidant response, which further suggests that the PAA-induced oxidative stress was not strong at the mucosa.

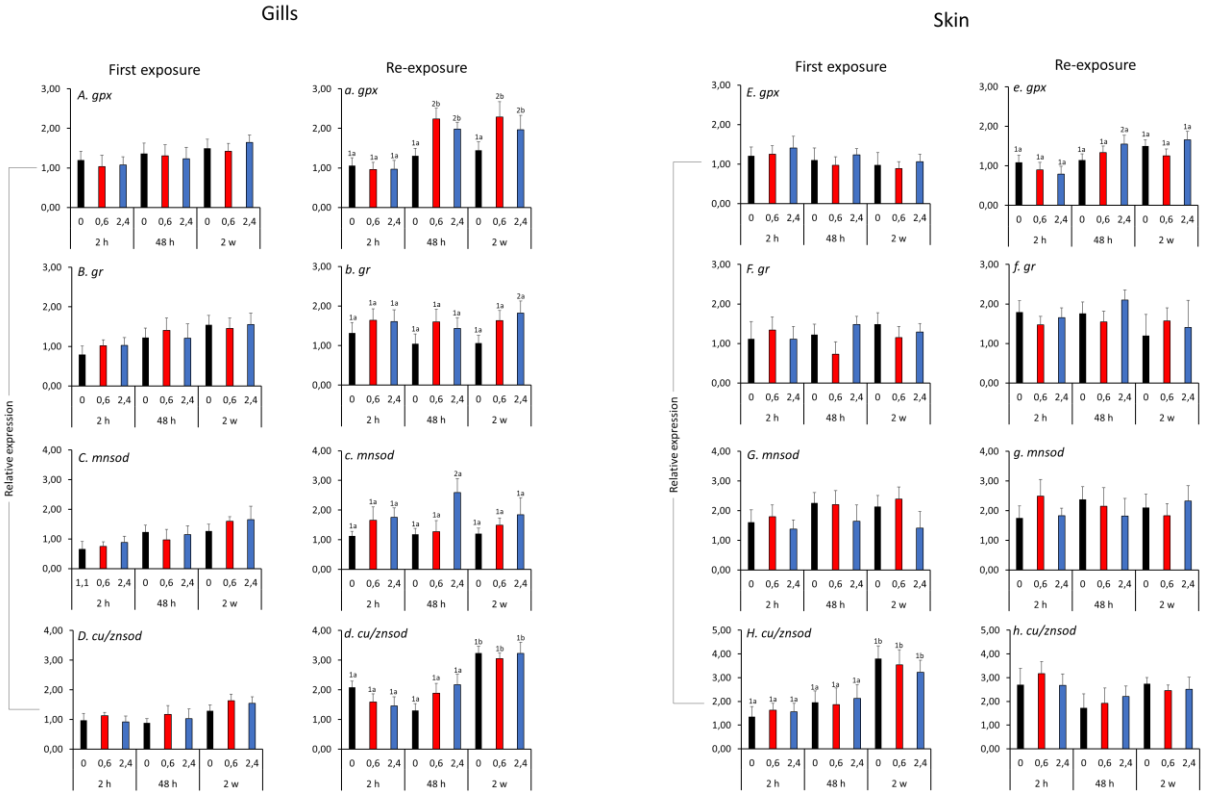

Figure 3. Expression profiles of antioxidant genes in the gills (A,a; B,b; C,c; D,d) and skin (E,e; F,f; G,g; H,h) of salmon from Experiment 1. Values are mean ± SE of eight individual fish. Different numbers signify significant differences between treatments within a sampling point, whilst different letters indicate significant differences within a treatment through time. gpx = glutathione peroxidase; gr = glutathione reductase; mnsod = manganese superoxide dismutase; cu/znsod = copper/zinc superoxide dismutase.

The gene expression profile in experiment 2 corroborated the results in experiment 1; that the antioxidant defence is more sensitive to PAA in the gills than in the skin (Figure 4). *Gpx* expression in the gills was significantly downregulated in no stress-PAA compared with the no stress-control 4 h after exposure (Figure 4.A). However, when stress status had been altered prior to PAA exposure, the level of *gpx* transcript in the gills was significantly higher in stress-PAA compared with stress-control, particularly in the early hours after exposure. The stressful episode can modulate the expression of *gpx* in salmon [49]. The elevated level of *gpx* expression in the gills of the stress-PAA group implies that stress prior to PAA treatment could increase the *gpx*-mediated antioxidant potential during oxidative stress. However, an opposite trend was an emblematic response in the expression of *gr* (Figure 4.B), *mnsod* (Figure 4.C), and *cu/znsod* (Figure 4.D) in the gills of fish subjected to stress prior to PAA exposure. The profiles revealed that gill transcription of antioxidant genes in the stress-PAA group was

significantly downregulated at 1 h (i.e., *gr*, *cu/znsod*) and at 2 w (i.e., *gr*, *mnsod*, and *cu/znsod*) after exposure compared with the stress-control group. In some cases, the level of *gr* and *cu/znsod* expression in the stress-PAA group was significantly lower compared with their counterparts in the no stress-PAA group. These conspicuous transcriptional changes in the antioxidant genes within the stress group illustrate that a stressful episode prior to PAA exposure interfered with the ability of gills to mount an antioxidative response during PAA-induced oxidative stress. There were no distinct overall transcriptional changes in the skin in experiment 2 and the random changes observed were related to the temporal dynamics of gene expression (Figure 4E–H). It appeared that the antioxidant markers were not strongly responsive to PAA, crowding stress, or their combination in the skin.

The overall results indicate that the antioxidant defence towards PAA was more responsive in the gills than in the skin. Moreover, fish with stress history prior to PAA exposure exhibited a different mucosal antioxidative response pattern to PAA compared with the non-stressed fish, highlighting the potential confounding and compounding roles of crowding stress in the antioxidant defence. The gills have a large surface area in contact with the water and are less structurally complex than the skin, which has multiple layers [71]. Moreover, PAA and its intermediate products have a low molecular mass that might be gill-permeable and diffuse into the fish [47, 48]. Therefore, this might explain, at least in part, the striking regulation of the antioxidant system in the gills relative to the skin.

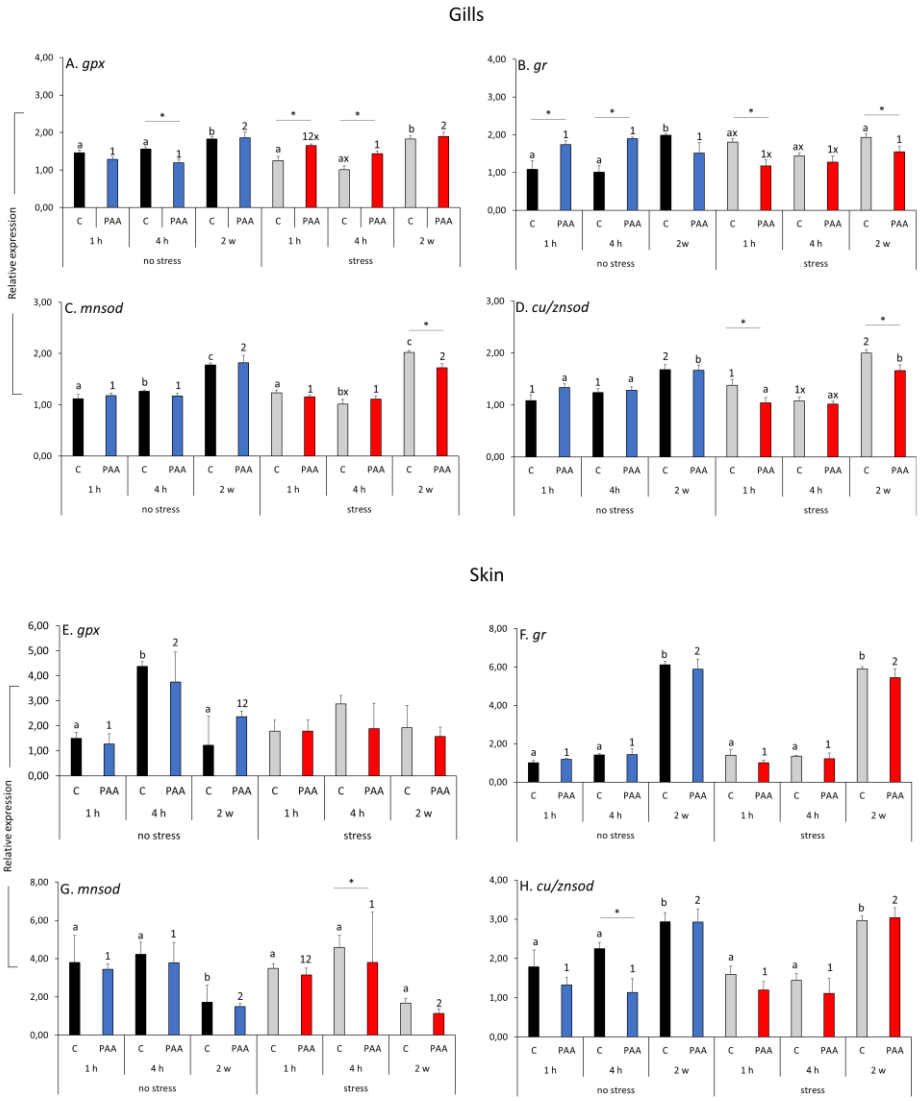

Figure 4. Expression profiles of antioxidant genes in the gills (A–D) and skin (E–H) of salmon from Experiment 2. Values are mean ± SE of eight individual fish. Different letters denote significant differences within control groups through time, while different numbers indicate differences within PAA exposed groups through time. The same notations are used for no stress and stress groups. x designates that the level of a particular group significantly differs between no stress and stress groups, whereas # indicates a significant difference between the control and PAA-exposed group at a particular time point

Conclusions

Peracetic acid is a disinfectant with great promise as a prophylaxis and chemotherapeutant in aquaculture. This study shows that salmon smolts attune their systemic and mucosal defences to counteract the physiological demands of the presence of PAA, a potential oxidative stressor, in the water. PAA likely triggered systemic oxidative stress, but salmon addressed the ROS imbalance by producing circulating antioxidants. The classical stress indicators in the plasma were affected by PAA. Previous exposure history to PAA did not dramatically interfere with the stress responses, and the fish were able to recover quickly after re-exposure. Crowding stress before PAA treatment, however, did influence some of the stress indicators, particularly the level of glucose and lactate. Mucosal antioxidant defences were also affected, where changes were prominently observed in the gills. There was a clear tendency that prior stress might interfere with the mobilisation of mucosal antioxidant defences under increased ROS. The results of the present study add valuable insights into the physiological

consequences of PAA exposure in salmon. The adaptive responses documented here reveal that PAA, though possibly triggering stress responses, can be used for salmon within the concentrated tested in the current study, with minimal physiological consequences, but attention must be given to confounding factors. Moreover, the data presented here have implications for the use of PAA as a routine disinfection in recirculating aquaculture system.

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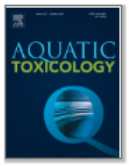

Oxidant-induced modifications in the mucosal transcriptome and circulating metabolome of Atlantic salmon

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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.

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 [18, 19, 72]. Nonetheless, chemotherapeutics remains the only available alternatives in some cases [73, 74]. 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 [75, 76]. It is imperative that an integrative approach is adopted for streamlining the use of chemicals (e.g., parasiticides) in aquaculture [31].

Peracetic acid (CH3CO3H, 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 [37, 38]. It is the peroxide of acetic acid and is commercially available in an equilibrium mixture with acetic acid, H2O2 and water. PAA has high oxidising potential and fat solubility [77], 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 [39, 78]. 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 the chemiosmotic function of the lipoprotein cytoplasmic membrane and transport through the dislocation or rupture of cell walls [27, 28]. PAA has stronger disinfecting power than H2O2 because of the former's fat solubility [29]. The combination of PAA and H2O2 is synergistic [30].

While earlier studies documented the potency of PAA against numerous pathogens and toxicity towards a number of farmed fish species [27, 39, 79], 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 [80, 81]. 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 [37, 47, 48]. 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 [82], reported in another salmonid species (rainbow trout) [80, 83, 84], and biocidal activity against *Neoparamoeba perurans,* the causative agent of AGD [85]. 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 [86]. 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.

Materials and Methods

Ethics statement

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

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 [81]. 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.

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 [81]. 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, 2020 #60}. 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, 2017 #61}.

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 [87]. 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 to 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 1,000 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.

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 [88]. 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.

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.* [89]. Data-processing was carried

out in MZmine 2 [90] 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.

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.

Results and Discussion

PAA exposure does not elicit aberrant behavioural responses

PAA is recognised as a strong irritant and lacrimator in mice [91], 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 [48, 83], indicating that the treatment did not interfere with the growth potential and metabolism of salmon, as supported in part by the metabolomics data.

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 [92]. 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 (Figure 1A), with composite scores below 1, on a scale of 0 to 3.

We further evaluated variations in the individual 11 external parameters in the scoring scheme (Figure 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).

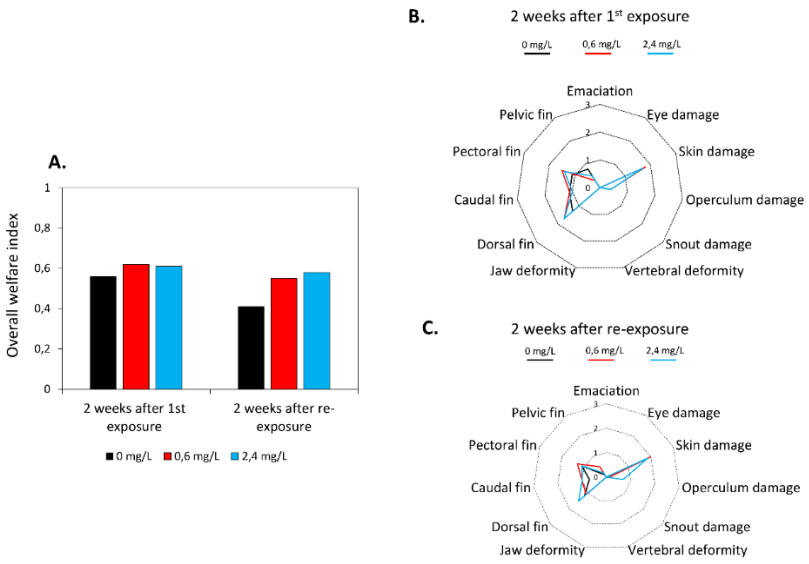

Figure 1. External welfare status of Atlantic salmon exposed to therapeutic doses of peracetic acid. A) The overall external welfare index of each treatment group based on the average composite score of 11 external welfare indicators, as presented individually in panels B and C.

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 [68]. 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 (Figure 2). 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) (Figure 2A). About 25 % of the DEGs in Cluster 1 are involved in mucosal tissue adhesion, differentiation, extracellular matrix and neural activity (Figure 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 [31, 80] and these radicals will eventually affect the oxidative state, triggering a cascade of immune effects to counter the physiological pressure [93, 94]. 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.

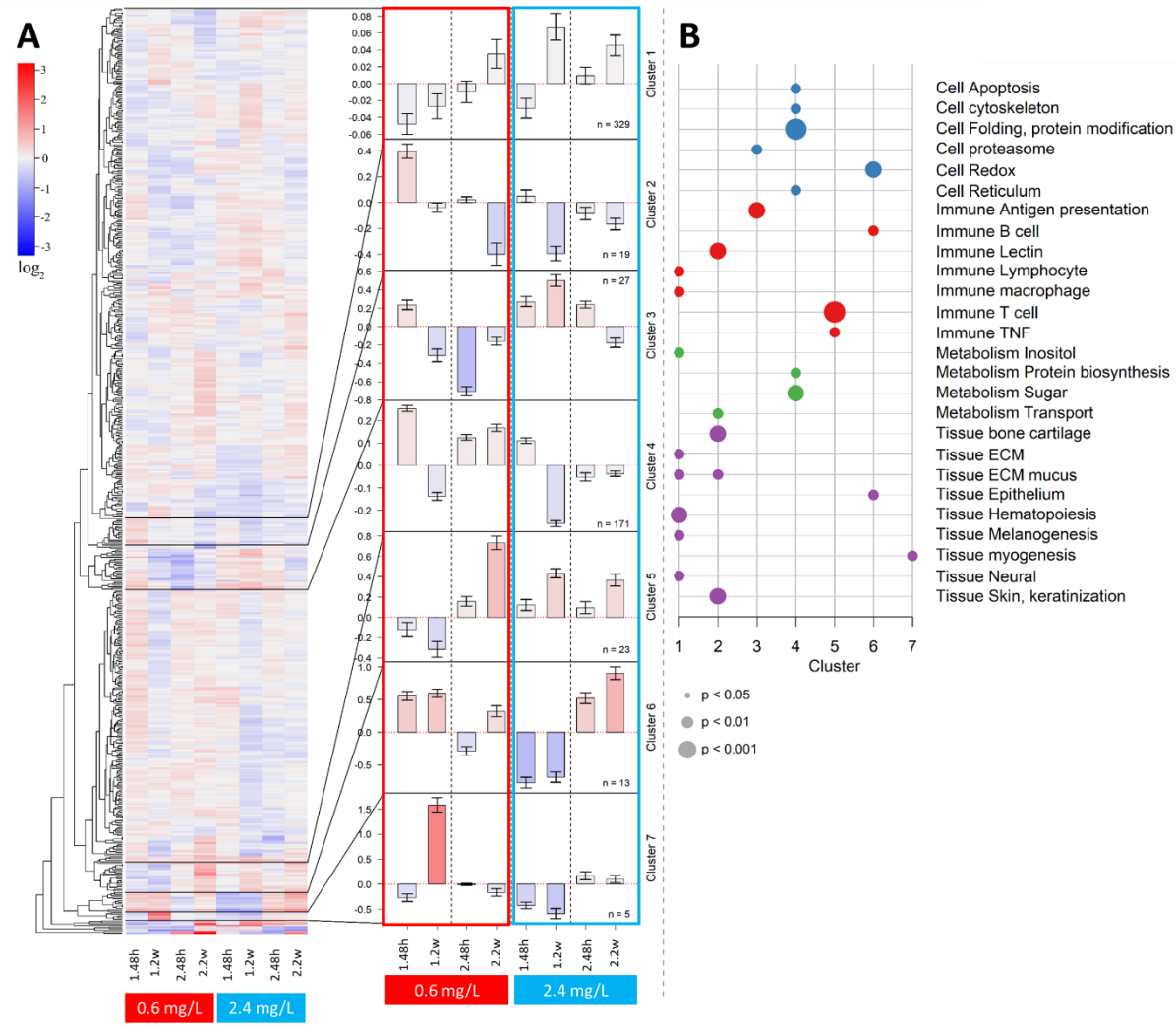

Figure 2. Gill transcriptome of Atlantic salmon exposed to therapeutic doses of peracetic acid. A) The heatmap on the left shows the down- and upregulation of DEGs in a colour gradient from blue to red. The dendrogram was split into seven sub-clusters and the mean values for genes within these clusters 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: 1.48h = 48 h after initial exposure; 1.2w = 2 weeks after initial exposure; 2.48h = 48 h after re-exposure; 2.2w = 2 weeks after re-exposure.

The second overly represented group is Cluster 4 with 171 DEGs (Figure 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 (Figure 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 (Figure 3). Moreover, the overall changes in the cutaneous expression in the PAA-exposed groups were considerably larger in magnitude compared with the gills. In terms of surface area to water contact ratio, the gills are larger than the skin [95]. 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 (Figure 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 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 (Figure 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 %) (Figure 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 [96, 97]. 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.

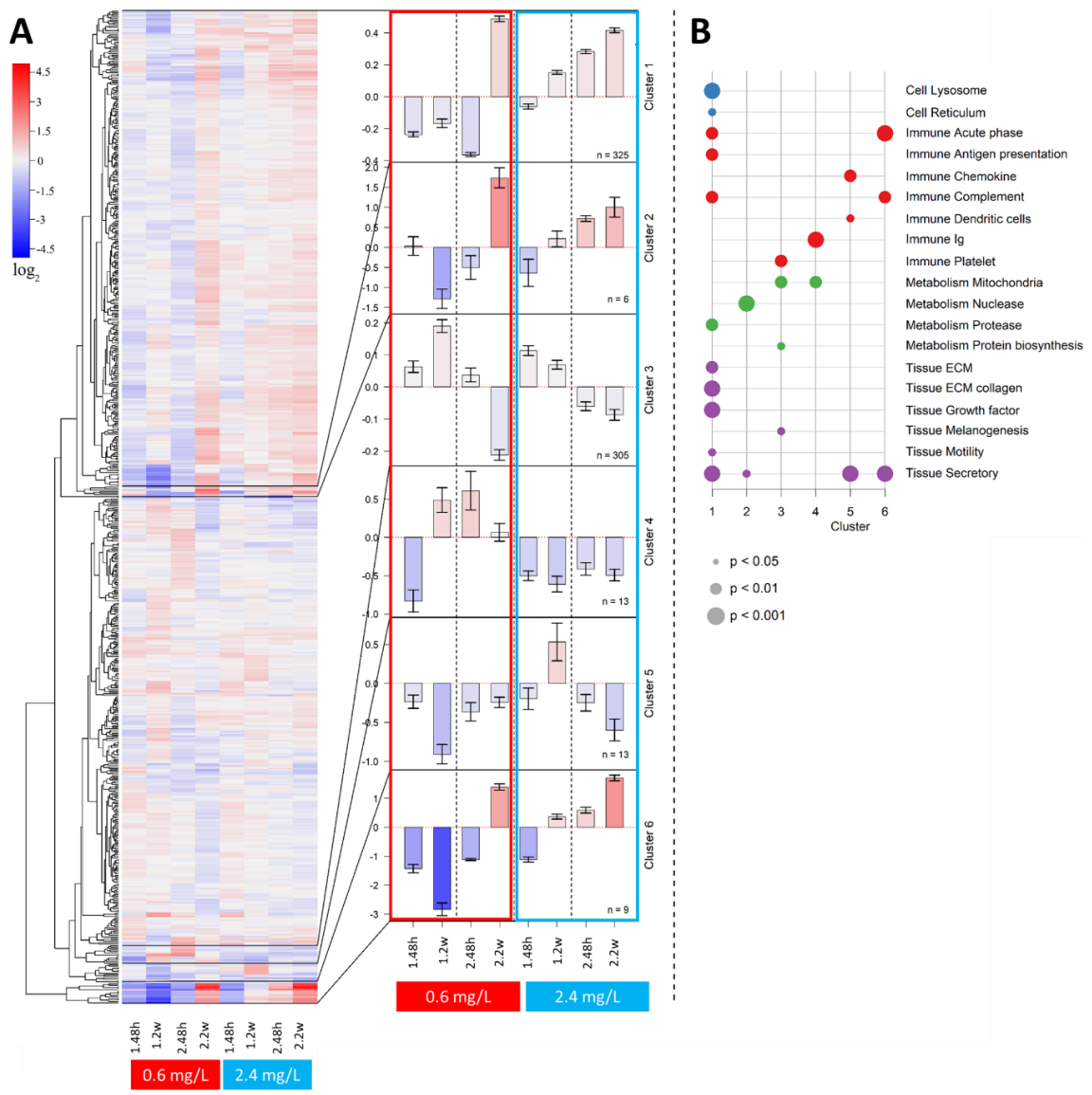

Figure 3. Skin transcriptome of Atlantic salmon exposed to therapeutic doses of peracetic acid. Analysis and representation for DEGs of skin samples were conducted in the same way as for the gills. For further information, see the caption of Figure 2.

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 [98]. 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 barrier mucosa after the treatments. *Spondin 1a* is also an epithelial mucus protein component [99] 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.

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*	↓↓	↑↑	↑↓	↓↑	↓↓	↑↑	↑↑	↑↑

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 [81]. 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 (Figure 4A) and the loading (Figure 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 (Figure 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 (Figure 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.

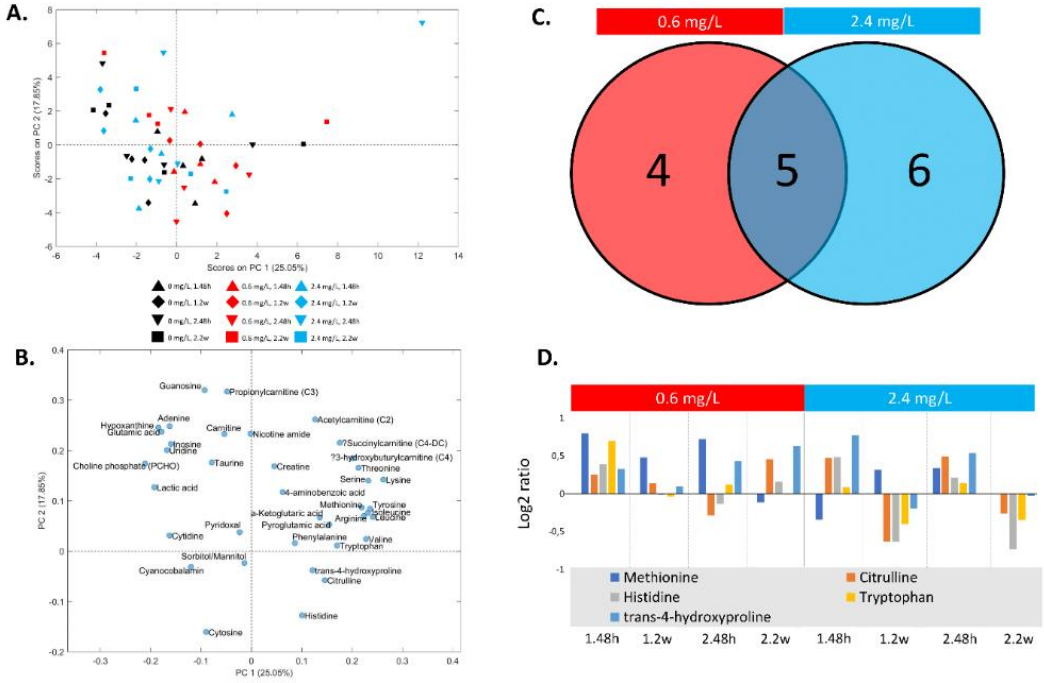

Figure 4. Plasma metabolome of Atlantic salmon exposed to therapeutic doses of peracetic acid. A) Score plot from the PCA model calculated from the relative concentrations of the variables in the identified compounds. Data are auto scaled. B) Loading plot from the PCA model calculated from the relative 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: 1.48h = 48 h after initial exposure; 1.2w = 2 weeks after first exposure; 2.48h = 48 h after re-exposure; 2.2w = 2 weeks after re-exposure.

Methionine (Figure 4D) is likely a key molecule in the response of salmon to low doses of PAA, elevated 48 h after each treatment. Methionine is an aliphatic, sulphur-containing, essential amino acid [100] that has been identified as a crucial regulator of the oxidative state in fish [101]. 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 [102]. 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 [103, 104]. 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 (Figure 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 [105]. 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 [106, 107]. 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 [81]. 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 [108, 109] 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 (Figure 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 [110]. 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 [111], which sheds light as to why PAA dramatically influenced the tissue extracellular matrix of the mucosa (Figures 2-3).

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 (Figure 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 [112]. Tryptophan deficiency is related to several physiological problems that have severe implications on fish growth [113]. 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.

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.

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

Publication 4

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Morphomolecular alterations in the skin mucosa of Atlantic salmon (*Salmo salar*) after exposure to peracetic acid-based disinfectant

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(A part of this publication was included in Sindre Haddeland's MSc thesis submitted to the University of Bergen)

ABSTRACT

Peroxygen-based chemotherapeutants are commonly used in treating ectoparasitic infections in fish. A majority of relevant studies address the effects of these substances on the targeted causative agents, but little is known about their physiological impacts on the host organism. This study documented the changes in the skin of Atlantic salmon exposed to peracetic acid (PAA), a peroxygen disinfectant with potent oxidative properties. Fish were exposed to three therapeutic concentrations of PAA-based disinfectant: 0, 0.6, and 2.4 ppm. The initial exposure time was 5 min, and two weeks after, fish were re-exposed to the same doses for 30 min. Skin colour was not dramatically affected by the PAA treatments. No gross pathologies, lesions, or wounds were observed in the sampled fish. Histological evaluation revealed that fish treated with PAA appeared to have rough epidermal surface compared with the 0 ppm group, especially after the re-exposure. Morphometrics of mucous cells did not markedly vary amongst the treatments, although the group treated with 2.4 ppm displayed a relatively larger mucous cells 2 weeks after the first exposure. Transcriptional analysis was conducted for key markers that were previously identified to be involved in the mucosal response to PAA, and the results revealed that proteolysis-related genes were modulated more remarkably during the first exposure than during re-exposure. These data revealed that therapeutic doses of PAA induced morphomolecular changes in the skin of salmon, although the magnitude of alteration was marginal.

An ideal therapeutant in aquaculture should not only be effective against the target agent but also it should not have inherently detrimental effects on the host organism or the environment. Many of the chemotherapeutants currently in use are effective against major aquaculture pathogens [19, 114, 115], but there remain substantial knowledge gaps in how they impact fish physiology, both short-term and long-term. One reason may be that the classical indicator of treatment success in aquaculture is the resolution of clinical signs of the disease, whereas the physiological responses of fish to the treatments are often not well documented. A better understanding of how fish respond to a treatment would provide opportunities for risk assessment, which would in turn, foster more sustainable use of chemotherapies in aquaculture.

Peroxygen-based disinfectants are frequently applied as bath chemotherapeutants in fish farming, and hydrogen peroxide (H_2O_2) is the most common one. These compounds are also referred to as oxidative biocides, and they influence cellular activity via different mechanisms, including peroxidation and disruption of membrane layers, oxidation of oxygen scavengers and thiol groups, enzyme inhibition, oxidation of nucleosides, impaired energy production, disruption of protein synthesis, and, ultimately, cell death [116]. Peracetic acid (PAA) a peroxygen compound with strong oxidative potential, is currently gaining popularity as a sustainable disinfectant in aquaculture because of its potency at lower concentrations and rapid degradation to neutral compounds [31, 38, 117]. Commercial PAA-products contain PAA, H_2O_2, water and acetic acid in an equilibrium mixture. Moreover, it has a broad spectrum of biocidal activity against pathogens that present significant challenges in fish farming, including *Ichthyophthirius multifiliis*, *Aeromonas salmonicida*, *Saprolegnia sp.* and *Flavobacterium columnare*, to name a few [118, 119]. However, physiological studies on PAA use in fish are limited.

At present, PAA-based disinfectant is being explored as an alternative therapeutant for amoebic gill disease (AGD), which is an ectoparasitic infestation in Atlantic salmon [120]. Earlier reports showed that PAA exposure resulted in minimal physiological alterations, and exposed fish were able to mount appropriate responses to the biological pressures of PAA [37, 81, 121]. However, our current understanding is incomplete, especially regarding how the skin mucosa—one of the barrier surfaces that directly interacts with the compound—responds to the presence of the oxidative compound. Thus, we report on how the skin of Atlantic salmon (*Salmo salar*) responded to different therapeutic doses of PAA by investigating histo-structural alterations and molecular responses following two exposures.

Salmon smolts (weight: 150.3 ± 5.6 g, mean ± SE) were purchased from Danish Salmon A/S (Hirtshals, Denmark) and reared in the aquaculture recirculation facility of DTU Aqua in Hirtshals. Sixty fish were stocked in each of the six 1-m^2 holding tanks (water volume≈600 L) with full-strength seawater (33 ppt) and were allowed to acclimate to laboratory conditions for three weeks. Water temperature was maintained at 15 ± 1 °C, dissolved oxygen at >80 % saturation and pH at 7.5-7.7. Fish were exposed to three different therapeutic doses of PAA: 0, 0.6, and 2.4 ppm. The chosen test concentrations were based on efficacy against major aquaculture pathogens [82], earlier applications in another salmonid species (rainbow trout) [80, 83, 84], and *in vitro* inhibitory activity against *Neoparamoeba perurans,* the causative agent of AGD, where PAA is being developed as a potential treatment [85]. PAA-based disinfectant (Divosan Forte™) was provided by Lilleborg AS (Oslo, Norway). The disinfectant is a stabilised PAA solution, and the actual concentration of PAA in the product was determined by a two-step titration at the DTU Aqua Laboratory [117]. Each treatment had two replicate tanks. For the first exposure, fish were exposed to PAA for 5 min, and two weeks after, the groups were exposed again to the same concentration but for 30 min. Briefly, fish from a holding tank were transferred to an exposure tank with similar water conditions and technical specifications. Water flow was stopped and PAA was added to the water to achieve the desired nominal concentration, and aeration was provided to facilitate mixing for the duration of each exposure (additional details of the trial are described in Soleng *et al.* [81]). After the exposure period, fish were returned to the holding tanks for recovery. For the chemical

behaviour of PAA in the water during the trial, including its degradation through time, readers are referred to [117].

Prior to tissue collection, fish (5 fish per replicate tank; starved for 24 h prior to sampling) were humanely euthanised by an overdose of 20 % benzocaine solution, and the whole body of each fish for sampling was then photographed in a box with controlled light conditions (Canon EOS 60S, manual settings f/11, 1/8s, ISO200, 23 mm). Individual photos were processed with an R-script to crop out an image of the skin in the centre of the fish with a width of 600 pixels and spanning over 60 % of the height of the body. The cropped pictures of skin were analysed by calculating the mean intensities for red, green and blue (RBG).

Skin samples from the dorsal region (~8 cm x 4 cm) were collected for histological and qPCR analyses. A small portion of the skin was suspended in RNAlater® (Sigma-Aldrich, USA) and left overnight to allow for penetration at room temperature followed by storage at -70 ^{o}C until RNA isolation. The remaining skin analyses focused on the mucosal epithelium. The skin sample was cut in two, with one half for Quantidoc's standard mucosal cell mapping (giving 1-2 square cm of surface area using tangential sectioning), and the other half for histological skin health scoring at Nofima (traditional transverse sectioning giving about 2 square microns of surface area). Both samples were preserved in neutral buffered formalin.

For Quantidoc skin samples, the tissues were embedded in paraffin, sliced tangentially into 3-µm-thick sections, and stained with Periodic Acid Schiff-Alcian Blue (PAS-AB) according to Quantidoc's protocol [122, 123]. All samples were scanned by a Hamamatsu slide scanner to obtain high-resolution digital images (NDPI format). Mucosal mapping with Veribarr™ and Mucomaster™ were used to analyse and estimate the volumetric density and the mean area of mucous cells in the skin mucosa. The mean area and volumetric density were used to calculate the barrier status of the mucosal epithelium as 1/(Area/Density)*1000, which indicates the quality of the barrier tissue.

Samples for skin health scoring were processed and photographed as described previously [124]. The section was scored by an impartial evaluator (no prior knowledge of sample treatment) using a 0 to 3-point system, with 0 indicating healthy skin and 3 indicating severely damaged conditions. Two key epidermal features were characterised: general appearance of the epidermis and epidermal surface characteristics (Supplementary File 1). Skin colour analysis and both histological characterisations were performed using samples collected 2 weeks after each exposure. We earlier reported that the effects of PAA on selected stress parameters were still persistent after 14 days post treatment [81], hence, the results here will offer additional insights into the short-to-mid-term effects of PAA.

The RNA isolation, complementary DNA (cDNA) synthesis, and quantitative polymerase chain reaction (qPCR) assay were described in detail in a previous publication [81]. Briefly, RNA from skin samples was isolated using Agencourt® RNAdvance™ Tissue Total RNA Purification Kit (Beckman Coulter Inc., CA, USA). Total RNA was reverse transcribed to cDNA using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) from a 200-ng RNA template. Quantification of the gene transcript by real-time qPCR was performed with a QuantStudio 5 Real-Time PCR system (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems). Ct values were generated using a threshold fluorescence of 0.1, and the relative gene expression was calculated by the delta-delta Ct method [125]. The genes measured included *haptoglobin-like* (*hpl*), *complement factor-like H* (*cfhl*), *chymotrypsin* B (*ctrb*), and *trypsin II* (*trp-ii*), previously identified from a microarray data set as being responsive to PAA treatment [120]. *Elongation factor 1 alpha* (*elf1a*) was used as an internal control. Primer sequences are provided in Supplementary File 2. Skin samples collected at 48 h and 2 weeks after each exposure were used for gene expression analysis.

PAA exhibits bleaching power [126], which might present potential side effects when salmon are bathed in this compound. Regardless of the treatment dose and duration, we found that skin colour (individual RBG and mean RBG) was not significantly affected by PAA except a transient response of the blue channel at two weeks after the first exposure, though not after the second exposure (Figure 1). The blue colour is a product of blue iridophores reflecting light and black melanophores absorbing light across the spectrum [127]. The blue colour of salmon skin that was exposed to 2.4 ppm for 5 min was significantly lighter than that of the other two treatment groups, suggesting either expansion of the iridocytes or reduction of melanocytes, as well as an interaction between skin colour and the stress response [128]. Although PAA treatment may have some colour-bleaching effect, the fish recovered, and re-exposure did not exacerbate the impact, suggesting that the response was likely an acute adaptation to the PAA bath treatment.

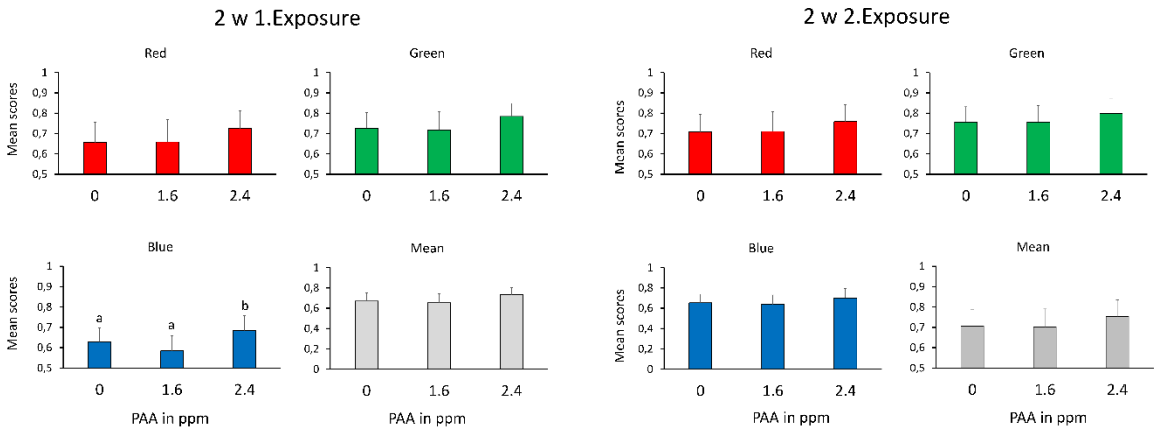


Figure 1. Skin colour analysis of PAA-exposed fish 2 weeks after each exposure. Fish were exposed to 3 PAA doses: 0, 0.6 and 2.4 ppm. Each fish for sampling (n = 10 fish per treatment group) were photographed, processed and the values are presented as mean score (mean ± SD) of individual colour panel (Red, Blue, Greed = RBG) as well as the overall RBG mean value of the picture. A higher value represents lighter/brighter colours, a lower value indicates a darker colour. Different letters indicate significant inter-treatment differences at P < 0.05 as inferred by one-way ANOVA followed by Holm-Sidak test.

No external skin lesions or wounds were observed on the fish sampled, although scale loss was prominent in all treatment groups, which can be ascribed to handling during exposure [120]. Traditional histopathology of the skin did not identify any major pathological alterations. Skin health scoring, which mainly evaluated the quality of surface structure revealed some minor changes following treatments (Figure 2A, B). The scores for general appearance were between 1-2, indicating parts of the epidermis were missing, for groups treated with PAA at 2 weeks after the second exposure (Figure 2D, E). However, there were no significant inter-treatment differences. Both the 0.6 and 2.4-ppm groups had scores of 1-2 in their epidermal surface appearance, indicating rough cells at the surface, and around 50 % of the evaluated epidermal surface was structurally compromised. It is not clear whether this cutaneous result after treatment is worsened by repeated exposure since we only examined 2 exposures.

The dynamic behaviour of mucous cells following treatment and in response to different environmental stimuli has clinical significance and have thus been used to characterise mucosal health in skin, gills and intestines [122, 123, 129]. The mean mucous cell area amongst the groups were 188.71±38.9 μm^2 at 2 weeks after the first exposure, while for the second exposure it was around 190.50±16.8 μm^2 (Figure 2C, F), which were within the normal range for skin mucous cells in this fish size (Quantidoc database). Though there was an apparent tendency that mucous cell area increased in size at increasing PAA concentration during the first exposure, there were no significant inter-treatment differences. Interestingly, an opposite tendency was documented during the second exposure,

nonetheless, the changes remained not statistically significant. The mucous cell density did not significantly vary amongst the treatment groups, either 2 weeks after the first or second exposures. Barrier status is a mathematical representation of the barrier quality of the epithelial surface as a function of mucous cell size and volumetric density. PAA treatments did not significantly affect the skin mucosal barrier status of salmon (Figure 2G), corroborating the skin health scoring (Figure 2D, E). The two methods – qualitative (*skin health scoring*) and quantitative (*mucosal mapping*), revealed the minimal changes associated with PAA exposure at therapeutic doses, thus indicate that the treatments did not compromise the quality of the skin epithelial layer.

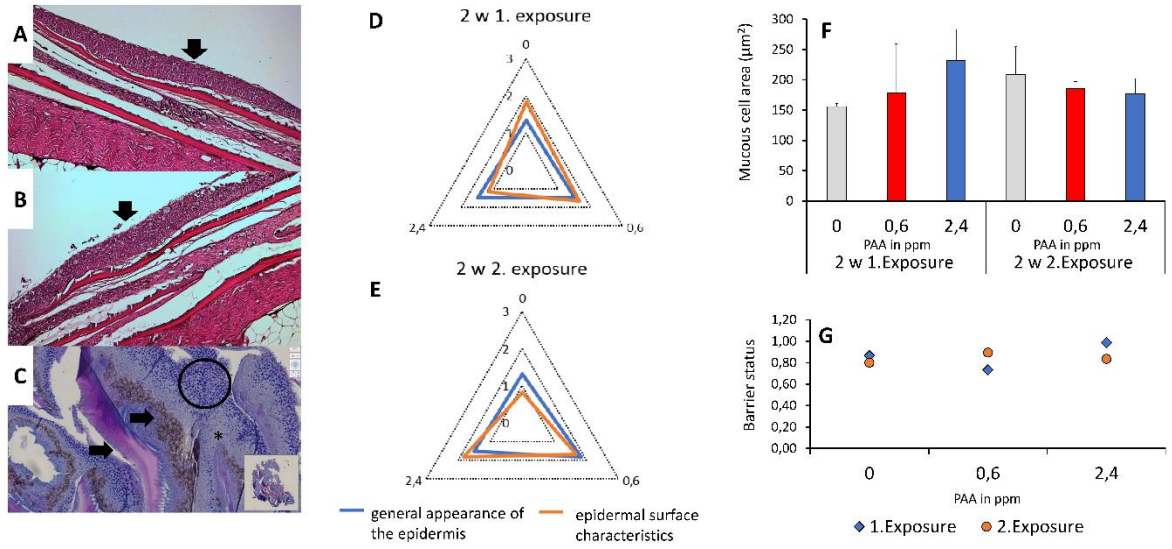


*Figure 2. Skin histomorphometry of PAA-exposed fish 2 weeks after each exposure. A-C: Representative photomicrographs of salmon skin exposed to PAA. A,B: Traditional cross-section of the skin stained with haematoxylin and eosin. A: Healthy-looking epithelium with a smooth surface and well-defined structure (arrowhead), typical in 0 ppm group; B: Moderately compromised epithelium with a rough surface (arrowhead), common in fish exposed to 2.4 ppm PAA. C: A tangential section of the skin from 0 ppm group, stained with AB/PAS to reveal the mucous cells (dark, blue-coloured cells, encircled). Black = pigment (arrowhead), pink = scales (arrowhead), medium blue = epithelium (asterisk, *). A typical appearance of skin with good barrier status as indicated by the area and volumetric density of the mucous cells.* ***D, E:*** *Radar charts showing the quality of skin epithelial surface as scored 0 to 3, with 3 as the worst.* ***F, G:*** *Mucosal mapping of the skin surface.* ***F:*** *Mean mucous cell area (mean±SD) and* ***G:*** *Barrier status, a mathematical representation developed by Quantidoc AS for the quality of mucosal barrier as a function of mucous cell area and density. No significant inter-treatment differences were identified by one-way ANOVA followed by Holm-Sidak test (P < 0.05).*

A global transcriptomic study on salmon identified several gene markers for PAA response in the skin [120], and the expression of four of them was quantified by qPCR in this study. In general, the transcription of marker genes in the skin was significantly modulated when fish were exposed to 2.4 ppm of PAA. In a previous study, fish exposed to the PAA concentrations used in the present study had elevated levels of systemic total antioxidant capacity, indicating that oxidative stress responses had been activated [81]. Besides their well-documented role in inflammation, *haptoglobins* are acute-phase proteins that participate in anti-oxidant response [130]. *Hpl* transcript level was significantly elevated in both PAA-exposed groups relative to the control group only at 2 weeks after the second exposure (Figure 3A). This profile suggests that *hpl* may not likely be involved in the acute response but rather during the physiological responses involved in the recovery from PAA exposures.

The mucus-secreting cell layer of salmon skin produces trypsin [131], which has been identified to be involved in the defence repertoire of the epithelial surface [132]. Two trypsin genes were identified to be responsive to PAA, *chymotrypsin B* (*ctrb*) and *trypsin II* (*trp-ii*) [120], and their expression in the present

study revealed that they may be more responsive during the first exposure than during the second exposure with longer duration (Figures 3B, C). Significantly elevated transcription was observed for *trpii1* in both PAA-exposed groups 2 weeks after the first exposure, while significantly higher expression of *ctrb* was observed 48 h after the first exposure, but only in the 2.4-ppm group.

Proteolytic responses are an important secondary defence against oxidative stress by destroying oxidised and damaged proteins, thus preventing intracellular accumulation [133]. It was interesting to observe the modulated responses from the two trypsin genes during the first exposure since a published companion study showed that the systemic alteration of oxidative stress was more pronounced after the second exposure [81]. Although both genes have other functions, their known involvement in oxidative stress allowed us to hypothesise that *trpii* and *ctrb* are very sensitive to PAA and might have active roles in the proteolysis-mediated response to protect the mucosa from acute PAA-induced oxidative stress. Their unresponsiveness following the re-exposure suggests that secondary exposure to PAA did not have an additive effect, and the mucosa recognised the signal as safe, at least in the proteolytic system. *Cfhl* did not exhibit significant inter-treatment variations, but temporal variability was apparent (Figure 3D).

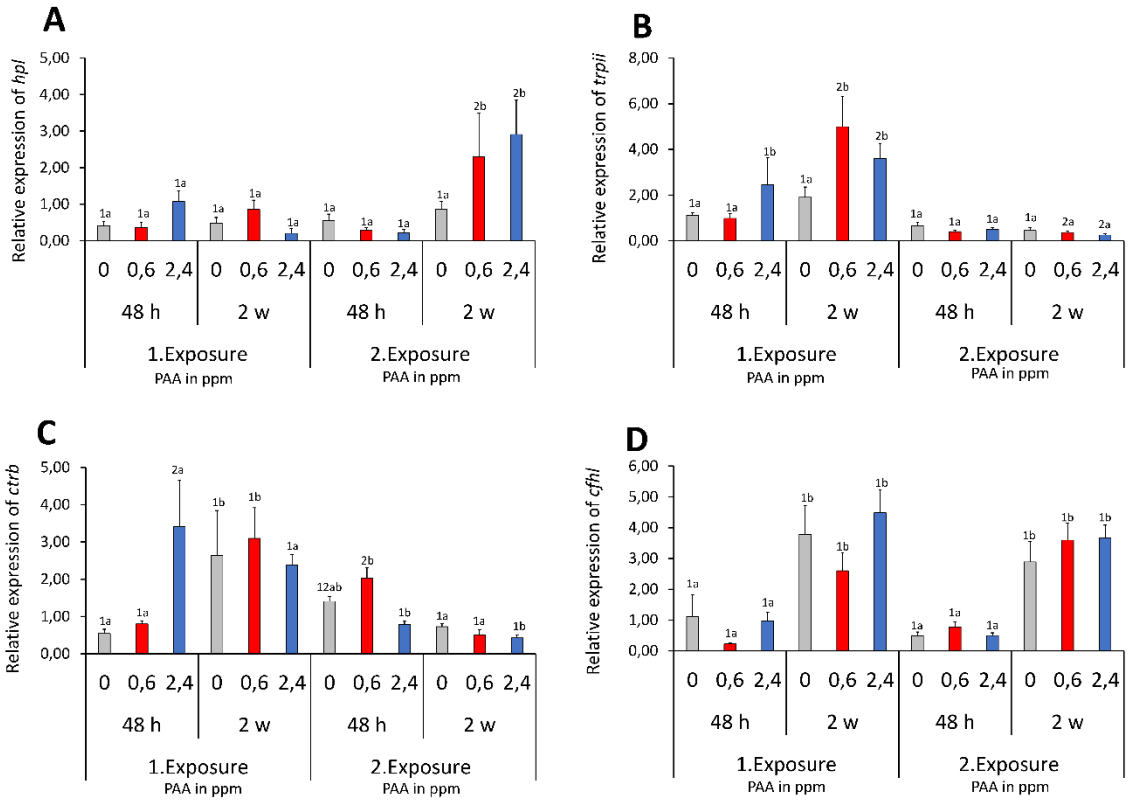
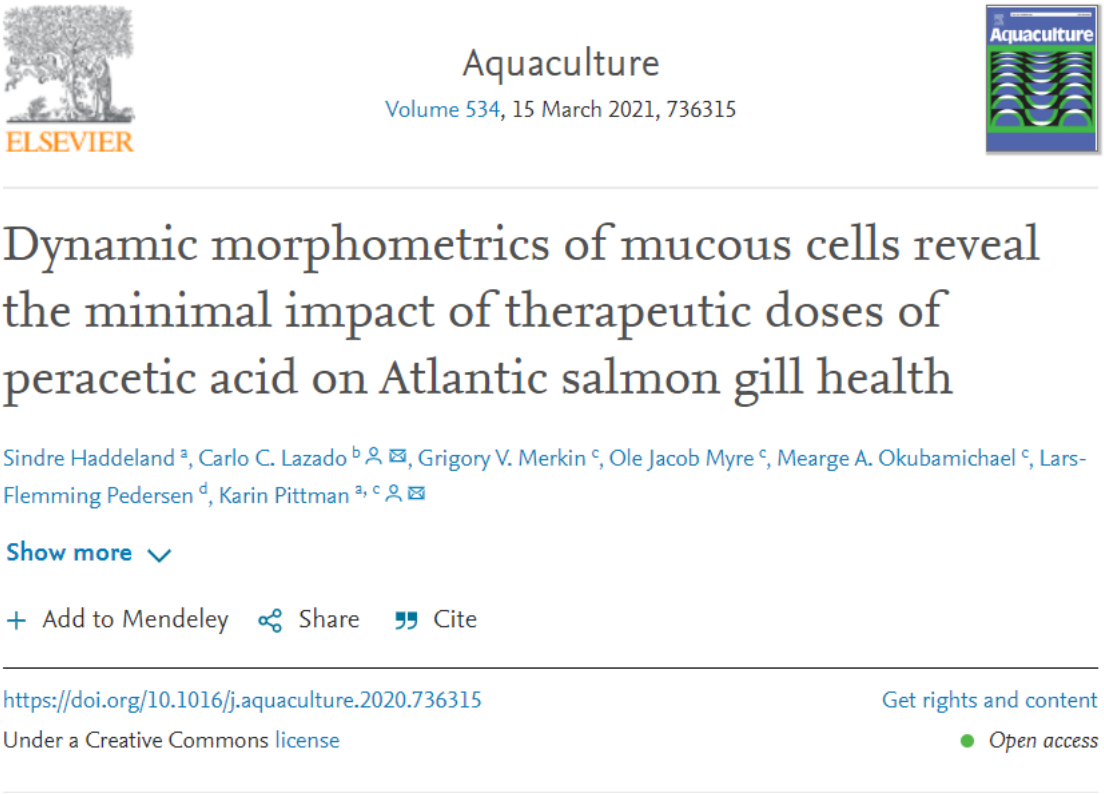


Figure 3. Gene expression profiles in the skin of salmon exposed to different levels of PAA (0, 0.6, 2.4 ppm), either for 5 min (1. Exposure) or 30 min (2.Exposure). Values are mean ± SEM of eight individual fish. Two-way ANOVA (P < 0.05) followed by Holm-Sidak test was used to identify differences between treatments and sampling points. Different numbers signify significant differences between treatments within a sampling point, whilst different letters indicate significant differences within a treatment through time. A: haptoglobin-like (hpl); B: trypsin II (trpii); C: chymotrypsin B (ctrb); D: complement factor-like H (cfhl).

In conclusion, the results of the present study indicate that the skin of salmon responded, though minimally, to varying therapeutic concentrations of PAA-based disinfectant. There were no pronounced and severe histostructural changes (i.e., surface quality and mucous cell morphometrics) after the exposures. In addition, the expression of four PAA response marker genes revealed no general

tendency though provided some indications that PAA exposure can differentially impact their transcription. Collectively, the magnitude of alternations related to PAA response in terms of skin colour, histostructure and gene expression was marginal, hence, substantiating further the earlier evidence that PAA does not present a significant challenge to the mucosal health of salmon in the tested concentration range. Since PAA-based disinfectants are available commercially in different strengths, composition and stabilisers, the changes documented here are to be considered for the tested product only. It would be interesting to compare different commercial PAA products to benchmark the responses of salmon skin to peracetic acid.

Publication 5



(This is part of Sindre Haddeland's MSc thesis submitted to the University of Bergen)

ABSTRACT

Mucous cells, the microscopic structural hallmark of mucosal surfaces, are highly responsive to environmental changes. Here we report how the gills of Atlantic salmon (*Salmo salar*) responded to peracetic acid (PAA), a potent oxidative disinfectant and a candidate chemotherapeutant in aquaculture, through the mucosal mapping showing mucous cell size, volumetric density and defence activity, coupled with two-way histopathological scoring strategies. Two hundred and forty smolts were exposed to therapeutic doses of PAA on two occasions. The initial exposure included a 5-min bathing at concentrations of 0, 0.6, 1.2, and 2.4 ppm PAA. After a two-week recovery, the treatment groups were re-exposed to the same PAA concentrations for 30 min. Gill samples were collected at 2 hours, 2 days, and 2 weeks after each exposure. The dynamic changes (i.e., size, volumetric density and defence activity) of the mucous cells were analysed on the distinct mucous cell populations in the gill filament and the lamella, as well as the lamellar thickness. Lamellar mucous cells were always significantly

smaller (<70 µm^2) and less dense (<2 % volume) than those in the filament (70-100 µm^2 and 8-11 % volume) giving defense activities of 0.1-0.4 for lamellae and about 0.6 – 1.4 for filaments, consistent with the functions of these branchial areas. A transient sub-acute mucous cell hypertrophy was a striking response in the gill lamella to PAA during the initial and re-exposure, particularly demonstrated by the groups exposed to 0.6 and 1.2 ppm where this size change was significant. Nonetheless, the recovery was quick, suggesting more an effect of general stress than dose of PAA. Similarly, a transient reduction in hyperplasia was noted as mucous cell density decreased on the filament, but generally significant hyperplasia was not detected, and volumetric density remained unaltered regardless of treatment doses and duration of exposure. The defence activity (combination of mean mucous cell area and volumetric density in a given tissue) of the lamella and the filament demonstrated a transient tendency to decrease after the initial exposure but was minimally affected by re-exposure. Lamellar thickness was not markedly affected by the highest PAA dose and overall was positively correlated with mucous cell size. The results from two independent histopathological scorings revealed that at least 93 % of the evaluated gill filaments per fish were categorised as healthy. Taken together, mucous cells in the gills of salmon responded with transient hypertrophy of mucous cells to therapeutic doses of PAA however the impact was minimal, and the mucosal morphometrics were in agreement with the marginal alterations in tissue structure and integrity. The data suggest that the PAA doses used in the study are safe for salmon and do not pose substantial impact on gill mucosal health.

Introduction

Mucous cells, the microscopic structural hallmark of mucosal surfaces, are highly responsive to environmental changes and are especially relevant to the health of the multifunctional gills [134]. Mucosal epithelia have protected aquatic organisms for about half a billion years [135] and constitute the primary barrier against pathogens and other harmful compounds and stimuli [68]. While the gill structure can be remodelled in response to a variety of impacts, histological indicators of gill disease highlight hypertrophy and hyperplasia of the epithelial and mucous cells [134, 136]. The convoluted 3D structure of the gill has been resistant to simple histological measures but the advent and application of mucosal mapping, a design-based stereological method [122, 123] provides an unbiased, standard and universally applicable measure of mucosal epithelia in skin and gills that is highly sensitive, represents many fold more surface area, is efficient and suitable for ecotoxicological studies as well as trials on stressors and diets [122, 123, 129, 137, 138].

The gill surface is estimated to 0.1–0.4 m^2/kg body weight, representing the largest organ-specific surface interacting with the environment and constitutes approximately 50 % of the total surface area of the fish [95, 139]. The two segments of the gill, filament and lamellae, have distinct mucous cell populations and discrete responses, with the filament having larger and denser cells and the respiratory surface of the lamellae having fewer and smaller mucous cells (Dang et al. 2019, 2020). Gill tissues react quickly to unfavourable environmental conditions [140] and lesions on the gills are visible ahead of behavioural changes [141]. Hence, the rapid and sensitive reaction to external changes makes gills an ideal organ for fish health monitoring [142] and for ecotoxicological studies [137, 143].

Stressors, such as therapeutically employed oxidants, are frequent in commercial fish farming and may have both short- and long-term effects on the remodelling of basic gill structures. Peracetic acid (PAA, CH_3COOOH) is a highly reactive peroxygen compound with a broad antimicrobial spectrum [38]. It is considered a promising aquatic disinfectant with low environmental risk because of the absence of persistent toxic or mutagenic residuals and byproducts, minor dependence on pH fluctuation, and short contact time to deliver a potent action [28]. It is commercially available as an equilibrium mixture with acetic acid, hydrogen peroxide, and water [31]. PAA produces hydroxyl radicals (•OH) and “active” oxygen from the photolysis upon decay and they are known as powerful oxidizing agents. These substances play a central role in the antimicrobial activity of PAA. Elevated levels of environmental

radicals may pose health and welfare issues as they can cause oxidative stress [81, 144], the imbalance between the level of oxidants and the ability of the organism to sequester the radicals [145]. Oxygen metabolites have been documented to impact the physiology and morphology of mucous cells in both mammalian [146, 147] and fish [148, 149] models, however, objective quantification of their response is currently lacking.

PAA is currently being explored as a candidate chemotherapeutant for amoebic gill disease in Atlantic salmon [81]. Here we investigate the consequences of repeated therapeutic doses of PAA and apply Mucosal Mapping to evaluate the gill tissue response in filaments and lamellae of salmon smolt.

Materials and Methods

Ethical statement

The experimental trial was performed in accordance with national and EU legislation (2010/63/EU) on animal experimentation.

Fish and acclimation

Atlantic salmon smolts (*Salmo salar*) (average weight 150 ± 9.5 g; mean ± standard deviation) were purchased from a local supplier (Danish Salmon, Hirtshals, Denmark) and transported to the recirculation aquaculture facility of DTU Aqua (Hirtshals, Denmark). Sixty fish were stocked to each of the 8 1-m^2 holding tanks (volume approximately 600 L, density *ca* 15 kg/m^3) in a seawater recirculating aquaculture system. The fish were acclimated for 3 weeks under the following laboratory conditions: temperature – 15±1 ^oC; dissolved oxygen – 80-90 % saturation; salinity – 33-34 ppt; photoperiod – 16L:8D (0600-2200). The fish were fed (Biomar, EFICO Enviro, 4.5 mm) at a ratio of 1-1.5 % total biomass per day which was administered via a belt feeder.

Peracetic acid exposure

Fish from the holding tanks (8) were transferred to their corresponding exposure tanks (8), with similar conditions (i.e., density, volume, water quality). After 10 mins, peracetic acid-based disinfectant (PAA; Divosan Forte™, Lilleborg AS, Norway) solution was added with aeration to achieve the following final concentrations in duplicate tanks: 0 (seawater), 0.6, 1.2, and 2.4 ppm. After an exposure to these doses lasting only 5 mins, fish were returned to their original holding tanks. Feeding was resumed two days after the PAA exposure. Fish recovered for two weeks, after which the fish were re-exposed to the same PAA concentration for 30 min. Post exposure the salmon were again kept for 2 weeks in the original tanks before final sampling. Additional details of the trials were described in earlier publications [81, 144].

Sample collection

Sample collection was performed prior to exposure, and at 2 h, 2 days and 2 weeks after each exposure experiment. Five fish were taken from each replicate tank and were humanely euthanised with an overdose of 20 % benzocaine solution. The length and weight were measured, and an external evaluation of fish appearance was done before invasive sampling. For this study, the second gill arch from the right gill was carefully sampled from each fish, placed in a labelled histocassette and preserved in 10 % neutral buffered formalin (Sigma-Aldrich, Darmstadt, Germany).

Sample processing and Mucosal Mapping analysis

The gill samples were processed for evaluation following Quantidoc's standard Mucosal Mapping protocol. Briefly, gills were dehydrated, embedded in paraffin, sliced *tangentially* in 3 µm sections and

stained with PAS – Alcian Blue. Stained sections were digitally scanned by NanoZoomer 2.0-RS, Hamamatsu Photonics K.K. (Japan) to high-resolution NDPI image format. Mucosal mapping was done according to Pittman *et al.* [122, 123] with calibrated dedicated semi-automatic software developed for stereological image analysis of mucosa by Quantidoc AS (Veribarr™ and Mucomaster). The volumetric density in % of mucous cells in the epithelium (D) and the mean area of mucous cells at the equator (µm²; A) on the gill filament (GF) and lamella (GL) were analysed and used to calculate the defence activity of the mucosal epithelia, or how the amount of mucus cells has changed, in each tissue according to the formula: 1/(A/D)*1000. These variables of volumetric density and defence activity are necessarily distinguished from the unfortunately misleading numerical density commonly used and the simple counts of cells as a variable. The application of design-based stereology recognises the complex 3D structure of gills and compensates for the 2D limitations of a histological slide.

The mean width of the lamellae gives some indication of the diffusion distance (the distance oxygen travels from water to blood) [150]. This was measured on 20 random locations in the lamellae of gills in the control and 2.4 ppm PAA groups using VIS image analysis software (Visiopharm, Hoersholm Denmark).

Semi-Quantitative histopathology

Gill sections were evaluated by two methods and two independent groups of evaluators. First, histopathological case scoring was performed following a previously published strategy [151], with modifications [143]. The evaluation was carried out by randomly selecting five locations in a gill section (i.e., 2 upper half, 2 lower half and 1 middle of the whole gill section). A total of 100 lamellae were evaluated per fish. Cases of clubbing, lamellar fusion, hyperplasia, hypertrophy, lifting, hyperaemia, aneurysm and necrosis were documented. Lamellae that did not show any histopathological changes as described above were denoted as “healthy”. The second strategy involved the assessment of histopathological alteration index (HAI) following the method of Poleksic and Mitrovic-Tutundzic [140] where gill lesions are given a score according to the severity of the alteration. Both strategies were performed by blind evaluation.

Statistics

A Linear mixed effect model (lme) was used to test differences in mean area and defence activity as well as lamellar thickness at each sampling time (R studio, Massachusetts, USA). A quasi-generalized linear model (GLM quasi) was used to compare the difference in volumetric density between treatments. A Pearson correlation test was done for mucous cell area and lamellar thickness with significance set at $P \leq 0.05$.

Results

There was no recorded mortality during the trial and no significant differences amongst the treatment groups in length, weight, and K-factor. Unfortunately, the samples taken prior to PAA exposure were subject to transport and storage problems and could not be used for this analysis (Soleng et al 2019). Statistical comparisons are therefore made to the control group and between treatments.

Mucous cell morphometry in the gill lamella (GL)

GL mucous cell area

The mucous cell area on the lamella varied from 27.62 μm^2 to 81.7 μm^2, with a mean mucous cell area of 49.98 μm^2. Two hours after a 5-minute exposure, the range of mean mucous cell sizes in GL was

49.05 µm^2 to 52.19 µm^2 with no significant difference between the control and the 2.4 ppm PAA group (Figure 1A, *left panel*). However, the GL mucous cell area of the group exposed to 0.6 ppm PAA was significantly larger while those exposed to 1.2 ppm PAA group did not significantly differ from the control.

Two days after 1st exposure for 5 minutes, the mean mucous cell size was about the same in all groups. At 2 weeks, gills exposed to 2.4 ppm PAA exhibited significantly smaller mucous cells than the 1.2 ppm and the 0.6 ppm groups. No significant differences were observed within the groups between 2 days and 2 weeks after the 1st exposure (Figure 1A, *left panel*).

Two hours after the 2nd exposure, this time for 30 minutes, the mucous cell area in the groups exposed to 1.2 ppm and 0.6 ppm PAA were significantly larger than in the control group and 2.4 ppm PAA group (Figure 1A, *right panel*). At 2 days post exposure, 0.6 ppm PAA resulted in significantly larger mucous cells than in the control and the 2.4 ppm groups. No significant dose-related differences were observed 2 weeks after 2nd exposure (Figure 1A, *right panel*).

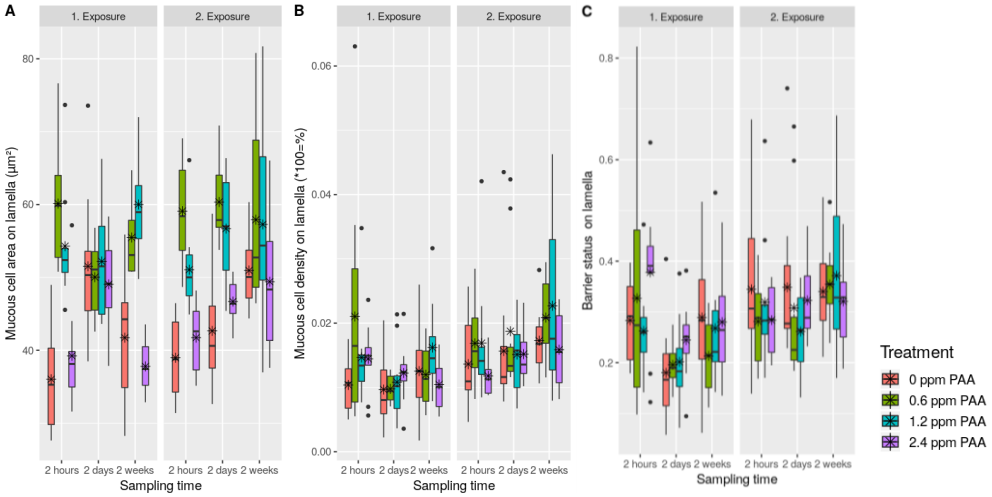


Figure 1. Temporal morphometry of mucous cells on the gill lamella of fish treated with therapeutic doses of PAA. Fish were exposed to PAA at 2 occasions. A = mean mucous cell area at the equator; B= volumetric density of mucous cells in mucosal epithelium; C= defence activity (barrier status) of mucosal epithelium. n=10 fish per treatment per sampling point, two tanks per treatment.

GL mucous cell density

The GL mucous cell ranged from 0.97 % to 1.22 % volumetric density of the lamellar epithelium (Figure 1B). The initial exposure had no significant density impact, but the 0.6 ppm group displayed the highest variance with densities ranging from 0.8 % to 3.5 % and a mean of 2.1 % (Figure 1B, *left panel*).

The second PAA exposure, this time of 30 minutes duration, did not significantly influence the GL mucous cell density in any dose or time point (Figure 1B, *right panel*).

GL defence activity

Two hours following the initial exposure the mean defence activities ranged from about 0.25 to 0.4. However, 2 days after exposure all groups had non-significantly reduced defence activity relative to those 2 h after exposure (means between 0.17 to 0.25). The defence activity (Figure 1C, *left panel*) returned to nearly initial levels 2 weeks after the 1st exposure (means between 0.2 and 0.3).

The 2nd exposure had no substantial impact or dose-related response on the mean GL defence activity (Figure 1C, *right panel*).

Mucous cell morphometry in the gill filament (GF)

GF mucous cell area

The mucous cell area on the filament varied from 56.9 µm^2 to 141 µm^2 with a mean mucous cell area of 85.3µm^2. Dose-related significant differences following 1st exposure were not detected in the mean size of the filament mucous cells (Figure 2A, *left panel*).

Following the 2nd and longer exposure, mucous cells in the gill filaments displayed similar response patterns to those of the 1st exposure. Nonetheless, exposure to the highest dose, 2.4 ppm, induced significantly larger mucous cell sizes at 2 days relative to 2 h after the 2nd exposure. Two weeks after 2nd exposure, all groups displayed the same filament mucous cell sizes as those immediately following the repeated treatment with no dose-related changes in response (Figure 2A, *right panel*).

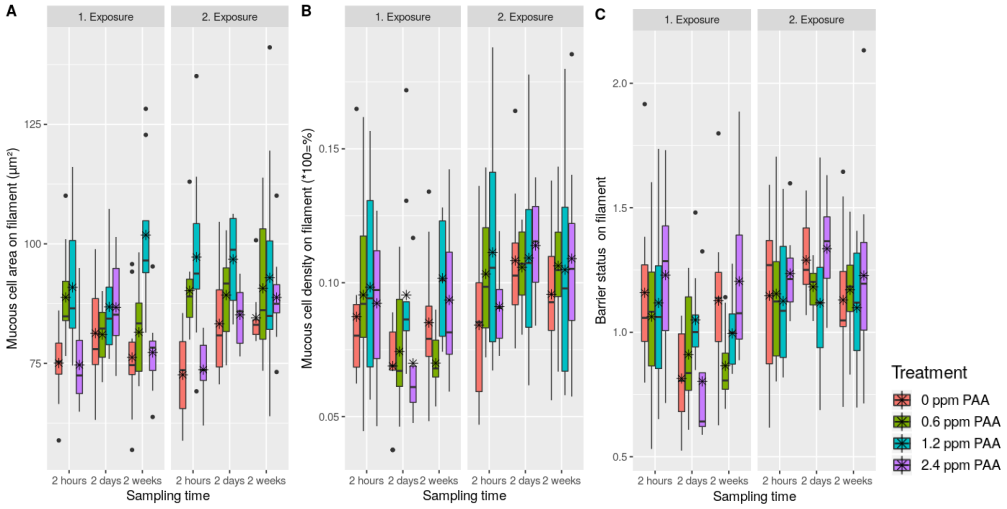


Figure 2. Temporal morphometry of mucous cells on the gill filament of fish treated with therapeutic doses of PAA. Fish were exposed to PAA at 2 occasions. A = mean mucous cell area at the equator; B = volumetric density of mucous cells in mucosal epithelium; C = barrier status (defence activity) or the combination of area and density of mucosal epithelium. An individual box plot represents measurements from 10 individual fish (n=10) of a treatment per sampling point, two tanks per treatment.

GF mucous cell density

The mean mucous cell density varied from 8.73 % to 10.57 % of the mucosal epithelium with no significant dose-related differences (Figure 2B). A small reduction in density 2 days post 1st exposure in the 2.4 ppm group was not significantly different.

Exposing the fish to PAA at longer durations did not significantly affect the volumetric density of mucous cells in the gill filaments at any timepoint (Figure 2B, right panel).

GF defence activity

Mean defence activity varied between 1 and 1.25 for all doses immediately after the 1st exposure (Figure 2C, left panel). The longer-term response pattern for all groups showed a decrease in defence activity at 2 days post exposure, particularly for the high dose group (not significant) but returned to nearly initial values by 2 weeks post exposure. The control group (0 ppm) showed significantly elevated defence activity 2 weeks post exposure compared to 2 days suggesting a response to a stressor rather than treatment dose. There were no dose-related differences in filament defence activity during the 2 weeks after 2nd exposure, suggesting a stabilization of response (Figure 2C, *right panel*).

Lamellar thickness

The lamellar thickness in the control and high dose groups varied between 7.8 µm and 19.6 µm with a mean of 12.22 µm (Figure 3A). Oddly, only the control group thickened significantly from 2 hours to 2 days after the 1st exposure. There was no significant difference between the control and the 2.4 PAA group 2 h after the 2nd exposure, though the gradual increase over time was not statistically significant (Figure 3A, right panel).

The relationship between mucous cell size and the physical space it needs to occupy in the double epithelial layer of the lamella is demonstrated by the significant positive correlation between this and the lamellar thickness (*r*=0.47) (Figure 3B).

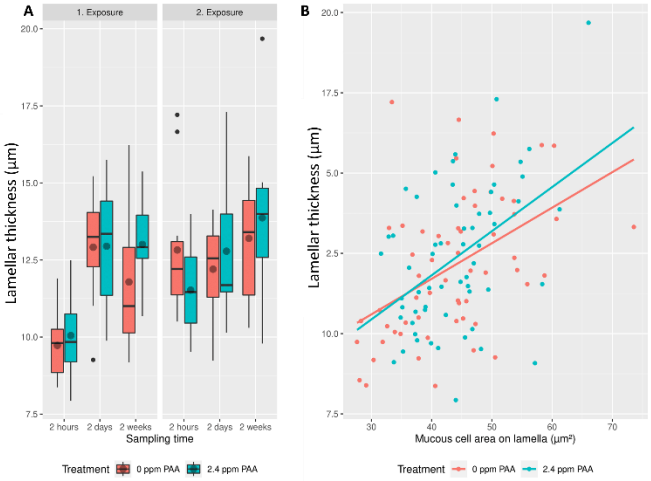


Figure 3. A) Gill lamellar thickness and B) its correlation with mucous cell area. N=120 individual fish, 60 per treatment group.

Relationship between the mucous cells on the gill lamella and filament

Mucous cells were generally larger in the filament than in the lamella at all doses, exposures and sampling times. The mucous cell density was even more distinct between the two gill sites, where the filament mean density was 9.49 % and the lamellar mean density had a mean volumetric of 1.48 % of the mucosal epithelium (Figures 1B,2B). Mucous cell volumetric density on the filament was significantly higher than the lamella in all instances.

Histopathological alterations

The majority of the gills analysed (n=225, 93.8 %) were healthy and had a HAI-score between 0-10 indicating functionally normal gills. The remainder were moderately damaged gills (n=14, 5.8 %) or moderate to heavily damaged gills (n=1, 0.4 %). Two weeks after the 1st exposure, more alterations were induced by 2.4 ppm PAA. However, all gills were histologically categorised as functionally normal by the end of the trial period (Figure 4).

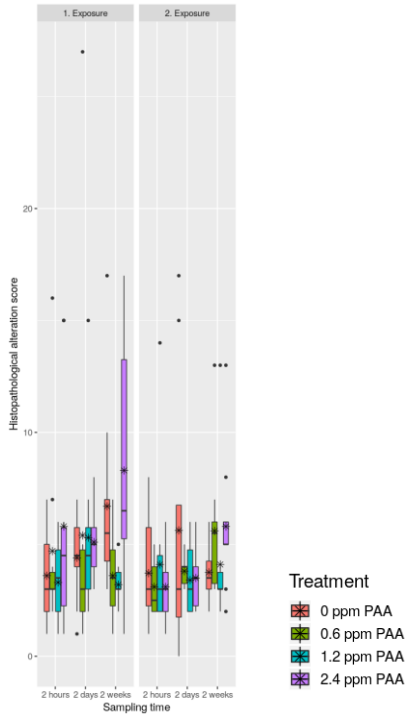


Figure 4. Histopathological alteration index for the gills of the treatment groups. 60 gills were analysed per treatment group, making 240 gills for all the trial.

These results agreed with the semi-quantification of common histopathological characteristics (Figure 5A). Clubbing and lifting were the most prevalent observations, accounting for 0.5 – 3 % of the cases (Figure 5B). Hyperplasia was significantly more 2 weeks after 2nd exposure than the same time point after the 1st exposure but there was no relationship with dose. Lamellar clubbing during the 1st exposure increased as a function of PAA dose.

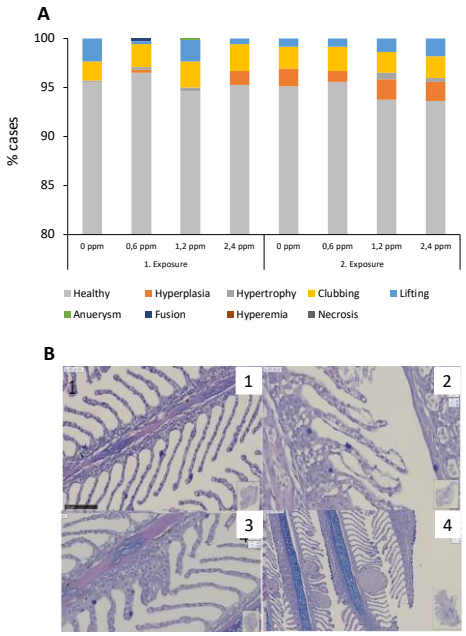


Figure 5. Quantification of histopathological cases in the gills at 2 weeks after first and second exposure. (A) Histopathological cases are reported at % relative to the total number of evaluations. (B) some of the histopathology observed in the gills of experimental fish. 1= healthy gills, 2=lifting, 3= hypertrophy, 4= aneurism.

Discussion

Several studies have already demonstrated that mucous cells respond rapidly to environmental changes, with changes in size of mucous and epithelial cells as the most common characteristics of complex gill disease [136]. These reproducible changes can be objectively measured using mucosal mapping to illuminate even subtle effects of potential therapeutics like PAA and other interventions [68, 122, 123, 129, 137, 152].

Changes in mucous cell morphometries exhibit a dose dependent response to PAA

Exposure to the highest dose, 2.4 ppm, induced significant hypertrophy in filament mucous cells at 2 days relative to 2 h after the 2nd exposure, suggesting a latent membrane-wide development. The second highest dose 1.2 ppm PAA exhibited some hypertrophy 2 weeks after exposure. Beyond that, varying doses of PAA from 0 to 2.4 ppm did not significantly change the mucous cell sizes in the gill filament (mean 85.3 µm^2). These were smaller than those on the gill filament of commercially produced salmon (mean 97.14 µm^2) exposed to 1500 ppm of H_2O_2 [153], but comparable with filament mucous cell sizes in shorthorn sculpins (*Myoxocephalus scorpius*) exposed to an environmental gradient of heavy metals (i.e., lead and zinc) (87.63 µm^2 [129]. The mucous cell density on the filament ranged from 3.7 % to 18.8 % of the mucosal epithelium with a mean density of 9.49 % with no inter-treatment differences. A sub-acute response, a significant *decrease* in defence activity on the filament, was found 2 days after fish were first exposed to the highest dose of 2.4 ppm PAA. However, this effect was only detected after the initial exposure, not the second exposure, and the fish recovered. This change may offer insight into the influence of previous exposure on the effects of PAA on mucous cell size. A study in rainbow trout demonstrated that repeated exposure to PAA lead to a form of physiological habituation [37] and such a response had been likewise identified in salmon [154]. The behavior of mucous cells in the filament is pointing to a similar form of response to PAA. Since even the control group with 0 ppm PAA exhibited similar transient changes in the filament, it suggests that the mucosal changes are not induced by PAA up to 2.4 ppm but rather by other generalized stressors, for example, handling during treatment.

Some hypertrophy of mucous cells was also found in the gill lamellae. Both medium doses (1.2 and 0.6 ppm) induced significant hypertrophy of lamellar mucous cells 2 hours after the initial and re-exposures. Lamellar mucous cell sizes in this study (mean 49.98 µm^2) were smaller than those found by Rantty (70.26 µm^2) [153] on salmon exposed to H_2O_2 but larger than in salmon of about 100 g in a controlled feeding trial (<35 µm^2; [155]. Healthy wild smolts have very few, spatially distributed lamellar mucous cells of about 30 µm^2 (Quantidoc unpublished data). This acute response to PAA in the respiratory surface was independent of dose or exposure time, again suggesting a conserved response to brief challenges and minimal impact of repeated doses of PAA up to 2.4 ppm. In earlier studies where fish were exposed to environmental stressors and pollutants [129, 153], a common response to the insults was a *reduction* of mucous cell density. Stress from acetic exposures can stimulate secretion of mucus [156, 157], and short-term stress and acetic exposure can reduce mucous cells in the skin and the mucosal barrier in general [138, 158]. Both PAA and H_2O_2 generate an increased volumetric density of mucous cells a couple of days after exposure followed by a return to baseline level two weeks after. This further supports the protective and adaptive mechanisms of the mucous cells [141].

The impact of these mucous cell measures extends into the physiology of growth because the cells occupy space between the respiratory epithelial membranes. The lamellar thickness is positively correlated with mucous cell size in our study. Ultsch & Gros [159] hypothesised that an increase in mucous around the gill will decrease the oxygen diffusion efficiency by increasing the physical space between the outer membrane and the central capillaries. The estimated maximum diffusion distance (thickness divided by 2; mean 6.1 µm) was in agreement with previous estimates for salmon gill diffusion

distances [95, 160]. There was no clear PAA dose-related change in gill mucosal thickness in this study, suggesting unchanged respiratory parameters.

Mucous cells from the filament and lamellae demonstrate a distinct response to PAA

Mucous cells reside in both the filament and lamella of the gills. Most mucous cell morphometry studies in fish do not often differentiate their response to stimuli, as the traditional manual approach is tedious and laborious. In addition, the traditional 2D sections often give a misleading impression of the actual variation in 3D structure of the gills. The Mucosal Mapping addresses this issue. The technique has facilitated extensive measurements of mucous cells and has identified that the two populations of the mucous cells in the gills often responded differently based on thousands of gill samples analyzed from different experiments and localities; thus, the distinction of the two proposed populations is highlighted in this paper. Our results show that the two mucous cell populations in the gill filament and in the gill lamellae demonstrated distinct responses to PAA. These include significant transient hypertrophy but not hyperplasia in both segments. A transient decrease in hyperplasia (decrease in defence activity) was found only in response to 2.4 ppm, suggesting that higher doses of PAA may have measurable impacts on the gills. The significantly larger and denser mucous cells of the filament *vs* those in the lamellae support the hypothesis of two separate populations of mucous cells in the gills, where the filament mucous cells are associated with excretion of ions, minerals and metals [129, 161] and may reflect an upregulation of metabolism or an increased need for excretion of metabolic products [155] and those in the lamella are more integrated with functions of respiration and mucosal immunity. Thus, studies pooling cell populations from both segments may be overlooking valuable information which would be apparent when using separated measurements (Dang et al. 2020). Future studies must explore in depth the functional relevance of the distinct responses of these two mucous cell groups in the gills of fish.

Visualising the distinction between these cell populations may be key to advancing aquaculture sustainably. While traditional histological sections of gills show patchiness in mucous cell distribution and direct comparison is visually difficult, a standardised illustration of mucous cell size, volumetric density and defence activity is given below (Figure 6). In this case, Quantidoc's Dicer App v2 illustrates average mucous cells in gill lamellae (Figure 6A, B) and in gill filament (Figure 6C,D) in a standardised 10 thousand square microns of epithelium at densities found in this study. The illustrations underscore the tissue differences in size, density and defence activity between healthy lamellae and healthy filaments.

Figure 6. Standardised representations of mucous cells in the gill lamellae and gill filament in this study, generated using Quantidoc's Dice App v2. A-B) Gill lamellar mucous cells shown at mean size of 50 µm2 and volumetric densities of 1 % (A) to 1.2 % (B) in 10 000 µm2 of epithelium. C-D) Gill filament mucous cells shown at a mean size of 85 µm2 and volumetric densities of 8.7 % (C) to 10.6 % (D) in 10 000 µm2 of epithelium.

Mucosal mapping complements traditional histopathology

Both histopathological scorings employed in the present study indicated that 90 % of the gills remained in a healthy state following PAA exposures although infrequent pathologies were observed. Alterations of gill tissue can be caused by pathogens [162], pollution [163] and particles [164], among other stimuli. Gills exposed to suspended materials have previously shown increased mucous production, hypertrophy and hyperplasia [165] and branchial histopathological alterations can reduce mucosal function [143, 166, 167]. Alterations can vary from minor (clubbing and lifting of the epithelium) to serious (necrosis of gill epithelium) and can be divided into direct damage of environmental stimuli, and defence mechanisms (lifting, hypertrophy and hyperplasia) resulting in reduced oxygen absorption in the gills [141, 168]. The histopathological scores and the limited hypertrophy strongly corroborate that application of PAA at therapeutic doses used in the present study does not pose significant gill health concerns. Both histopathological scorings and mucous cell dynamics highlight the minimal, mostly transient and fast recovery rate from PAA-induced changes.

Conclusions

Taken together, the results of the present study revealed that mucous cells in the gills of Atlantic salmon responded minimally to therapeutic doses of PAA. Nonetheless, the changes can be characterized as adaptive responses to general stressors and the levels of oxidants present in the immediate environment, without significantly damaging gill health. A number of the significant changes in the cellular morphometries were observed during the 1st exposure, indicating the possibility that the gill mucous cells respond to the oxidant following an initial rapid reaction to PAA, and the recovery was likewise quick. These mucosal responses are in agreement with an earlier report on the physiological adaptation of salmon to repeated exposure to PAA [81]. Mucosal mapping could reliably distinguish between the two populations of mucous cells, one in the filament and one in the lamellae, each with distinct transient hypertrophy, which caused no measurable lasting impact on functionality of healthy salmon gills. It would be interesting to explore in the future whether the changes documented in the present study are specific to a particular PAA trade product or are a universal response to PAA-based disinfectant. The variable compositions of commercial PAA products provide limitations to the observations in the present study. Moreover, the safe thresholds for PAA vary among fish species, and factors such age, physiological status, mode of delivery, exposure duration, among others, will affect how fish respond to the oxidant challenge [83, 169, 170]. Hence, the responses observed here are only valid within the parameters described in the exposure trial.

5.2.2 Effects of crowding

The results from this trial appeared on 2 separate publications:

Soleng, M., Johansen, L.H., Johnsen, H., Johansson, G.S., Breiland, M.W., K., Rørmark, L., Pittman, Pedersen, L.F., Lazado, C.C. 2019. Atlantic salmon (*Salmo salar*) mounts systemic and mucosal stress responses to peracetic acid. *Fish & Shellfish Immunology*. 93, 895-903. (*NB: Presented in 5.2.1*)

Lazado, C.C., Sveen, L., Soleng, M., Pedersen, L.F., Timmerhaus, G. 2021. Crowding reshapes the mucosal but not the systemic response repertoires of Atlantic salmon post-smolts to peracetic acid. *Aquaculture*. 531, 735830.

Publication 6

Crowding reshapes the mucosal but not the systemic response repertoires of Atlantic salmon to peracetic acid

Carlo C. Lazado [a], Lene R. Sveen [a], Malene Soleng [b], Lars-Flemming Pedersen [c], Gerrit Timmerhaus [a]

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ABSTRACT

Knowledge of the impact of aquaculture chemotherapeutants on fish physiology is scarce. This is particularly relevant for peracetic acid (PAA), a widely used oxidative disinfectant in aquaculture. The chemical behavior in water is well studied but knowledge about the physiological consequences for fish is limited. The present study investigated the transcriptomics, morphology, and physiology of Atlantic salmon (*Salmo salar*) responses to PAA and explored how crowding prior to exposure influenced these responses. Post-smolts were subjected to crowding by reducing the water volume thereby increasing the density for 1 h before they were exposed to 4.8 ppm PAA for 30 minutes. The exposed fish were allowed to recover for 2 weeks (w), with samplings carried out at 4 h and 2 w post-exposure (p.e.). There were four treatment groups in total: no crowding/control; no crowding/PAA; crowding/control; and crowding/PAA. The physiological changes were documented at the mucosal (i.e., skin and gills) and systemic (i.e., plasma) levels. The overall external welfare score was in good status in all experimental groups. The treatments did not dramatically affect the number of mucous cells in both the skin and the gills. Branchial histomorphology was in a fairly good condition, despite the increased occurrence of epithelial lifting in the crowded groups at 2 w p.e. The gill transcriptome was affected by crowding, PAA, and their combinations more than the skin, as manifested by the number of differentially expressed

genes (DEG) in the former. In general, individual stimuli and their combinations elicited strong transcriptional responses in the gills at 4 h p.e. and a marked recovery was observed 2 w thereafter. Crowding altered the dynamics of transcriptional response to PAA especially at 4 h p.e. and the two mucosal tissues demonstrated a contrasting profile – a higher number of DEGs in the gills without crowding history, while higher skin DEGs were observed in the group subjected to crowding prior to exposure. Plasma metabolomics identified 639 compounds, and the metabolomic changes were affected mainly by crowding and sampling time, and not by PAA exposure. The results revealed the ability of salmon to mobilize physiological countermeasures to PAA exposure that were differentially influenced by crowding, and that such an effect was remarkably exhibited at the mucosa rather than in the circulating metabolome.

Introduction

Aquaculture is one of the fastest-growing food-producing sectors in the world and is envisioned to be the key driver in meeting the need for aquatic food products among the increasing global population [171]. In particular, the global Atlantic salmon (*Salmo salar*) aquaculture industry has grown dramatically over the last years, reaching almost 2.5 million tons in 2018 – a 5 % increase from the previous year. Norway is the world leader in salmon farming, with a contribution of about 50 % of the annual global production [172].

However, the prominence of Atlantic salmon in the global aquaculture scene is threatened by several bottlenecks, and diseases remain a perennial issue. For some time now, the industry's daunting challenge has been the ectoparasitic salmon louse (*Lepeophtherius salmonis*) [173, 174]. These caligid copepods attach to the skin and feed on mucus and blood, resulting in skin erosion, damage, osmoregulatory failure, immune suppression and increased risk of secondary infection, and chronic stress [173, 175, 176]. Another ectoparasitic infection is amoebic gill disease (AGD) caused by *Neoparamoeba perurans*, a widespread condition affecting salmonids farmed in the marine environment [9]. AGD is characterised by raised, multifocal white mucoid patches on the gills, resulting in respiratory distress, and then, eventually, in death when the infection has severely progressed [177]. Anti-parasitic chemotherapeutants are the most common methods to control these parasitic infections, with hydrogen peroxide (H_2O_2) being a popular choice. Traditionally, H_2O_2 has been considered as posing a low environmental risk because it rapidly disassociates into water and oxygen and does not bioaccumulate in the environment [178, 179]. However, its excessive use in recent years has raised some serious concerns, and the frequency of treatment has been implicated in the development of resistance to the chemotherapeutant [180, 181]. These concerns are also prompted by a significant caveat about the lack of knowledge of the physiological consequences of peroxide use in salmon, as earlier approaches focused on the impacts on the causative agent and the disappearance of clinical signs. Therefore, the contemporary approaches aimed at identifying alternative treatments must provide evidence of how a chemotherapeutant affects the host organism.

Peracetic acid (PAA, CH_3CO_3H) is a strong oxidant and is commercially available as an equilibrium mixture with acetic acid (CH_3COOH) and hydrogen peroxide (H_2O_2). One of its main advantages is its broad spectrum of inhibitory activity against many microorganisms – it exhibits bactericidal, virucidal, fungicidal, and sporicidal activity [28, 182]. Other than this beneficial attribute, the absence of residual or toxic and/or mutagenic by-products, no requirement for dechlorination, present low dependency on pH, and short contact time has been essential in defining PAA as a more sustainable peroxide-based disinfectant in fish farming [183]. PAA and H_2O_2 are in the family of oxidative disinfectants, and the former has the attributes of a potential alternative chemotherapeutant for the latter; not only does PAA degrade relatively faster than H_2O_2 [184] but its effective dose against many aquaculture pathogens is also lower than H_2O_2 [29, 39, 46]. The chemical behavior of PAA in both freshwater and seawater matrices is well-described [38, 184] and the toxicity of PAA towards several aquaculture fish has been

reported [39]. Most of the studies documenting its physiological impacts on fish have focused on rainbow trout (*Oncorhynchus mykiss*), where PAA exposure has been demonstrated to trigger oxidative stress, though the trout were able to respond to the oxidant by activating physiological adaptive mechanisms including immunity and the neuroendocrine axis [37, 58, 80]. Using a limited panel of known markers for stress, we have earlier reported that salmon post-smolts were able to mount systemic and mucosal responses to PAA concentrations ranging from 0.6 to 4.8 ppm [81]. Nonetheless, there remains a conundrum regarding the extent to which PAA influences the physiology of salmon, as system-wide physiological assessment has yet to be conducted.

Despite being identified as a major welfare risk (i.e. high incidence of mechanical wounds, scale loss) [185, 186], crowding is an inevitable production procedure in salmon farming, such as during vaccination, transport, grading, de-licing, and chemotherapeutic bath treatments [92]. This process may pose behavioural and physiological changes. Hence, crowding effects must be accounted for when one is assessing the impacts of husbandry manipulations such as bath treatments. Salmon can mount stress responses to PAA [81]. However, it is not yet ascertained how pre-treatment stress from crowding influences the concerted physiological response to subsequent PAA exposure.

The present study documented the health and welfare impacts of PAA exposure in Atlantic salmon post-smolts and explored how crowding influenced these responses. The skin and gills, the target organs of the current study, represent two of the most important mucosal organs in fish, and their close interaction with the aquatic environment makes them susceptible to environmental changes and husbandry-related manipulations, which consequently affects overall health and welfare [68, 187]. In addition, we identified systemic-wide response by characterising the circulating metabolome. Using complementary platforms, we profiled the consequences of PAA treatments from the different levels of biological organisations. This approach allowed us to identify molecular signatures that may be used as biomarkers for PAA response.

Materials and Methods

Crowding and peracetic acid exposure

All fish handling procedures complied with the Guidelines of the European Union (2010/63/EU), as well as with Danish legislation. The experimental fish were purchased from Danish Salmon A/S (Hirtshals, Denmark). After smoltification, the fish were transported to the nearby experimental recirculation aquaculture (RAS) facility of DTU Aqua (Hirtshals, Denmark). Upon arrival at the facility, the fish were sorted and weighed. Then, 100 fish were stocked to each of the two 4 m^2 holding tanks (water volume ≈ 1500 L) in a seawater flow-through system. The fish were allowed to acclimate for 2 weeks under the following environmental conditions: salinity at 35 ppt, temperature at 11±1^oC, pH at 7.6 - 7.8, oxygen at > 85 % saturation, and photoperiod set at 24L:0D provided by an indirect light source. These conditions were maintained all-throughout the trial, from acclimation to recovery phase. Additional operational system information can be found in an earlier publication [81]. Commercial fish feed (Biomar, EFICO Enviro, 4.5 mm) was provided through a belt feeder at a daily ration of 1 – 1.5 % total biomass. There was no mortality during the acclimation phase.

Feeding was stopped 24 h before the experiment. The crowding-exposure experiment was designed to roughly simulate a treatment scenario in the field, in which salmon are usually subjected to handling, pumping, and crowding before peroxide treatment [92, 185], and likewise limit the number of fish used for experiment but still addressing the main objective on how crowding influenced responses to PAA. From the holding tanks, the fish were divided into 4 groups of 50 and were transferred to its corresponding closed-system 500 L exposure tank, achieving a density of roughly 15 kg/m^3. They were allowed to rest for about 15 min before the density and treatment manipulations were performed. For

the two fish groups subjected to crowding, the density was increased to 75 kg/m^3 through lowering of the water volume. Aeration was provided throughout the duration of the 1 h crowding. Thereafter, the water level returned to its initial level and the fish were allowed to recover for 15 minutes. One of the crowded groups was exposed to 4.8 ppm PAA nominal concentration. During this time, the other crowded group was exposed to 0 ppm (sham exposure with seawater). PAA (Divosan Forte™, PAA) was supplied by Lilleborg AS (Oslo, Norway). The actual PAA concentration of the commercial product had been verified by DTU Aqua Laboratory and was determined to be around 18%. Both bath treatments lasted for 30 min. The decay kinetics of PAA in the system were earlier described in a companion paper [184]. During the exposure trial, aeration was also provided to facilitate mixing and maintain the required DO level (>80 % saturation). For the fish groups that were not subjected to crowding, the following protocol was applied: After settling in for 15 min following transfer, one group was exposed to 4.8 ppm PAA while the other group was exposed to 0 ppm (seawater) PAA. The exposure likewise lasted for 30 min. After the exposure experiment, the fish were transferred to their corresponding 1 m^2 recovery tanks (water volume ≈ 600 L) connected to a recirculation system with full-strength seawater. Each group was divided into groups of 25 and allowed to recover in the recovery treatment tanks. Operational system parameters and environmental conditions were similar between acclimation and recovery periods.

Sample collection

Sampling was performed at 4 h (for plasma and RNA) and 2 w (for plasma, RNA, histology, skin color, and welfare scoring) after PAA exposure. Five fish (average weight at 4 h post-exposure: 131.3 ± 2.3 g mean ± SE; average weight at 2 w: 159.2 ± 11.3 g) were taken from each replicate tank and were humanely euthanised with an overdose of 20 % benzocaine solution. After the length and weight were measured, the whole body of each fish for sampling was photographed (Canon EOS 60S, f/11, 1/8s, ISO200, 23 mm) and the external welfare scoring was performed following the FISHWELL handbook [92]. Blood was withdrawn from the caudal artery using a heparinised vacutainer, centrifuged at 1000 x *g* for 10 min at 4 °C, and the plasma was collected and kept at –80 °C until analyses. A section of the dorsal skin and the second gill arch was dissected and divided into two portions. The portion for microarray was suspended in RNAlater (Ambion, USA), left at room temperature overnight for penetration and thereafter kept at -80 °C before RNA extraction. The other half was preserved in neutral buffered formalin for histological evaluation (CellPath, UK).

Microarray analysis

Total RNA was isolated from the skin and gills by the MagMAX TM-96 Total RNA Isolation Kit (Ambion). RNA concentration and quality were determined using a NanoDrop 8000 spectrophotometer (ThermoFischer Scientific, USA). RNA quality was further assessed using an Agilent® 2100 Bioanalyzer™ RNA 6000 Nano kit (Agilent Technology Inc., 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 functions, cell communication and junctions, and extracellular matrix, amongst many others [88]. This microarray is annotated into four major gene clusters: a *Tissue* cluster that includes genes involved in tissue structure, integrity, development, and architecture; a *Metabolism* cluster that constitutes genes important for metabolic processes; an *Immune* cluster that contains genes with a known function in innate and adaptive, cellular, and humoral immune responses; and a *Cell* cluster that comprises genes vital for cellular processes, development, communication, and signalling. Agilent Technologies manufactured and supplied the microarrays, reagents, and equipment used in the analysis. A One-Color Quick Amp Labeling Kit was used for RNA amplification and Cy3 labelling, and 200 ng of total RNA template was used per reaction. Thereafter, labelled RNA was subjected to fragmentation using the Gene Expression Hybridization Kit and hybridisation was carried out for 17 h in an oven thermostatted at 65 °C with a constant rotation speed of 10 rpm. Thereafter, the arrays were

washed in sequence with Gene Expression Wash Buffers 1 and 2 and were scanned through an Agilent SureScan Microarray scanner. Data processing was carried out in Nofima's bioinformatics package STARS.

Plasma metabolomics

Plasma proteins were initially precipitated using methanol followed by liquid-liquid extraction with chloroform and water before the aqueous phase was collected and dried under nitrogen flow. The analyses were carried out using a UPLC system (Vanquish, Thermo Fisher Scientific) coupled to a high-resolution quadrupole-orbitrap mass spectrometer (Q Exactive™ HF Hybrid Quadrupole-Orbitrap, Thermo Fisher Scientific). An electrospray ionization interface was used as an ionisation source and operated in both negative and positive ionisation modes. A QC sample was analysed in MS/MS mode for the identification of compounds. The LC method was a slightly modified version of the protocol described by [89]. Data were processed using Compound Discoverer 3.0 (Thermo Fisher Scientific). Identification and annotation of compounds were performed in four levels: Level 1: the most confident identifications, in which the annotations are based on three pieces of information – accurate mass, MSMS spectra, and known retention time obtained from reference standards analyzed on the same system; Level 2: annotations are based on two pieces of information and are further divided into two sublevels, i.e., Level 2a is based on the accurate mass and known retention time as obtained from reference standards analyzed on the same system, whereas Level 2b is based on the accurate mass and MS-MS spectra from an external library; and Level 3: annotations are based on library searches using the accurate mass and elemental composition alone.

Skin colour analysis

Individual photos were processed with an R-script to crop out an image of the skin from the belly to the back with a width of 600 pixels. The pictures were further processed by determining their mean color (RGB; Red Green Blue) values. The overall mean and the three color channels (red, green, blue) were measured as described earlier [138].

Quantitative histomorphometry

The gills and skin samples preserved in formalin were paraffin infiltrated following a 10-h-long sequential program of PBS, 50 %, 70 %, 96 %, and 3× 100 % ethanol, 3× xylene, and 2× paraffin (Leica TP1020). Embedded tissues were sectioned into 5 µm sections and stained with Periodic Acid Schiff-and Alcian Blue (AB-PAS, Sigma-Aldrich). Photographs were taken using Zeiss Axio Observer Z1 (Carl Zeiss).

For quantification of mucous cells in the gills, 6 frames, each of which consisted of 20 lamellae, were used. Quantification was defined into mucous cells at the lamellar base or filament and mucous cells at the lamella. For the skin, measurements were performed in 4 randomly selected regions, accounting for about 1700 µm per region. Two mucous cell populations were quantified based on their position in the epidermis: outer mucous cells in contact with *stratum superficiale*, and mucous cells in the *intermedium stratum*.

A semi-quantitative approach was employed to characterise the microscopic epithelial surface quality of the skin using a scoring method described earlier, with slight modification [124]. The section was scored by an impartial evaluator (no prior knowledge of sample treatment) using a 0- to 3-point system, with 0 indicating healthy skin with intact epithelial surfaces and 3 indicating severely damaged conditions characterised by a rough surface and the complete disappearance of the outer epidermal layer. For the gill sections, case scoring was performed following a previously published strategy [151], with modifications [188]. The evaluation was carried out by randomly selecting five gill filaments (i.e., two upper half, two lower half, and one middle of the whole gill arch section). A total of 100 lamellae were

evaluated per fish. Cases of clubbing, lamellar fusion, hyperplasia, hypertrophy, lifting, hyperaemia, aneurysm, and necrosis were documented. Lamella that did not show any pathological changes as enumerated above were denoted as “healthy”. If more than one pathology is present in the same lamella, the pathology which was the most prominent was accounted. If the scorer could not confidently differentiate the pathologies, then, the lamella was not included in the scoring and another lamella was chosen in the same pre-selected field.

Statistics

A Shapiro-Wilk test was used to evaluate the normal distribution, while a Brown-Forsyth test was used to check for the equal variance of the data from welfare scoring, skin color, and histological assessment. A one-way ANOVA was used to test for differences between treatment groups. A Holm-Sidak test was used to identify pairwise differences.

The mean intensities of all microarrays were equalized. Expression ratios (ER) were calculated by dividing the individual values for each feature by the mean value of the feature in all samples. The log2-ER were calculated and normalized with the locally weighted non-linear regression (lowess). Two comparisons were performed: 1) to study the effect of crowding alone (i.e., no crowding/control *vs* crowding/control); and 2) to study the effects of crowding to PAA response (i.e., no crowding/control *vs* no crowding/PAA; crowding/control *vs* crowding/PAA). Differentially expressed genes (DEG) were selected by criteria of significant log2-ER > |0.6|, $P < 0.05$.

For metabolome data, multivariate models (e.g., PCA models) were used to reveal treatment effects that affect many variables. In contrast, univariate statistics in the form of a *t*-test were used to show whether any single variable was significantly different between the two groups. Because the dataset contained a high number of variables, Benjamini-Hochberg correction was employed. The Benjamini-Hochberg critical value, (i/m)Q, was calculated for each compound. The largest *P*-value that has P<(i/m)Q is significant, as are all of the *P*-values that are smaller than this – even those that are higher than their Benjamini-Hochberg critical value.

Results and Discussion

Peracetic-acid-based products are gaining popularity in aquaculture as both disinfectants and chemotherapeutants. To support their application in Atlantic salmon, the present study documented the impacts of PAA exposure in salmon at the mucosal and systemic levels using gross pathology, histology, transcriptomics, and metabolomics. This suite of response variables allowed for the profiling of the impacts on salmon health and welfare from the different levels of biological organizations: gene – metabolite – cells – histostructure – organismal appearance. Salmon are subjected to crowding during parasite treatments and for other husbandry operations during a production cycle. Depending on the severity of the impact, such a protocol may influence their response to other husbandry manipulations or stressors [37], including peroxide bath treatment. We found that crowding prior to treatment was a potential confounding factor in the responses of salmon to PAA. PAA-based products are available in various mixtures of acetic acid and H_2O_2, as well as with different stabilizers. This particular feature of commercially available PAA outlines the limitation that the physiological responses documented here are specific to the product used in the present study.

The overall external welfare scores of experimental fish, regardless of the treatments, remained in good condition. All treatment groups had a composite score lower than 2, in an 11-indicator scoring scale of 0 to 3, where 3 indicated a highly compromised status [92]. Damages to pectoral fin, dorsal fin, and skin (i.e., mainly scale loss) were the notable indicators that received an average score of >1 in all treatment groups, though no significant inter-treatment differences were observed.

Key structural features of mucosal tissues are minimally affected by the treatments

The skin color analysis revealed that PAA exposure did not affect the skin color of salmon as the individual RGB channels and their mean values did not significantly vary amongst the experimental groups 2 w p.e. (Figure 1A-D). However, there was an apparent tendency for the PAA-exposed group that was not subjected to crowding to appear to have a slightly lighter skin color in all channels compared to the other groups. In an earlier publication, we have identified that PAA at a dose lower than what was used in this trial resulted in a transient increase in the blue channel of the salmon skin [138].

Microscopic epithelial surface quality scoring revealed that scores >2 (in a scale rating 0 to 3) were more prevalent in the group that was not exposed to crowding (Figure 1E-F). The majority of the fish from this group had a rough epithelial surface characterised by the lifting of the flat outer keratocytes in the epithelial layer (Figure 1E). The no crowding/control group was significantly lower skin health score from the no crowding/PAA group and the crowding/control group. It is rather difficult to provide a conclusive implication for such a distinct difference because, besides the limited number of fish, both groups had the same production history and no significant rearing deviations were noted during the 2-week recovery.

Histostructural evaluation of the gills showed a relatively clearer tendency than that of the skin (Figure 2), revealing that at least 93 % of the evaluated filaments looked healthy. Hyperplasia, hypertrophy clubbing, and lifting were the most common pathological changes documented (Figure 2A-E). PAA exposure did not drastically affect the histostructures of the gills because the profiles between control and PAA-exposed within the two groups (i.e., no crowding *vs* crowding) were similar. However, cases of epithelial lifting were significantly higher in groups with crowding history, and it seemed that subsequent exposure to PAA might exacerbate the pathology even more, indicating an additive effect of a secondary stressor. Epithelial lifting is one of the initial branchial reactions to a variety of pollutants [189]. Such a response to stressful conditions/the presence of contamination would result in an increased diffusion distance between water and blood, hence, giving rise to circulatory alterations [190]. Crowding carries a strong respiratory demand for fish [92], and the epithelial lifting that was still palpable even at 2 weeks post-treatment indicates a mid-term consequence for gill health, in which the present data set was unable to identify the recovery time.

Mucous cells are a ubiquitous element of the mucosal surface. They are the main producers of mucus, a glycopolymeric fluid that acts as a natural, physical, biochemical, dynamic, and semipermeable barrier at the mucosa [191]. Husbandry manipulations have been demonstrated to influence their numbers, which has implications for both the protective state of the mucosa and the quality of the aquatic environment [80, 192]. Quantification of mucous cells on the gill and skin epithelial surfaces revealed that neither crowding nor PAA, nor their combination, resulted in dramatic alterations, indicating a stable population of mucous cells on these surfaces, at least in the presence of the stimuli in the current study (Table 1). However, it is yet to be established whether this static population also results in stable exudation of mucus to cover the mucosa, thereby, maintaining a biophysical barrier. Nonetheless, this unchanged number of mucous cells perhaps demonstrates that a barrier element is maintained to provide a protective functional structure under varying conditions.

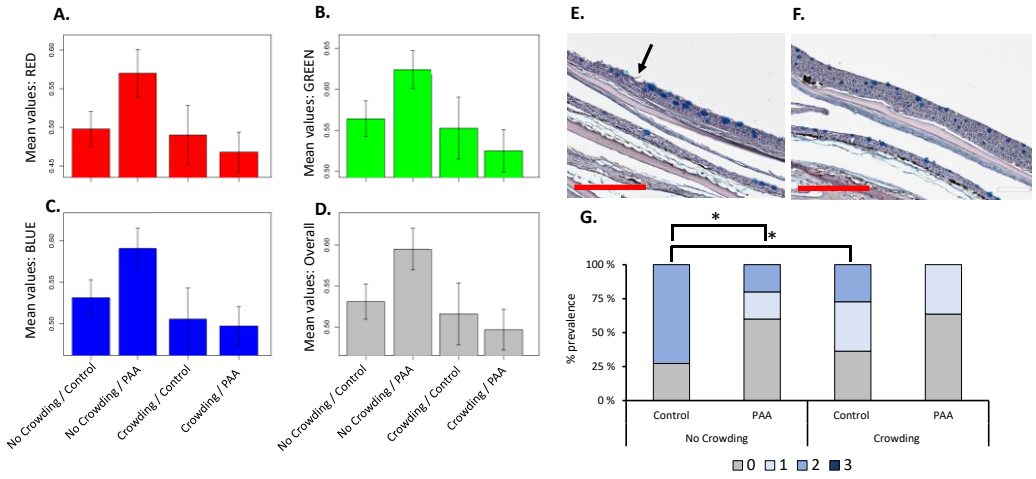

Figure 1. Macro- and micro-features of Atlantic salmon post-smolts skin 2 weeks after exposure to PAA with and without crowding history. Panels A-D: Skin color analysis revealing the individual RGB values (A-C) as well as the mean values (D). A higher value represents lighter/brighter colors; a lower value indicates a darker color. No inter-treatment differences were found at P < 0.05, as inferred from one-way ANOVA. Panels E-F: Representative photomicrographs of the skin of the control group without crowding history (E) and PAA-exposed fish with crowding history (F). Note the rough (arrow) surface of the skin surface of the control fish, which is corroborated by the quality of the skin epithelial surface (Panel G). The quality of the epidermal surface was scored by an impartial evaluator based on a 0-to-3 rating, where 0 means healthy/intact whereas 3 indicates severely compromised. Significant difference by pairwise comparison is indicated by an asterisk (*). Scale bar = 200 µm.

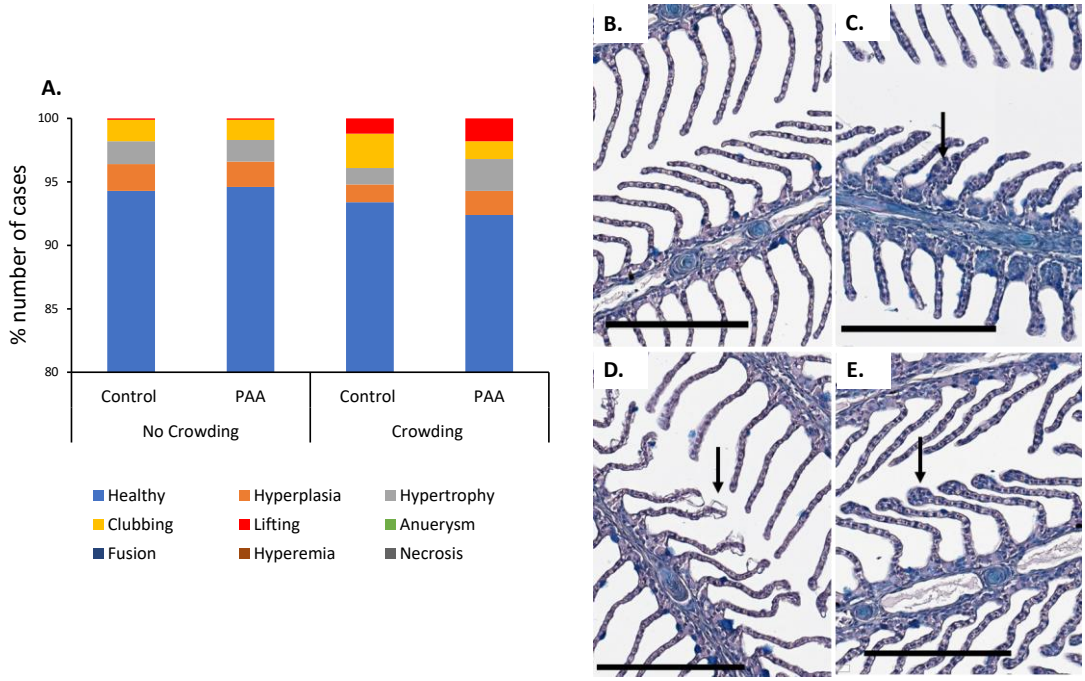

Figure 2. Histological scoring of branchial alterations in Atlantic salmon post-smolts 2 weeks after exposure to PAA with and without crowding history. Panel A: The prevalence of 9 common cases was quantified from 100 individual lamellae per fish. Only epithelial lifting was identified to exhibit inter-treatment differences, where the cases in the crowded group were significantly higher compared to those in the non-crowded group (note scale on Y-axis). Representative photomicrographs showing healthy gills (B) and common pathologies (arrow) such as hyperplasia (C), epithelial lifting (D), and lamellar clubbing (E). Scale bar = 200 µm.

Table 1. Mucous cell number in the gills and skin of Atlantic salmon post-smolts 2 weeks after exposure to PAA with and without crowding history.

		No Crowding		Crowding	
		Control	PAA	Control	PAA
Gills	Filament	8.8 ± 0.7	8.8 ± 1.2	7.9 ± 1.6	9.0 ± 1.1
	Lamella	7.1 ± 2.0	6.4 ± 1.0	8.4 ± 1.9	9.2 ± 1.9
Skin	Outer	26.3 ± 9.2	30.0 ± 6.5	25.4 ± 6.0	30.2 ± 3.2
	Inner	24.6 ± 18.8	28.2 ± 15.7	24.8 ± 20.4	37.1 ± 18.2

NB. Values are mean±SD from 10 individual fish. Please refer to section 2.6 for the strategies used to randomise measurements in each fish. No significant differences were observed amongst the treatment groups.

Crowding elicits a stronger transcriptomic response from the gills than the skin

It has been shown earlier in rainbow trout that the adaptive response to a secondary stress (i.e., chasing) was not altered by prior PAA exposure [37]. However, no data are available to indicate how stress (e.g., crowding) before treatment influences responses to subsequent PAA exposure. Salmon subjected to the crowding protocol in this study displayed a typical plasma cortisol increase after the treatment, indicating that stress responses have been mobilised [81]. The same group of fish from that earlier report was used in this study.

We first isolated the impact of stress alone on the mucosal transcriptome. The profiles revealed that crowding had a more remarkable effect on the gills than on the skin at both sampling points (Figure 3). In the gills, most of the crowding-induced DEGs were upregulated at 4 h p.e., where genes involved in immune response exhibited the highest gene counts (Figure 3A). At 2 w p.e., all the gene clusters were comparably represented. Moreover, there was a temporal shift in the overall profile – most of the DEGs (*ca* 66 %) were upregulated at 4 h p.e., whereas approx. half of DEGs (*ca* 53 %) were downregulated at 2 w p.e. The significant number of upregulated genes at 4 h p.e., including known stress-response genes *hsp70* and *hsp90α*, suggests a potential mobilisation of the adaptative stress response to the physiological disturbance from crowding. Moreover, *c-c motif chemokine 19 precursor-1* and *putative interferon-α/βreceptor α chain* were the two immune-related transcripts common at both time-points, implying the possible role that these molecules play in orchestrating the early and latent immune response associated with crowding. In the skin, 15 DEGs were identified at 4 h p.e., whereas 25 were identified at 2 w p.e. – substantially lower compared to the numbers in the gills (Figure 3B). From this, 87 % of the DEGs were downregulated at 4 h p.e., while only 28 % were downregulated 2 weeks after. Similar to the gills, *c-c motif chemokine 19 precursor-1* was the only identified DEG common at both time-points, highlighting the important function of this chemokine in both mucosal tissues in response to crowding. The function of *ccl19* is poorly understood in fish, though some evidence suggests that they exhibit canonical mammalian CCL19 functions including leukocyte trafficking, cell proliferation, and antiviral and antibacterial features [193, 194]. The emblematic modulation of their transcription following crowding provides new insights into their mucosal function in fish during crowding stress.

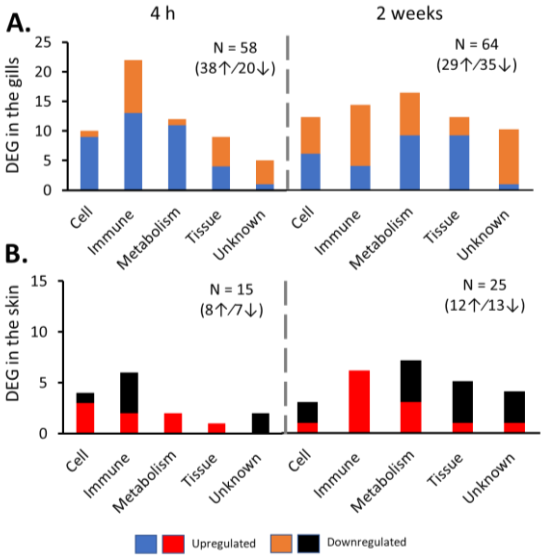

Figure 3. Differentially expressed genes (DEG) in the gills and skin of Atlantic salmon post-smolts 4 h and 2 weeks after crowding. The no-crowding control group was compared to the crowding control group to identify genes that were responsive to crowding alone. DEGs were identified with a criterion P<0.05 and log2 diff >0.6. The total number of DEG is provided together with the proportion of upregulated (indicated by ↑) and downregulated (by ↓) gene transcripts. The full list of DEGs is provided in Supplementary File 1.

The dynamics of mucosal molecular responses to PAA are differentially affected by crowding history

Evidence of global molecular responses is lacking in our current understanding of the physiological consequences of PAA exposure in fish [37, 48, 80, 83]. Here, we show that the transcriptome of the two mucosal tissues that directly interacted with PAA during treatment responded differently to PAA, with the gills exhibiting a stronger response than the skin (Figure 4). Such a general profile is similar to the effects of crowding alone (Figure 3).

The branchial transcriptomic response to PAA at both timepoints was more pronounced when fish did not experience crowding (Figure 4AB). At 4 h p.e., the number of DEGs in the no-crowding group was 30 % higher than that of the group that had experienced crowding. It could be possible that crowding dampened the ability of gills to respond to PAA, given that crowding is energy and metabolically demanding [195]. A significant portion of the molecular repertoire at the gill mucosa may have already been mobilised by crowding; hence, the ability to respond to another stimulus (i.e., PAA) likely diminished. A similar tendency was likewise observed at 2 w p.e., where the no-crowding history group exhibited a 54 % higher DEG than the group with crowding history. The number of DEGs at this timepoint was substantially lower than that at the earlier timepoint, indicating that the gills can consequently recover following an acute response to PAA. It was apparent that genes under cell and tissue clusters were markedly represented at 4 h p.e. in the no-crowding group, though such a tendency was not clearly exhibited in the group with crowding. The tissue cluster was the most represented in the no-crowding PAA-exposed group at this timepoint, where 77 % of the DEGs were upregulated, including genes involved in mucosal epithelial organisation, extracellular matrix integrity, and erythrocyte physiology. Six collagen genes (e.g., *collagen 6 α2*, *collagen 2 α1*) were significantly upregulated in this group. Interestingly, these transcripts were not found to be differentially affected in the crowded PAA-exposed group. It was earlier demonstrated in mammalian cardiac fibroblast that an increased reactive oxygen species (ROS) that eventually induced oxidative stress affected collagen synthesis [196, 197]. The increased expression of these collagen genes, as well as other genes involved in epithelial extracellular matrix integrity (e.g., *laminin subunit β-1*, *matrix Gla protein precursor*) suggests that the gills probably underwent a remodelling of extracellular matrix quantity and quality to counteract the presence of the

oxidant in the water, thus, playing a role in protecting the mucosal epithelium. Such a mechanism was restricted in the crowded PAA-exposed group. The histological data support such an interaction (Figure 3).

Haemoglobin is an important molecule that satisfies the demand for oxygen during aerobic metabolism by facilitating the dissolution of large quantities of gas and transport into the tissues [198]. Several genes crucial for erythrocyte function (e.g., *haemoglobin subunit alpha-4*, *haemoglobin subunit beta-4*) were significantly upregulated and represented in the gills of the no-crowding PAA-exposed group, though such a profile was not identified in the crowded group at 4 h p.e. PAA, an oxidant that produces free radicals in reaction, possibly carries a strong metabolic demand in the gills, hence, requiring efficient oxygen turnover. Crowding may interfere with, and probably limits, oxygen transport in the gills, thereby affecting a cascade of physiological processes, such as cellular respiration and metabolism, important when a secondary stressor is encountered (i.e., PAA).

It was earlier reported that known antioxidant genes in salmon gills were differentially modulated by PAA exposure, which was crucial in protecting the mucosa from oxidative stress [81]. Other mediators of the redox balance identified in the microarray profile revealed that PAA negatively modulated their expression – all the identified redox-related genes (e.g., *glutathione transferase omega-1, glutathione S-transferase P*) were downregulated regardless of crowding history. This indicates that PAA exposure could result in redox imbalance in the gills. Nonetheless, there was probably an effective feedback, as shown by other upregulated mediators [81], hence, enabling antioxidative homeostasis.

Thirteen DEGs were common in the gills of both groups at 2 w p.e., 6 of which have known immune functions, including *C-C motif chemokine 19 precursor-1*, *interleukin 22*, *myeloperoxidase*, *inducible nitric oxide synthase*, *myeloperoxidase precursor*, and *TNF decoy receptor*. Interestingly, all these genes were upregulated in the crowded group, whereas their counterparts in the non-crowded group were downregulated. This indicates that crowding influenced the common immunological response to PAA that persisted after 2 weeks. Genes important for erythrocyte physiology, particularly haemoglobins, were similarly over-represented and upregulated in the non-crowded PAA-exposed group 2 w p.e.; none were identified in the other group. It would be interesting to explore, in the future, the cost of oxygen delivery of PAA exposure in combination with crowding, as the pronounced difference in the presence of key mediators of branchial erythrocyte physiology at 2 weeks after exposure between the 2 groups indicates interference in this crucial process.

The number of DEGs in the skin was substantially lower than that in the gills, indicating that despite its close contact with the water matrix, the skin was less responsive to PAA (Figure 4C, D). Nonetheless, the overall skin transcriptomic profile indicates that early-phase response (i.e., 4 h p.e.) to PAA was more remarkable when fish experienced crowding before treatment. Most of the DEGs identified at this timepoint for both groups were downregulated, including *caspase*, *inducible nitric oxide synthase, putative sodium hydrogen exchanger 3b,* and *cytochrome P450 1A1*. Chemokines were modulated in the group with crowding history but not in the other group, where 3 c-c chemokine transcripts (e.g., *C-C motif chemokine 20 precursor* (2 genes), *C-C chemokine receptor type 7*) were downregulated. These signalling molecules play roles in orchestrating an inflammatory response, and the result indicates that crowding before PAA exposure negatively interfered with these effector molecules. ROS influence GTP proteins – an interaction that has implications for oxidative stress-related pathologies [199]. Four genes (e.g., *Ras GTPase-activating protein nGAP, guanylate-binding protein*) involved in GTP signalling were found only in the group subjected to crowding, and 3 of them were downregulated. The presence of PAA-triggered systemic oxidative stress response as reported earlier [80, 81], and the modulation of GTP signalling molecules may be involved as intermediates in scheming out the oxidative response process. At 2 w p.e., the number of DEGs in the skin of the no-crowding group was 41 % higher than that of the crowding group, which was an opposite trend in comparison to 4 h p.e. This profile revealed

a bimodal response in the skin – crowding may have primed the immediate response to PAA, while the response to PAA of a group without prior crowding exhibited a slight delay. However, the majority of the DEGs in the no-crowding group were downregulated, whereas upregulation was the general profile in the group exposed to crowding. Many of the downregulated genes in the no-crowding group were key genes in cytoskeletal dynamics (i.e., myosins, troponins), suggesting that PAA exposure may likely impact microtubule polymerisation and trafficking, as the identified genes have known functions in these processes [200, 201]. The genes common in both groups at this timepoint were all upregulated, including *nuclear factor interleukin-3-regulated protein*, *arrestin domain-containing protein 2*, *growth arrest and DNA-damage-inducible protein GADD45 beta*, *CCAAT/enhancer-binding protein delta-2*, and *TRAF2 and NCK interacting kinase a*. This set of transcripts contains perhaps the core genes involved in the skin response to PAA, as their modulation was not dependent on crowding history.

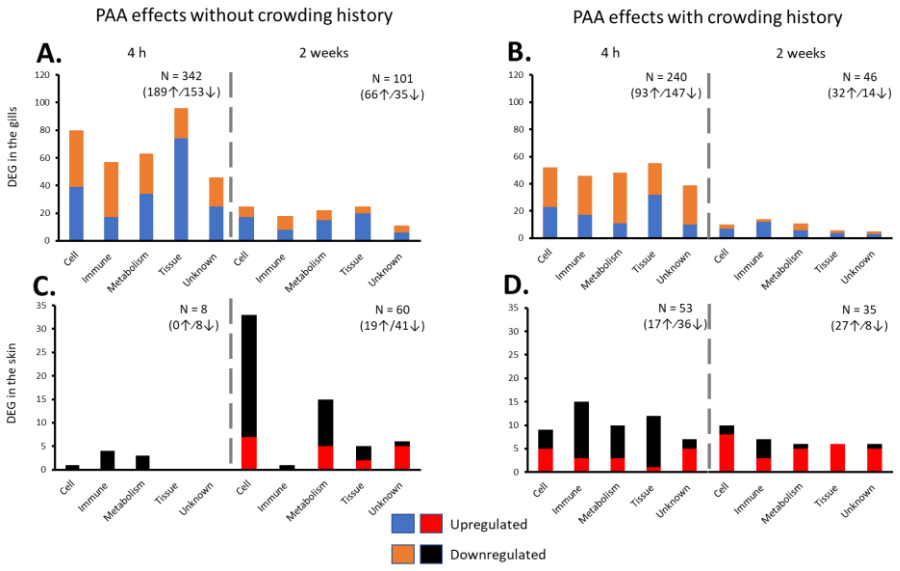

Figure 4. Differentially expressed genes in the gills and skin of Atlantic salmon post-smolts 4 h and 2 weeks after PAA exposure, with and without crowding history. PAA-exposed and control groups with no crowding history were compared to identify genes responsive to PAA treatment (Panels A, C). The same was done in the group subjected to crowding prior to PAA treatment (Panels B, D). The total number of DEG is provided together with the proportion of upregulated (indicated by ↑) and downregulated (by ↓) gene transcripts. The full list of DEGs is provided in Supplementary File 1.

Circulating metabolome provides insights into the systemic response to an oxidative agent

Lastly, we investigated the systemic impact of PAA and crowding, alone or in combination, by subjecting the plasma to metabolomic profiling. Analysis of the samples resulted in the detection of 639 compounds; of these, 138 were annotated on Level 3, 66 on Level 2b, 12 on Level 2a, and 42 on Level 1. The score plot from a PCA model calculated on the compounds annotated on levels 1, 2a, or 2b in the reduced dataset shown in Figure 5A demonstrated no clear separation amongst treatment groups. Inspection of groupings in higher-order PCs shows some treatment-related clusters in PC5 and PC6 (Figure 5B), indicating that crowding and sampling time had a more substantial effect than PAA treatment. Though quite minimal, PAA effect was more distinguishable in the group subjected to crowding before exposure.

The univariate data analysis identified 11 compounds, including guanine, xanthine, guanosine, disperse orange 3, 4-hydroxybutyric acid (GHB), 2-amino-1-propanol, N-benzylformamide, 4-hydroxybenzaldehyde, tyrosine, methionine sulfoxide, and laurolactam, that were significantly affected by the treatments (Table 2). These significantly affected metabolites support the PCA models

(Figure 5A, B) showing that the most significant differences were related to the effects of crowding and sampling time, and not PAA. Exposure to PAA affected only the concentration of 2-amino-1- propanol, which increased regardless of crowding history. It is difficult to reach a conclusion about the relevance of the modulation of 2-amino-1- propanol plasma level in relation to PAA, as, besides being annotated to Level 2b, no known biological function has yet been identified in fish. Hence, the physiological importance of its modulation following PAA exposure regardless of crowding history is worthy of future investigation. Crowding alone affected the levels of six compounds, including guanine, guanosine, 4-hydroxybutyric acid (GHB), Nbenzylformamide, 4-hydroxybenzaldehyde, and tyrosine, at 4 h p.e. However, the effects disappeared 2 w p.e. Tyrosine is a common precursor to hormones and neurotransmitters with essential roles during stress response in fish [202]. The plasma free tyrosine levels have been found to increase during acute stress in fish, suggesting the importance of tyrosine during a stress episode [203, 204]. Such a similar mechanism may be employed by salmon exposed to crowding stress. Exposure to PAA in crowded fish resulted in significant changes in guanine, guanosine, xanthine, and disperse orange 3, of which both guanine and xanthine were annotated to Level 1. Considering that xanthine can be created from guanine, these results indicate that the combination of crowding and PAA exposure may interfere with this specific pathway. DNA bases, specifically guanine, are very much susceptible to oxidation due to their having a low redox potential [205]. In addition, DNA damage associated with oxidative stress is mediated by guanine [206]. Therefore, the significant changes to these compounds, specifically guanine, reveals that crowding may influence the systemic oxidative potential, where the compound plays a vital role as mediator of the adaptive response. We have reported earlier that crowding before PAA exposure restricted the potential to produce antioxidants in the plasma [81]. Hence, the changes identified here may partly explain such a phenomenon. It is important to note that guanine is the sole compound affected by crowding alone and its combination with PAA, highlighting its potential as a biomarker for PAA exposure in salmon. Overall, the metabolome profiles indicate that PAA exposure did not result in substantial metabolomic disturbances.

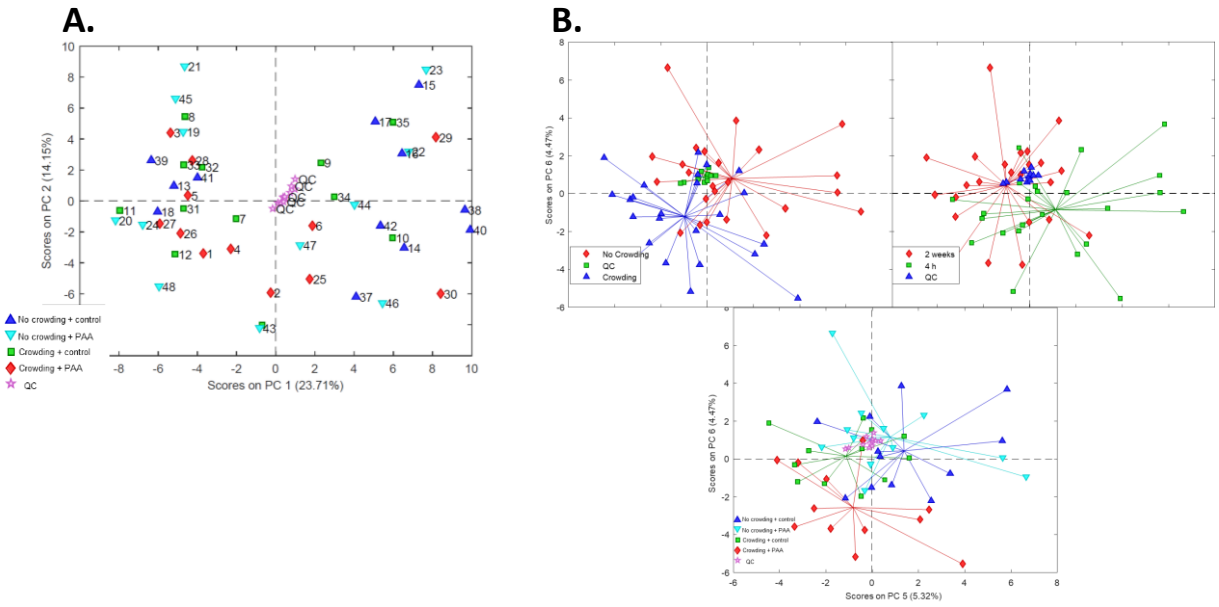

Figure 5. Plasma metabolomes of Atlantic salmon post-smolts 4 h and 2 weeks after PAA exposure with and without crowding history. Panel A: Score plot from the PCA model calculated on the relative concentrations of the variables in the reduced dataset. Data have been auto scaled. Panel B: Score plots from higher PCA models derived from the relative concentrations of the variables in the reduced dataset, showing the treatment of data, depending on crowding history, sampling point, and their combinations.

Table 2. Plasma metabolites significantly affected by at least one of the factors in the study

Annotation level	Metabolite ID	Factor				
		Effect of PAA exposure in crowded fish	Combined effects of crowding and PAA exposure	Effects of crowding	Effects of crowding when exposed to PAA	Effect of PAA exposure in non-crowded fish
1	Guanine	N;N*	**Y**;N	**Y**;N	N;N	N;N
2a	Guanosine	N;N	**Y**;N	**Y**;N	**Y**;N	N;N
1	Xanthine	N;N	**Y**;N	N;N	N;N	N;N
2b	Disperse orange 3	N;N	**Y**;N	N;N	N;N	N;N
2b	Methionine sulfoxide	N;N	N;N	N;N	N;**Y**	N;N
2b	Laurolactam	N;N	N;N	N;N	N;**Y**	N;N
2b	4-Hydroxybutyric acid (GHB)	N;N	N;N	**Y**;N	N;N	N;N
2b	2-Amino-1-propanol	N;**Y**	N;N	N;**Y**	N;N	N;**Y**
2b	N-Benzylformamide	N;N	N;N	**Y**;N	N;N	N;N
2b	4-Hydroxybenzaldehyde	N;N	N;N	**Y**;N	N;N	N;N
1	Tyrosine	N;N	N;N	Y;N	N;N	N;N

Notations: *The first letter indicates the response at 4 h, while the second letter denotes the response at 2 weeks post-exposure. **Y** = means the change was statistically significant, *P*-value < 0.05; N = means the change was not statistically significant, *P*-value > 0.5

Conclusions

The global response repertoire presented here contributes to a better understanding of the physiological consequences of PAA use in fish. Salmon post-smolts responded to PAA exposure by activating different mucosal and systemic molecules, many of which are relevant in defence, structural integrity, oxygen transport, and oxidative stress. The gills were notably more responsive than the skin to the PAA dose used, especially at a molecular level. We have demonstrated that the ability of salmon to respond to PAA was differentially affected by crowding, a common production protocol employed during peroxide treatment at sea in salmon farming. Nonetheless, such an interfering factor was more pronounced at the mucosa, particularly the gills, as compared to the circulating metabolome. Assessment of the impacts from different levels of biological organizations provides a much broader resolution of the physiological consequences of PAA, thereby underlining the health and welfare aspects of its use in salmon. Taken together, the response to PAA at the tested concentration and temperature was localized (i.e., mucosal) and did not result in a dramatic systemic metabolomic dysregulation. These results further support the use of PAA as a beneficial aquaculture treatment with minimal adverse welfare impact on treated fish. In a commercial situation, negative impacts can likely best be minimized by careful management of fish crowding protocols. It would be interesting to explore in the future the influence of fish size and temperature on the responses of salmon to PAA.

5.2.3 Effects of periodic high dose PAA treatment

The results from this section appeared on 2 separate publications and 1 currently under revision.

Lazado, C.C., Voldvik, V., Breiland, M.W., Osório, J., Hansen, M. S., Krasnov, A. 2020. Chemical oxidative stressors alter the physiological state of the nasal olfactory mucosa of Atlantic salmon. *Antioxidants*. 9 (11), 1144.

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Oxidative Chemical Stressors Alter the Physiological State of the Nasal Olfactory Mucosa of Atlantic Salmon

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ABSTRACT

The olfactory organs of fish have vital functions for chemosensory and defence. Though there have been some ground-breaking discoveries of their involvement in immunity against pathogens in recent years, little is known about how they respond to non-infectious agents, such as exogenous oxidants, which fish encounter regularly. To this end, we employed Atlantic salmon (*Salmo salar*) as a model to study the molecular responses at the nasal olfactory mucosa of a teleost fish when challenged with oxidants. Microarray analysis was employed to unravel the transcriptional changes at the nasal olfactory mucosa following 2 types of *in vivo* exposure to peracetic acid (PAA), a highly potent oxidative agent commonly used in aquaculture: Trial 1: periodic and low dose (1 ppm, every 3 days over 45 days) to simulate a routine disinfection; and Trial 2: less frequent and high dose (10 ppm for 30 min, every 15 days, 3 times) to mimic a bath treatment. Further, leukocytes from the olfactory organ were isolated and exposed to PAA, as well as to hydrogen peroxide (H_2O_2) and acetic acid (AA) – the two other components of PAA trade products – to perform targeted cellular and molecular response profiling. In

the first trial, microarrays identified 32 differentially expressed genes (DEG) after a 45-day oxidant exposure. Erythrocyte–specific genes were overly represented and substantially upregulated following exogenous oxidant exposure. In Trial 2, in which a higher dose was administered, 62 DEGs were identified, over 80 % of which were significantly upregulated after exposure. Genes involved in immune response, redox balance and stress, maintenance of cellular integrity and extracellular matrix were markedly affected by the oxidant. All chemical stimuli (i.e., PAA, H_2O_2, AA) significantly affected the proliferation of nasal leukocytes, with indications of recovery observed in PAA- and H_2O_2-exposed cells. The migration of nasal leukocytes was promoted by H_2O_2, but not much by PAA and AA. The three chemical oxidative stressors triggered oxidative stress in nasal leukocytes as indicated by an increase in the intracellular reactive oxygen species level. This resulted in the mobilisation of antioxidant defences in the nasal leukocytes as shown by the upregulation of crucial genes for this response network. Though qPCR revealed changes in the expression of selected cytokines and heat shock protein genes following *in vitro* challenge, the responses were stochastic. The results from the study advance our understanding of the role that the nasal olfactory mucosa plays in host defence, particularly towards oxidative chemical stressors.

Introduction

Oxidative stress is a physiological state in an organism in which the redox balance is altered, as characterised by an increase in the levels of reactive oxygen species (ROS) but normal or low amounts of antioxidants, which may be due to compromised neutralisation property and/or scavenging potential [207, 208]. Fish, like many other organisms, have an extensive repertoire to counteract oxidative stress [208, 209]. The integrated antioxidant systems, which include enzymatic and nonenzymatic antioxidants, are at the forefront of blocking the harmful effects of ROS [207]. Redox imbalance associated with oxidative stress promotes genetic instability, changes in gene expression patterns, alterations in cellular signalling cascades/cell metabolism, and disruption of the cell cycle, leading to several pathophysiological conditions [210, 211].

Oxidants can be endogenously produced or derived from external sources. Endogenous ROS are produced from molecular oxygen as a result of normal cellular metabolism [207], and ROS are constantly produced in all living cells in which roughly up to 1 % of an animal's total oxygen consumption may be attributed to ROS generation and detoxification [212]. Exogenous ROS may come from various sources, and their impacts on redox status have consequences on cell viability, activation, proliferation, and organ function. Farmed fish encounter an increased flow of exogenous ROS several times during a lifetime, as many husbandry practices employ ROS-generating compounds either as a form of disinfectant or water treatment, or as a chemotherapeutant [62, 81, 181, 213-215]. The antimicrobial activity of ROS towards opportunistic and pathogenic microorganisms underlines their use in providing fish with a favourable rearing environment [216]. Nonetheless, our knowledge of the physiological alterations associated with exogenous ROS, mainly from ROS-generating agents being used in fish farming, is fragmentary.

Mucosal organs of fish are multifunctional; besides their role in defence, they carry a multitude of other physiological functions [68, 217]. They are often considered the first line of defence because these structures interact with the water matrix where several biological and chemical challenges present themselves regularly. In recent years, there has been a dramatic development in the study of the physiology and immunology of mucosal surfaces in fish, driven mostly by their warranted importance in maintaining the health of farmed fish [68, 218].

The nasal olfactory system plays a role not only in chemoreception but also in immune defence, as it is considered an ancient component of the mucosal immune system of vertebrates [219]. It is a highly specialised sensory organ for the detection and identification of minute quantities of chemicals in the

environment [220, 221]; and because water constantly circulates through the nasal cavities, they are continuously prompted with environmental challenges [222]. The mucosal regions of the fish olfactory lamellae have different cellular elements such as goblet cells, sustentacular cells, olfactory sensory neurons, and, most importantly, a rich assemblage of immune cells [222-224]. Vertebrate olfactory sensory neurons rapidly sense chemical stimuli in the environment and transduce signals to the central nervous system [221]. The nasopharynx-associated lymphoid tissue (NALT) protects the teleost olfactory organ from water-borne pathogens, just as for airborne pathogens in terrestrial animals [219]. Several recently published studies have demonstrated how viral and bacterial stimulations affect the immunological repertoire of the nasal mucosa in fish. They reveal a very distinct microenvironment that can mount a localised immunity and, at the same time, influence distant immune functions [217, 219, 222, 223, 225]. Non-infectious agents such as exogenous oxidants are delivered via water and are expected to pass through the nasal cavity of fish. In mammalian models, oxidative stressors are highly potent modulators of the nasal epithelium, and the interaction could induce morphological and pathological alterations [226-228]. However, in fish, the influence of exogenous oxidants on the nasal olfactory mucosa is barely explored, despite its common use.

This study explored the impacts of oxidative chemical stressors on the nasal olfactory mucosa of Atlantic salmon (*Salmo salar*). We employed both *in vivo* and *in vitro* strategies to unravel the molecular changes in the nasal olfactory mucosa when challenged with exogenous oxidants relevant in fish farming. In this study, we employed peracetic acid (PAA) as the main oxidant, as it is currently being developed as a chemotherapeutant (i.e., for amoebic gill disease, AGD; disinfection against *Yersinia ruckeri*) for salmon [86, 213], and the results here are expected to help underline its potential for use. Both *in vivo* trials were designed to simulate the prospective use of PAA as either a routine disinfectant (Trial 1) or a treatment for a parasitic infection (Trial 2). In addition, *in vitro* trials were conceived to understand the physiological state of a specific cell type at the mucosa in response to not only PAA but also hydrogen peroxide (H_2O_2) and acetic acid (AA). These two compounds are present in equilibrium with PAA in its trade product.

Materials and methods

Oxidant exposure experiment

All fish handling procedures described in this paper followed the Guidelines of the European Union (2010/63/EU) and the *in vivo* exposure trials received approvals from the Norwegian Food Safety Authority (FOTS IDs 20831, 19321). All key personnel have a FELASA C certificate to conduct experimentation on live animals. Two independent *in vivo* trials (Figure 1) were performed in which the application of the exogenous oxidant was based on its proposed use for that particular stage of Atlantic salmon production. It was ensured that all fish used in the experiments (both *in vivo* and *in vitro*) did not have a history of oxidant exposure. Peracetic acid is available under different trade products and two commercially available PAA-based disinfectants were used in this study. All three major components of PAA trade (i.e., PAA, hydrogen peroxide and acetic acid) products have disinfection power, though PAA is the most potent and contributes to the main disinfection property of PAA-based disinfectants.

Trial 1 was conducted at Nofima Centre for Recirculation in Aquaculture (NCRA; Sunndalsøra, Norway) and was aimed at evaluating the impacts of periodic low-dose oxidant exposure on the transcriptome of the nasal olfactory mucosa. This experiment was designed to mimic the use of the oxidant as a routine water disinfectant in a recirculating aquaculture system [80]. Briefly, each of the four 3.2 m^3 octagonal tanks in a recirculation system was stocked with 735 smolts with an average weight of around 90 g. After four weeks of acclimatisation, the oxidant in the form of a peracetic acid-based disinfectant (Perfectoxid, Novadan ApS, Denmark) was directly applied to each tank at a nominal concentration of 1 ppm every 3 days for 45 days, making a total of 15 applications in the duration of the trial. This mode

of application was patterned on a previous PAA experiment conducted in rainbow trout, a closely related species of salmon [80]. Moreover, the concentration is within the range safe for use in salmon [210, 229]. It was ensured that the application of PAA on each occasion was between 0900-1000 to avoid temporal effects. The following parameters were maintained during the trial: water flow rate at 100 L min^{-1}, salinity at 11.6 ± 0.5 ‰, temperature at 12.8 ± 0.6 °C, pH at 7.5, dissolved oxygen > 90 % saturation, photoperiod at 24 L: 0 D, and a continuous feeding regime (Nutra Olympic 3 mm, Skretting, Averøy, Norway).

Trial 2 was performed at Havbruksstasjonen i Tromsø (HiT; Tromsø, Norway) and was designed to unravel the changes in the nasal transcriptome after repeated but less frequent exposure to higher doses of oxidant. PAA is currently being explored as a potential treatment for amoebic gill disease (AGD), a gill health issue affecting mostly seawater-adapted salmon [81, 213]. The trial was designed to simulate an oxidant exposure as a treatment protocol for AGD [86]. Forty fish with an average weight of 80-90 g were stocked into a 500 L circular tank in a flow-through system. There were six tanks in total: three for the control group and three for the oxidant-exposed group. Fish were allowed to acclimatise for a week before the first oxidant treatment was performed. Fish were fasted for 24 h prior to each treatment occasion. Oxidant treatment was performed as follows: Water flow in the tank was stopped. Thereafter, the oxidant (Divosan Forte™, Lilleborg AS, Norway) was added to the water column to achieve a final concentration of 10 ppm. This concentration was 2x higher than the concentration we earlier tested and reported [210]. Aeration was supplied to enable mixing and to maintain the oxygen level. After 30 min, the water flow was opened, and over 90 % of the water was replaced within 10 min. Feeding was continued a day after the exposure. This exposure protocol was performed every 15 days and there were three exposure occasions in the whole trial. The following parameters were maintained during the trial: water flow rate at 6-7 L min^{-1}, salinity at 35 ‰, temperature at 12.0 ± 1 °C, dissolved oxygen > 90 % saturation, photoperiod at 24 L: 0 D, and a continuous feeding regime (Nutra Olympic 3 mm, Skretting, Averøy, Norway).

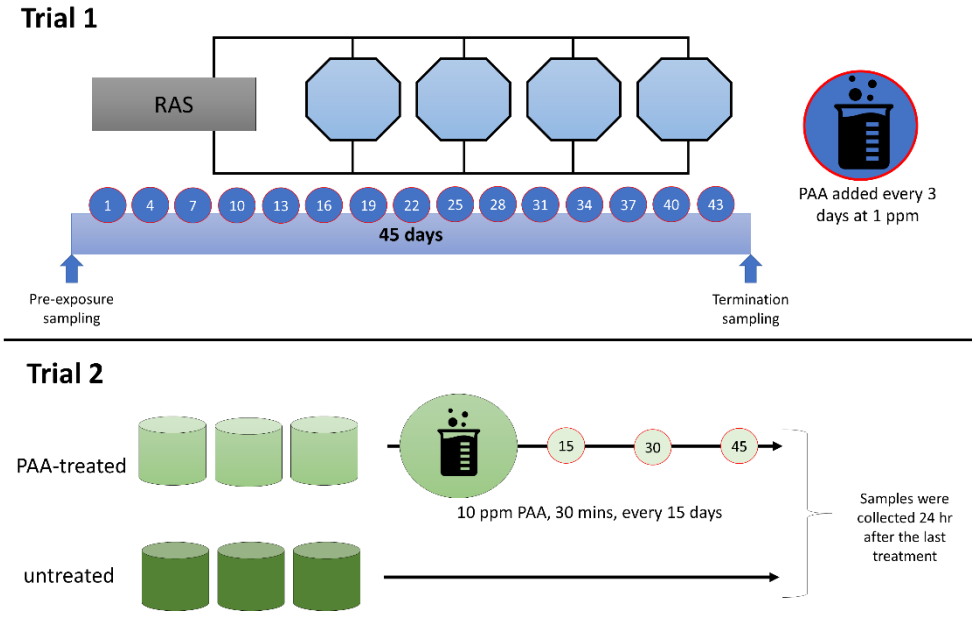

Figure 1. Diagrammatic summary of the in vivo trials. Trial 1 aimed to profile the impacts of periodic and low dose PAA application (1 ppm, every 3 days over 45 days); while Trial 2 was designed to investigate the nasal responses following less frequent and high dose PAA treatment (10 ppm for 30 min, every 15 days, 3 times). Details of each trial are described in section 2.1.

Olfactory organ collection

All fish for sampling were humanely euthanised with an overdose of either Tricaine methanesulfonate (Trial 1; MS222, PHARMAQ Ltd, Norway) or Benzocaine (Trial 2; Benzoak®, ACD Pharmaceuticals AS, Norway). Percussive stunning was avoided because it triggered the influx of blood to the head, including the olfactory epithelium. Olfactory organ was collected by opening the nostrils to expose the outermost section of the nasal mucosa. The rosette from the left side was then dissected out, immediately suspended in RNA*later*™ (Thermo Fisher, USA), kept at room temperature overnight for penetration, and then stored at -70 °C until RNA isolation. In Trial 1, olfactory rosettes (n = 8 per time-point) were collected before and 45 days (i.e., 24 h after the last PAA application) after the start of periodic oxidant exposure. In Trial 2, the tissue samples (n = 9, per group) were collected 24 h after the 3rd exposure.

Isolation of leukocytes from olfactory organ

Leukocytes from olfactory organ were isolated from 15 freshwater-adapted salmon (*ca* 80-90 g) with similar genetic background, following a previously published method [225], with slight modifications. Briefly, fish were humanely euthanised with an anaesthetic overdose (Aqui-S, MSD Animal Health, Norway). The olfactory organs from both sides were dissected and immediately placed in a modified L-15 (supplemented with 5 % foetal bovine serum, 1 % Penstrep, 1 % HEPES) on ice. Rosettes from all fish were combined, and the tissues were cut into small pieces (0.5-1 cm) and mechanically dissociated by incubating the tissue suspension at 4 °C for 30 min with constant agitation. The cell supernatant was collected and temporarily stored at 4 °C. The remaining tissue fragments were suspended in modified L-15 medium, and the process of mechanical dissociation was repeated four times. The collected supernatant from the four recurrences was combined and stored at 4 °C. The remaining tissue fragments were suspended in phosphate-buffered saline (PBS) with EDTA (1 mM) and DTT (0.9 mM) and incubated at 4 °C for 30 min with constant gentle agitation. The PBS supernatant was thereafter discarded. Enzymatic digestion was carried out by incubating the remaining fragments in collagenase solution (0.15 mg/mL in L-15, with 1 % Penstrep) for 2 h at room temperature (20 °C) with agitation. The supernatants from mechanical dissociations and enzymatic digestion were combined, gently passed through a 100 µm filter, and spun down at 300 *g* for 10 min. The cell pellet was washed and resuspended in modified L-15, laid over a 34%/51% Percoll® (Sigma-Aldrich, Norway) gradient, and centrifuged for 30 min at 400 *g* at 4 °C. The cell layer between the gradients was carefully transferred to a tube with modified L-15 medium, centrifuged for 10 min at 400 g at 4 °C, and suspended in new modified L-15 medium. Cell viability and number were determined by CellCountess™ II (Thermo Fisher). The cells were seeded out onto a 12-well plate (Corning® CellBIND® Surface, Sigma-Aldrich) at a density of 2 x 10^5 cells per well and incubated at 13 °C.

In vitro exposure trial

The cells were allowed to adhere for 48 h before the exposure was performed. The leukocytes were exposed to three chemical stressors at physiological concentrations – 100 µM PAA, 100 µM H_2O_2, and 100 µM AA – for 30 minutes. The concentrations were based on several preliminary *in vitro* trials and the concentration was selected because they were able to trigger significant increase in intracellular ROS, thereby providing an indication that the internal redox balance had been altered by the tested oxidants. Each treatment group had four independent wells. Untreated cells served as control and were handled similarly as the treatment groups, though no chemical stressor was added. After 30 min, the media were removed, cells were washed gently with modified L-15, and 300 µL of the same media was added to each well. After 24 h, the media were removed. The cells were suspended in lysis buffer (ZYMO *Quick*-RNA™ Microprep kit, USA) and scraped, and the cell suspension was stored at 70 °C until RNA isolation.

Proliferation and migration assays

Nasal leukocytes were isolated from 12 freshwater-adapted salmon (ca 80-90 g) following the method described in section 2.3 and seeded onto a 96-well plate (Corning®) at a density of 10^5 cells per well. After the cells were allowed to settle and adhere for 24 h, they were exposed to PAA, H_2O_2, and AA at a physiological concentration of 100 µM for 30 min and washed. New media were added and the exposed cells were allowed to recover in the incubator. Unexposed cells, serving as control, were likewise washed, and new media were added. Cell proliferation (proxy for cytotoxicity) was measured using the CyQUANT Proliferation Assay Kit (Thermo Fisher) 24 and 48 h after the challenge. Each treatment group, including the unexposed control group, had six replicate wells. Rate of proliferation was expressed relative to the control group of that time-point.

The effects of the chemical stressors on cellular migration were determined by the CytoSelect™ Cell Migration Assay kit (Cell Biolabs, Inc., USA). Freshly isolated nasal leukocytes were suspended in modified L-15 medium without serum. The lower receptacle of the migration chamber was added with L-15 media containing either PAA, H_2O_2, or AA in a final concentration of 100 µM. Wells with L-15 medium containing 10 % FBS served as the positive control for chemotaxis while L-15 medium alone was designated as a negative control. Each treatment, including the controls, had been assigned three wells. Thereafter, the cells were added to the upper receptacle of the migration chamber at a density of 2 x 10^5 cells. The migration chamber was incubated for 24 h at 13 °C before the migratory cells were dislodged from the membrane, lysed and labelled with CyQuant® GR dye solution, and fluorescence was read at 480 nm/520 nm.

Intracellular ROS quantification

Nasal leukocytes were isolated, cultured, and treated with the chemical stressors as described in detail in sections 2.4 and 2.5. The intracellular level of reactive oxygen species (ROS) in the treated cells, including untreated control, was quantified using the OxiSelect™ Intracellular ROS Assay Kit (Cell Biolabs, Inc.) at 24 and 48 h after challenge. The level of ROS is given as a proportion of fluorescent dichlorodihydrofluorescein (DCF).

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

The RNA from both olfactory tissues and nasal leukocytes were isolated using a commercially available kit (*Quick*-RNA™ Microprep kit). RNA concentration was measured in a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, DE, USA), and the quality of the samples for microarray was further assessed using an Agilent® 2100 Bioanalyzer™ RNA 6000 Nano kit (Agilent Technology Inc., Santa Clara, CA, USA). All samples had an RNA Integrity Value higher than 8.8.

A High Capacity RNA-to-cDNA Reverse Transcription kit (Applied Biosystems, USA) was used to prepare the complementary DNA from the nasal leukocyte samples using 300 ng RNA input following a synthesis protocol of 25 °C for 10 min, followed by 37 °C for 120 min and then 5 min at 85 °C. The expression of selected antioxidant defence, cytokines, and heat shock protein genes was quantified using the PowerUp™ SYBR™ Green master chemistry (Applied Biosystems) in a QuantStudio5 real-time quantitative PCR system (Applied Biosystems). The qPCR reaction mixture included 4 µL of diluted cDNA, 5 µL SYBR™ Green Master (Thermo Fisher), and 1 µL of the forward and reverse primer. All samples were run in duplicate, including minus reverse transcriptase and no template controls. The thermocycling protocol included pre-incubation at 95 °C for 2 min, amplification with 40 cycles at 95 °C for 1 s and at 60 °C for 30 s, and a dissociation step series of 95 °C for 15 s, 60 °C for 1 min, and 95 °C for 15 s. Amplification efficiency was calculated from a five-point standard curve of 2-fold dilution series of pooled cDNA. The expression of the target genes was normalised using three reference genes, namely e*longation factor 1a* (*eef1a*), *acidic ribosomal protein* (*arp*), and *β-actin* (*actb*) [230].

Microarray analysis

Olfactory rosettes from Trials 1 and 2 were subjected to microarray analysis using Nofima's Atlantic salmon DNA oligonucleotide microarray SIQ-6 (custom design, GPL16555), which contains 15 K probes for protein-coding genes involved in immunity, tissue structure, integrity and functions, cell communication and junctions, and extracellular matrix, amongst many others [88]. Agilent Technologies manufactured and supplied the microarrays, reagents, and equipment used in the analysis. Using 100 ng of total RNA template per reaction, RNA was amplified using a One-Color Quick Amp Labeling Kit, and thereafter Cy3 was labelled. Subsequently, fragmentation of the labelled RNA was carried out using a Gene Expression Hybridization Kit and hybridisation followed in an oven thermostatted at 65 °C with a constant rotation speed of 10 rpm for 17 h. The arrays were washed in sequence with Gene Expression Wash Buffers 1 and 2 and were scanned through an Agilent SureScan Microarray scanner. Data processing was carried out in Nofima's bioinformatics package STARS.

Data handling and statistics

The significant difference in the transcript level of the target marker genes between before and after periodic oxidant exposure in Trial 1 and between the unexposed-control and oxidant-exposed groups in Trial 2 was determined by Student's t-test for independent samples; the threshold of differential expression in microarray analyses was 1.75-fold. The level of significance was set at 5 % ($p < 0.05$).

A Shapiro-Wilk test was used to evaluate the normal distribution and a Brown-Forsyth test to check for equal variance of the proliferation assay and gene expression data set. Two-way ANOVA was then employed to investigate significant differences amongst treatment groups over time. In addition, the Holm-Sidak test was used to identify pairwise differences. One-way ANOVA was used for migration assay data. All statistical tests were performed using SigmaPlot 14.0 Statistical Software (Systat Software Inc., London, UK), with a level of significance set at $P < 0.05$.

Results

Transcriptomic changes in the olfactory rosettes from Trial 1

After 45 days of periodic low-dose oxidant exposure, microarray analysis identified 32 differentially regulated genes (DEG) in the nasal olfactory rosette (Table 1). The numbers of upregulated (16/32) and downregulated genes were equal (16/32). Erythrocyte-specific genes were the most represented group, with nine transcripts/variants identified as being upregulated in response to the exogenous oxidant. Genes involved in immune response such as *c-c motif chemokine 28*, *interleukin 13 receptor alpha-2*, *defensin beta 4*, *ig heavy chain*, and *mannose-specific lectin-like*, were all downregulated after 45 days of oxidant exposure. Two genes encoding cytokeratins were downregulated. Three out of four genes with metabolic functions were likewise downregulated.

Table 1. Some of the differentially expressed genes in the olfactory rosette of salmon from Trial 1. Transcripts are annotated for their known or predicted function. Expression data are ratios of means of 45 days after the exposure to pre-exposure.

Annotation	Name	Fold
Cytoskeleton	*Keratin, type I cytoskeletal 20*	-2,69
Cytoskeleton	*Keratin, type I cytoskeletal*	-3,16
DNA replication	*DNA replication licensing factor MCM6*	2,34
Chemokine	*C-C motif chemokine 28*	-4,33
Cytokine receptor	*Interleukin 13 receptor alpha-2*	-1,83
Antibacterial	*Defensin beta 4*	-2,60
B cell	*Ig heavy chain*	-3,69
Lectin	*Mannose-specific lectin-like*	-2,20
Lipid metabolism	*Phosphoethanolamine/phosphocholine phosphatase*	-2,43
Protease	*Duodenase-1*	-2,20
Protease	*Serine protease 23-like*	1,76
Energy metabolism	*Pyruvate dehydrogenase kinase isozyme 2*	-2,12
Tissue ECM mucus	*Mucin-2*	2,14
Erythrocyte	*Hemoglobin subunit beta-1*	2,58
Erythrocyte	*Hemoglobin subunit alpha (5)*	2,80
Erythrocyte	*Hemoglobin subunit alpha-4-like (2)*	5,40
Erythrocyte	*Hemoglobin subunit beta*	1,76

[a]For genes with several variants (number enclosed in parentheses), mean values are presented.
NB: The complete list of DEGs is given in Supplementary File 2.

Transcriptomic changes in the olfactory rosettes from Trial 2

Sixty-two DEGs were identified in the olfactory rosettes from fish exposed to an oxidant on three occasions, 56 of which, accounting for 82 % of the DEGs, were upregulated (Table 2). From this group, genes related to immunity, including cytokines and effectors, were largely represented with 14 upregulated transcripts. Genes with innate immune functions constitute a considerable number in the DEG panel. Genes with known involvement in cellular structural integrity such as *keratin* and *plekstrin* were likewise upregulated. A similar effect was observed on genes encoding extracellular proteins (e.g., *fibronectin*, *mucin5b*). Several genes involved in various metabolic pathways were represented in the DEGs panel, such as those involved in amine, amino acid, calcium, and xenobiotic metabolism. 3/4 DEGs of lipid metabolism were downregulated following oxidant exposure. Exogenous oxidant exposure upregulated the expression of genes with function in cellular processes such as DNA replication, signalling, and protein folding/modification including the *heat shock proteins*. Oxidant-induced changes in cellular redox balance were likewise manifested with two DEGs.

Effects of oxidative chemical stressors on leukocyte proliferation and migration

The proliferation of nasal leukocytes 24 h after the challenge was significantly affected by PAA, as well as by its two main components, H_2O_2 and AA (Figure 2A). Cellular proliferation reduced by at least 0.5-fold in all treatment groups and no inter-treatment differences were observed. After 48 h, nasal leukocytes exposed to PAA and H_2O_2 slightly recovered, and the proliferation rate did not significantly differ from that of the control group. However, the effect of AA on proliferation was still persistent after 48 h, when the proliferation index in the group was 0.6-fold lower compared to control. Moreover, a significant difference was observed between the AA-exposed and two other treatment groups.

PAA and AA did not significantly affect the migration potential of the nasal leukocytes (Figure 2B). On the other hand, H_2O_2 promoted the migration of nasal leucocytes with a significant increase compared to control. A comparison of treatments revealed that H_2O_2-induced migration was significantly different from PAA but not from AA.

Table 2. Some of the differentially expressed genes in the olfactory rosette of salmon from Trial 2 are annotated for their known or predicted function. Expression data are ratios of means of the 10 ppm PAA-treated group to the unexposed/control treated group. Samples were collected 24 h after the 3rd exposure.

Annotation	Name	Fold
Cytoskeleton	*Keratin 14*	1,83
Cytoskeleton	*Keratin 4*	2,05
Cytoskeleton	*Keratin cytoskeletal 17*	3,48
Cytoskeleton	*Pleckstrin 2*	2,11
DNA replication	*DNA replication licensing factor MCM6*	1,75
Protein folding	*14-3-3 protein alpha*	1,82
Protein folding	*Heat shock cognate 70 (2)*	3,63
Protein folding	*Heat shock protein 90, alpha (2)*	3,53
Signaling	*Guanine nucleotide binding protein*	2,06
Redox homeostasis	*Glutathione reductase, mitochondrial*	2,26
Redox homeostasis	*Redox-regulatory protein FAM213A*	-2,67
Signaling	*Tyrosine phosphatase type IVA, member 1 (2)*	1,97
Cell Surface	*Vacuole membrane protein 1*	2,12
B cell	*IgH-locus*	1,90
Cytokine receptor	*Interferon-alpha/beta receptor alpha chain*	1,93
Cytokine receptor	*Interleukin 13 receptor alpha-2*	1,96
Cytokine receptor	*Interleukin-1 receptor type II (2)*	2,37
Effector	*Differentially regulated trout protein 1*	2,85
Effector	*Ornithine decarboxylase (3)*	2,28
Effector	*Thrombin-like enzyme cerastocytin*	2,85
Lymphocyte	*T-lymphocite maturation associated protein*	2,72
T cell	*CD276 antigen-like*	2,66
TNF	*TNF decoy receptor*	1,90
Effector	*Spermidine/spermine N1-acetyltransferase 1*	4,07
Amino acid metabolism	*Methionine adenosyltransferase II, alpha b*	1,97
Calcium metabolism	*Protein S100-A1*	2,22
Iron metabolism	*Ferritin, heavy polypeptide 1b (5)*	2,34
Lipid metabolism	*Mid1-interacting protein 1-like*	-1,93
Lipid metabolism	*Phospholipid transfer protein*	-1,92
Lipid metabolism	*Short-chain dehydrogenase/reductase 3*	-2,18
Mitochondria	*Malic enzyme 3, NADP*	-1,87
Protease	*Calpain 9 (2)*	2,24
Steroid metabolism	*Cholesterol 25-hydroxylase-like protein A*	1,92
Sugar metabolism	*Glycogen debranching enzyme*	-1,90
Xenobiotic metabolism	*Glutathione S-transferase P-like*	2,44
Tissue ECM	*Fibronection*	1,74
Collagen	*Collagen alpha-2(VI) chain*	-3,01
Mucus	*GMP Giant mucus protein*	1,85
Mucus	*Mucin-5B (2)*	1,91
Mucus	*Arylalkylamine N-acetyltransferase*	-1,92
Epithelium	*Epithelial membrane protein 2-like*	1,75
Secretory	*Gastrotropin-like*	2,83
Lipid metabolism	*Globoside alpha-1,3-N-acetylgalactosaminyltransferase 1-like*	2,62

[a]For genes with several variants (number enclosed in parentheses), mean values are presented.

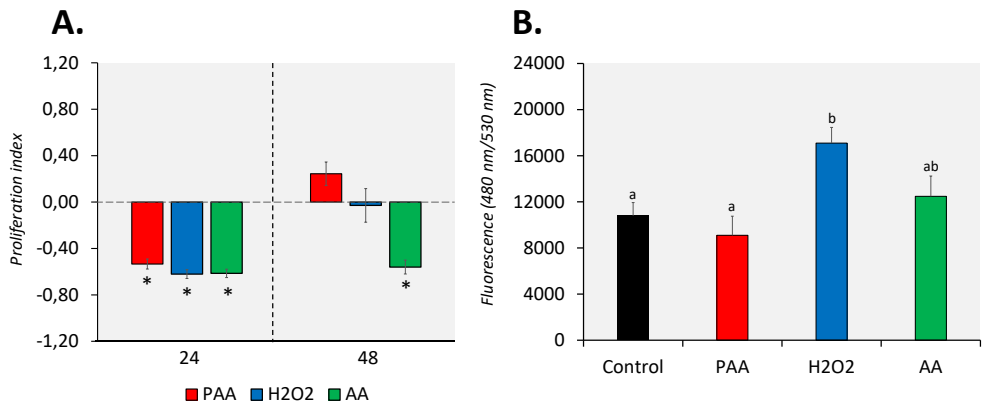

Figure 2. Effects of oxidative chemical stressors on the (A) proliferation and (B) migration of nasal olfactory leukocytes. For the proliferation assay, cells were isolated and cultured for 2 days before they were treated with 100 µM of PAA, H_2O_2, and AA for 30 min. Proliferation was quantified 24 and 48 h after challenge. Asterisk (*) denotes that proliferation was significantly affected relative to the unstimulated group. Results are presented as index of proliferation, where we expressed the proliferation relative to the control, unstimulated group. Two-way ANOVA followed by the Holm-Sidak test identified statistical difference amongst treatment groups over time. For migration assay, cells were allowed to migrate to a chamber containing the chemical stressor at a 100 µM concentration for 24 h. The positive control group has foetal bovine serum (FBS) as a chemoattractant. One-way ANOVA was used for migration assay data. Different letters indicate significant difference at P<0.05. Results were presented as mean±SD of 5 wells, with cells from 12 fish.

Level of ROS in chemically stressed cells

The three chemical stressors significantly increased the intracellular ROS level of nasal olfactory leukocytes 24 h after challenge (Figure 3). The highest increment was identified in H_2O_2-exposed cells, followed by PAA and AA. In addition, the intracellular ROS of PAA- and H_2O_2-exposed cells were significantly higher than in the AA-exposed group at this time-point. After 48 h, the ROS level in PAA and AA-exposed cells displayed no significant difference from the control. The intracellular ROS in H_2O_2-exposed cells remained elevated 48 h after challenge. Moreover, the level was significantly higher compared to the control and AA-exposed cells but not to the PAA-exposed cells. Though not statistically significant, the level in PAA- and H_2O_2-exposed cells was apparently lower than the measured level at 24 h post-challenge.

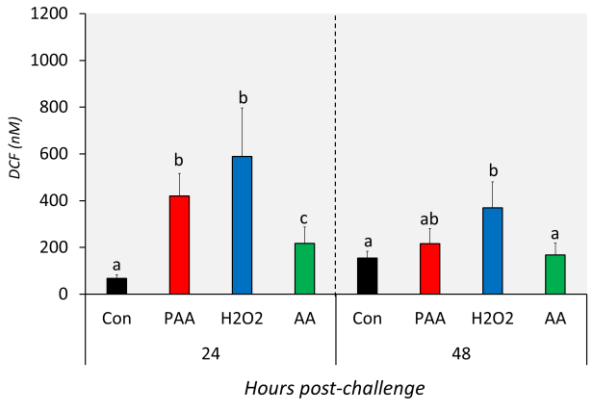

Figure 3. *Levels of intracellular reactive oxygen species in nasal olfactory leukocytes exposed to different oxidative chemical stressors. The level was measured at 24 and 48 h after exposure to 100 µM of PAA, H_2O_2, and AA for 30 min. The control group was handled similarly, but without any chemical stimulation. Two-way ANOVA followed by the Holm-Sidak test identified statistical difference amongst the treatment groups over time. Different letters indicate a significant difference at P<0.05 between groups within a time-point. There was no time-related difference within a treatment group. Results were presented as mean±SD of 5 wells, with cells from 12 fish.*

Changes in the expression of antioxidant defence genes in the nasal olfactory leukocytes

The expression of six genes with known function in antioxidant defence was differentially affected by the three chemical stressors (Figure 4). The transcript level of *gpx* was significantly higher in PAA- and H_2O_2-exposed cells compared to control at both time-points (Figure 4A). Such an increase was only identified in AA-exposed cells 48 h after challenge and the expression was significantly higher than the level at 24 h post-challenge. Chemically induced stress resulted in quite the opposite temporal expression profiles for *gr* and *gsta*. An elevated level of *gr* transcripts was observed in PAA- and H_2O_2-exposed groups at 24 h post-challenge while *gsta* expression was significantly altered after 48 h (Figure 4B, C). In both cases, the expression relative to the other time-point was significantly higher. The transcription of both genes in AA-exposed cells remained unaltered at both time-points. *Cat* expression was significantly upregulated in H_2O_2-exposed cells at both time-points, whereas for the PAA-exposed group, a significant increase was observed only 48 h post-challenge (Figure 4D). There was also a significant difference in *cat* expression in the PAA-exposed group between the two time-points. AA did not elicit a significant transcriptional change from *cat*. *Cu/zn sod* was significantly downregulated in the AA-exposed group as compared to control at 24 h, but not at 48 h after challenge. While *cu/zn sod* expression was unaltered in the PAA-exposed group at both time-points, a significant increase was observed in the H_2O_2-exposed group but only after 48 h. *Cu/zn sod* expression in H_2O_2-exposed cells at 24 h was likewise higher compared to the level at 48 h. The overall expression of *mnsod* displayed no significant alterations, except in PAA-exposed cells 48 h after challenge, where the expression was at least three times higher than the control and other treatment groups.

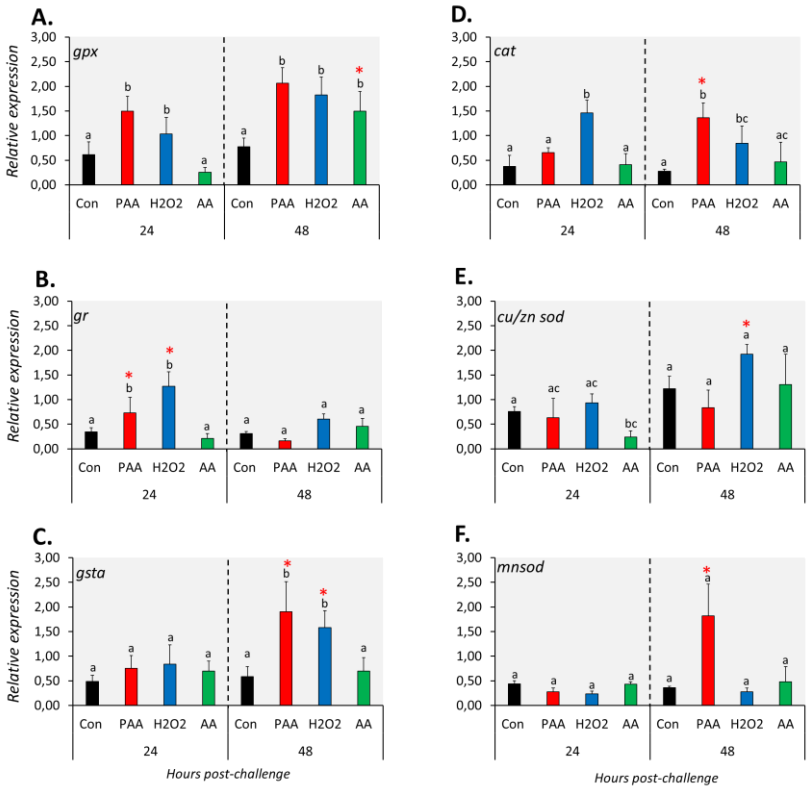

Figure 4. Changes in the expression of antioxidant defence genes following exposure to oxidative chemical stressors. The transcript level of six selected genes (i.e., gpx, gr, gsta, cat, cu/zn sod, mnsod) was quantified by RT-qPCR at 24 and 48 h after exposure to 100 µM of PAA, H_2O_2, and AA for 30 min. The control group was handled similarly, but without any chemical stimulation. Two-way ANOVA followed by the Holm-Sidak test identified statistical difference amongst treatment groups over time. Different letters indicate a significant difference at P<0.05 amongst the treatment groups within a time-point. Asterisk (*) denotes that the level in a treatment group differs between the two time-points. Results were presented as mean±SD of four wells, with ca 10^5 cells from 15 fish.

Changes in the gene expression of cytokine and heat shock proteins in the nasal olfactory leukocytes

The expression of *il1β* increased significantly in PAA- and H_2O_2-exposed groups compared to control 24 h after challenge, but such a change was no longer observed at 48 h (Figure 5A). On the other hand, AA exposure did not alter *il1β* expression at both time-points. Generally, *il10* expression was not significantly affected by the oxidative chemical stressors, except in AA-exposed cells at 24 h post-challenge, where a significant downregulation was observed. The transcript level of *il13r1a* was significantly lower in PAA- and AA-exposed cells compared to control 24 h after challenge, though the change did not persist until 48 h (Figure 5C). The expression of *ifn* was unaltered in most treatment scenarios at both time-points (Figure 5D).

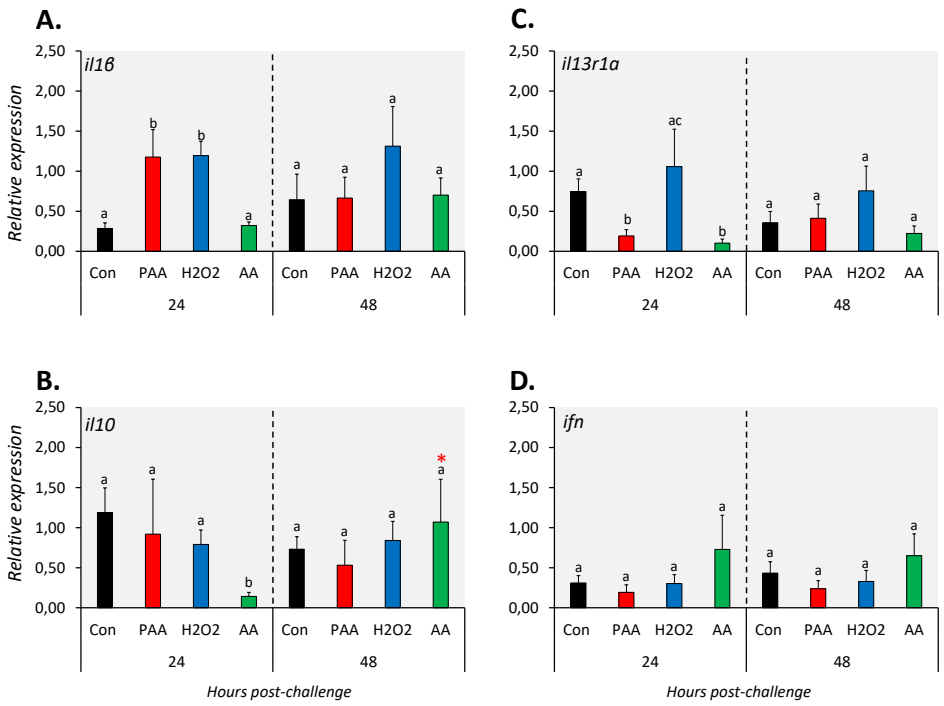

Figure 5. Changes in the expression of cytokine genes following exposure to oxidative chemical stressors. The transcript level of 4 selected genes (i.e., il1β, il10, il13r1a, ifn) was quantified by RT-qPCR at 24 and 48 h after exposure to 100 µM of PAA, H_2O_2, and AA for 30 min. The control group was handled similarly, but without any chemical stimulation. For the explanation on statistics and notations, please refer to Figure 4.

The three chemical stressors did not significantly change the expression of *hsp70* 24 h post-challenge (Figure 6A). After 48 h, however, *hsp70* expression was significantly elevated in all treatment groups compared to control, and the highest increment was identified in H_2O_2-exposed cells. *Hsp90* expression was not significantly altered in PAA- and H_2O_2-exposed cells at both time-points (Figure 6B). A significant downregulation was detected in AA-exposed cells at 24 but not at 48 h post-challenge, and the expression was higher in the latter than with the former time-point.

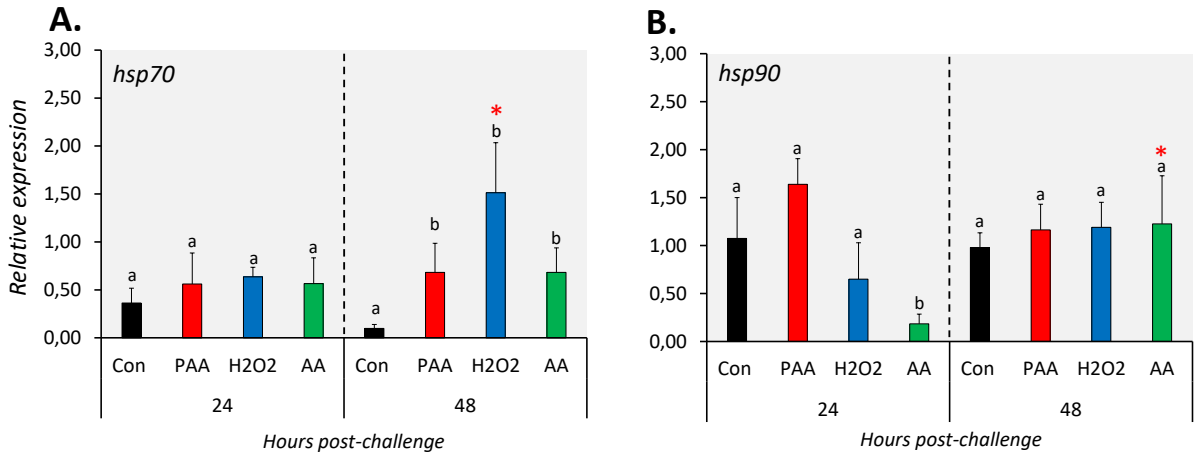

Figure 6. Changes in the expression of genes coding for heat shock proteins following oxidative chemical challenge. The transcript level of two selected genes (i.e., hsp70, hsp90) was quantified by RT-qPCR at 24 and 48 h after exposure to 100 µM of PAA, H_2O_2, and AA for 30 min. The control group was handled similarly, but without any chemical stimulation. For the explanation on statistics and notations, please refer to Figure 4.

Discussion

The present study reveals, through *in vivo* and *in vitro* exposure trials, how the nasal olfactory mucosa of Atlantic salmon mobilised its defence repertoires when challenged with oxidative chemical stressors. To our knowledge, this is the first report that demonstrated the molecular changes initiated by chemically induced oxidative stress in the nasal mucosa of a teleost fish. Using a nasal leukocyte model, it was further shown how the constituent oxidants of the tested therapeutics alter the cellular redox balance and the associated response mounted by a specific group of cells at the nasal olfactory mucosa to these challenges.

The two *in vivo* exposure studies uncovered the molecular repertoire of the nasal olfactory mucosa when challenged with either a periodic-low dose or less frequent-high dose of the oxidative agent PAA. In the first trial, we addressed the nasal consequences in the application of oxidant as a routine disinfectant in salmon. No conclusive implications can be drawn as to whether the exogenous oxidant was a stimulator or an inhibitor of nasal mucosal physiology as the ratios of upregulated and downregulated genes were equal after 45 days of exposure. However, two groups – haemoglobins and immune genes – displayed a trend in response to the exogenous oxidant. Haemoglobin (Hb) is a predominant component in erythrocytes responsible for oxygen transport to the different tissues in vertebrates [231]. The *hb* transcripts were upregulated in the nasal mucosa following periodic low-dose oxidant exposure, and both α and β subunits were represented. In murine models, it has been demonstrated that overexpression of Hb affected a network of genes involved in O_2 homeostasis, which subsequently resulted in the suppression of oxidative stress [232, 233]. Exposing HepG2 cells to H_2O_2 induced the expression of both haemoglobin α and β, and their overexpression likewise resulted in cellular protection against oxidative stress [234]. The upregulation of several haemoglobin transcripts in response to periodic low-dose oxidant challenge was probably a protective mechanism of the nasal mucosa against oxidative stress. Despite the limited number, there was an indication that intermittent oxidant exposure may negatively regulate nasal immunity. The constitutive presence of oxidant in the environment may trigger several responses from an organism – continual mounting of protective action, accommodation, or habituation (i.e., decreasing response through time). It was reported earlier that periodic application of oxidant (i.e., PAA) in trout – a species closely related to salmon – somehow resulted in a dampening response, which could be indicative of habituation [47, 80]. Such a consequence was likewise implicated in salmon post-smolts [229]. This partly sheds light on the downregulation of these immune genes after several weeks of exposure. We cannot disregard the potential oxidant-mediated immunosuppression in the nasal mucosa, as some of these transcripts have earlier been implicated in compromised immunity under oxidative stress in higher animal models [235, 236].

Trial 2 provided a relatively clearer picture of how an oxidant administered at a higher dose, but less frequently, altered the nasal transcriptome as shown with a higher number of DEGs and a prominent regulatory profile. It was evident that oxidant treatment resulted in dysregulation of nasal redox balance, and hence triggered mucosal oxidative stress. In earlier publications, we have demonstrated that antioxidant defences in mucosal tissues (i.e. gills and skin) were remarkably altered by a similar oxidant [81, 213], though delivered at a much higher concentration. The mobilisation of these antioxidant defences following oxidant exposure highlighted the capability of mucosal surfaces to muster physiological responses to increased environmental ROS, thereby protecting the mucosa from eventual oxidative damage, as supported by *in vitro* cell works demonstrated here as well. A substantial upregulation in immune-related genes was also observed, which likely offered insights into the complementarity of the immune and antioxidant defence mechanism at the mucosa during oxidative stress. There is a tight relationship between oxidative stress and immunity, and often the co-regulation of these two defence mechanisms provides robust responses during oxidative challenges [209, 237]. One of the immune effector molecules that were markedly regulated was *ornithine decarboxylase*, a gene coding for an enzyme responsible for catalysing the conversion of ornithine to putrescine, the first

and rate-limiting step in the synthesis of putrescine and the polyamines spermidine and spermine [238]. It is important to highlight that *spermidine/spermine N1-acetyltransferase 1*, a key molecule in amine metabolism, and with a counteractive function against oxidative stress, was significantly upregulated too. Increased expression of *ornithine decarboxylase* had been demonstrated in human hepatoma HUH7 cells subjected to chemically induced oxidative stress [239]. The upregulation observed in the present study is indicative of a similar function for protection against oxidative damage. Heat shock proteins play a role in several cellular processes that occur during and after exposure to oxidative stress [240]. There are indications, both in this trial as well as from previous studies using this oxidant, that it can induce transient oxidative stress [80, 81, 213]. The upregulation of both *hsps* in the nasal mucosa following oxidant exposure suggests intervention in oxidative stress-triggered changes by correction of conformation or selecting and directing aberrant proteins to the proteasome or lysosomes for degradation, in which these molecules are known to be key regulators. Besides the activation of several effector molecules that provide an interconnected response to oxidant-triggered oxidative stress, it is compelling to observe that several genes for molecules for cytostructural and extracellular matrix were represented in the list of upregulated genes. Previous studies using this oxidant found histostructural changes, though minimal, in the gill and skin mucosa [138, 213]. We can speculate that the upregulation observed here may play a role in maintaining the structural integrity and barrier functionality under oxidative challenge. The upregulation of two genes responsible for extracellular matrix, *GMP giant mucus protein* and *mucin 5b*, implies that mucus physiology is affected by the oxidant and that the changes in these two genes underline their function in providing a layer of protective defence at the mucosa. Two related genes have been shown to participate in modulating the mucus layer of the olfactory epithelium in mammalian models [241, 242], and they likely exert a similar function in the nasal mucosa of salmon. However, this must be functionally ascertained in the future. DEGs with known functions in lipid metabolism were downregulated in the oxidant-challenged nasal mucosa. Though it is difficult to conclude whether oxidative stress triggered an imbalanced lipid metabolism in the nasal mucosa because of a limited panel of DEGs under this category, it is interesting to note that such interaction has been reported in other animals [243, 244]. The downregulation observed in these lipid metabolic mediators indicates that oxidant exposure may interfere with this process, though the magnitude remains an open question.

We then focused on one specific cell type at the nasal mucosa to investigate how the cells respond to the oxidant, as well as to the two other constituents of PAA trade products. The proliferation of nasal olfactory leukocytes was affected by the three chemical stressors 24 h after exposure at an almost similar rate. However, such an influence was no longer observable 48 h after exposure in PAA- and H_2O_2-exposed cells. This indicates that the effects on cellular proliferation following PAA and H_2O_2 stimulation could be transitory, and the cells were able to recover quickly. AA was more powerful in inhibiting cellular proliferation, as the effects persisted until 48 h. Nasal olfactory leukocytes exhibited migratory potential, as all factors resulted in migration of the cells, though at varying levels. Cell migration plays an important role in many normal biological and pathophysiological processes, and oxidant can either promote or inhibit migration [245]. H_2O_2 administered individually and not in mixture with PAA and AA modulated the migration of the nasal olfactory leukocytes, suggesting its potent chemoattractant function, as demonstrated by earlier studies in other animal models [246, 247]. The migratory potential of nasal olfactory leukocytes is vital in orchestrating a cellular response when environmental ROS levels reach a concerning concentration or when pathophysiological response had been caused by oxidative stress.

We further asked: *If the oxidants triggered changes in the cellular phenotypic response* (i.e., proliferation and migration), *can they also induce oxidative stress*? The oxidant used had been shown to prompt oxidative stress at the systemic [81] and tissue [81, 213] levels, but this has yet to be demonstrated at the cellular level. Exposure to the three chemical oxidative stressors resulted in an increase in the intracellular concentration of ROS, which indicates that it induced redox imbalance; hence, oxidative

stress occurred. Both H_2O_2 and AA have long been identified as inducers of oxidative stress [62, 246-248]. Here, we have shown that PAA and H_2O_2 were far more potent inducers of intracellular ROS production than AA in nasal leukocytes. Interestingly, for both PAA and AA, induction of intracellular level was transient because, after an elevated level 24 h after exposure, the concentration returned to the normal/control level. This was not observed in H_2O_2-exposed cells; their intracellular ROS was still at an elevated level 48 h after exposure, though the level was slightly lower compared to that at 24 h. This indicates that cells exposed to PAA and AA have a faster capability to abate an increased intracellular ROS level than H_2O_2-exposed cells, which further suggests that H_2O_2 has a higher and more persisting impact as an oxidative stressor on the nasal leukocytes. Different oxidants could trigger different mechanisms of oxidative stress induction [207, 249], and somehow the observations in the present study are in agreement with this differential regulatory impact.

The increased intracellular ROS incited by the three chemical stressors initiated a response from the antioxidant system of the nasal olfactory leukocytes. The expression of enzymatic antioxidants was predominantly upregulated, indicating their key role towards the threats of the oxidative stressors. The overall expression profile of these antioxidant genes indicates that PAA and H_2O_2 were more potent triggers than AA, which, to some extent, is in agreement with the results in terms of how the oxidative stressors affected the intracellular ROS level. Antioxidant markers *gpx*, *gr*, *gsta*, and *cat* were perhaps the key response molecules, as their expression was significantly elevated in at least one time-point in PAA- and H_2O_2-exposed cells. The response profile can be divided into two arbitrary groups based on their elevated temporal expression – early (i.e., *gr*) and late (i.e., *gsta* and *cat*) oxidant responders. *Glutathione peroxidase* metabolises H_2O_2 to H_2O and the reduced glutathione provides an antioxidant function by resetting the redox status in tissues [250]. *Gpx* has been implicated in the responses of fish to environmental toxicants that can trigger oxidative stress [251], and this mechanism may also be working in nasal leukocytes. We have previously documented oxidant-induced alteration in *gpx* expression in the gills and skin of salmon; hence, the results provide further evidence that it is a vital molecule for the mucosal antioxidant system in this fish species [81]. It is interesting to note that *glutathione reductase gr* was upregulated both in the cells and as one of the DEGs identified in Trial 2. *Gr*, as an antioxidant, is responsible for the regeneration of reduced glutathione during detoxification of peroxides and free radicals formed in mitochondria, thus maintaining the redox potential of the cell [252]. The upregulation of *gr* in both instances, as well as in previous oxidant studies in salmon [81, 215], provides strong support for its canonical function in mucosal antioxidant defence, that is likely ubiquitously regulated by the oxidative chemical stressor. There was no apparent tendency for AA-induced changes in the antioxidant repertoire in the nasal olfactory leukocytes, though both *gpx* and *cu/zn sod* were responsive.

The overall profile in the expression of cytokine markers and heat shock protein genes could not be conclusively established, though the stochastic changes provide insights into how oxidative stressors may likely influence these molecules. The relationship between oxidants and inflammatory response is well-established in mammalian systems [253], though such interaction is less understood in fish, especially concerning exogenous oxidative stressors. We have shown that *il1b* expression was modulated by PAA and H_2O_2 24 h after exposure. An earlier publication reported that increased IL-1β stimulated glutathione production, thereby protecting neurons from oxidative damage [254]. The present data could not decisively ascertain whether such a directional effect was also initiated in the nasal leukocytes; however, the upregulation of *il1b* and the two genes of glutathione metabolism offer a potential link. In Trial 2, we have identified several cytokines genes that were upregulated following the oxidant challenge. It is possible that this increase in expression facilitated the migration of inflammatory cells to the epithelial surface where the oxidant was in close contact. One of the areas that must be explored in the future is the early phase of inflammatory response which was not captured by the current data. Both *hsp70* and *hsp90* were significantly upregulated in the olfactory rosette in Trial 2, but a similar

change in the cell experiment could not be observed. The mode of oxidant application may play in part in this apparent difference.

Fish encounter environmental oxidants during production, as several husbandry practices rely on the use of oxidative chemical compounds, such as during water disinfection [80] or disease treatment [181]. Application frequency may vary, from continuous to periodic, each depending on its intended use (i.e., disinfectant vs therapy). PAA is a potent oxidant, though the window of safe dose is limited [255]. The present study contributes to a better understanding of how the nasal olfactory mucosa of Atlantic salmon, one of the least explored mucosal tissues with regards to redox physiology, mount physiological and immunological responses when prompted with exogenously generated oxidative challenges. Oxidative stress is a physiological imbalance that requires a coordinated response to protect the organism from oxidative damage and, eventually, facilitate repair and recovery. The nasal mucosa of salmon can activate different molecules that may likely participate in the adaptive responses to oxidative stress. Nasal immunology is one of the emerging fields in fish immunology research, and the several oxidant-responsive genes identified in the paper are potential molecules for in-depth functional characterisation for their role in the nasal microenvironment, mainly towards non-infectious agents. One area that is interesting for future experiment is on whether exogenous antioxidants (e.g., in-feed antioxidants) can mitigate the effects of the chemical oxidative stressors by augmenting the natural antioxidant system of fish.

Publication 8

antioxidants

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Article

Multiomics Provide Insights into the Key Molecules and Pathways Involved in the Physiological Adaptation of Atlantic Salmon (*Salmo salar*) to Chemotherapeutic-Induced Oxidative Stress

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ABSTRACT

Although chemotherapeutics is used to treat infections in farmed fish, knowledge on how they alter host physiology is limited. Here we elucidated the physiological consequences of repeated exposure to the potent oxidative chemotherapeutic peracetic acid (PAA) in Atlantic salmon (*Salmo salar*) smolts. Fish were exposed to the oxidant for 15 (short exposure) or 30 (long exposure) minutes every 15 days over 45 days. Unexposed fish served as the control. Thereafter, the ability of the remaining fish to handle a secondary stressor was investigated. Periodic chemotherapeutic exposure did not affect production

performance, though survival was lower in the PAA-treated groups than in the control. Increased ventilation, erratic swimming, and a loss of balance were common behavioural manifestations during the oxidant exposure. The plasma reactive oxygen species levels increased in the PAA-treated groups, particularly after the 3rd exposure, suggesting an alteration in the systemic oxidative stress status. Plasma indicators for internal organ health were affected to a certain degree, with the changes mainly observed after the 2nd and 3rd exposures. Metabolomics disclosed that the oxidant altered several circulating metabolites. Inosine and guanosine were the two metabolites significantly affected by the oxidative stressor, regardless of exposure time. A microarray analysis revealed that the gills and liver were more responsive to the oxidant than the skin, with the gills the most sensitive. Moreover, the magnitude of the transcriptomic modifications depended on the exposure duration. A functional analysis showed that genes involved in immunity and ribosomal functions were significantly affected in the gills. In contrast, genes crucial for the oxidation-reduction process were mainly targeted in the liver. Skin mucus proteomics uncovered that the changes in the mucosal proteome were dependent on exposure duration and that the oxidant interfered with ribosome-related processes. Mucosal mapping revealed gill mucous cell hypertrophy after the 2nd and 3rd exposures, although the skin morphological parameters remained unaltered. Lastly, repeated oxidant exposures did not impede the ability of the fish to mount a response to a secondary stressor. This study provides insights into how a chemical oxidative stressor alters salmon physiology at both the systemic and mucosal levels. This knowledge will be pivotal in developing an evidence-driven approach to the use of oxidative therapeutics in fish, with some of the molecules and pathways identified as potential biomarkers and targets for assessing the physiological cost of these treatments.

Introduction

The continuous growth of fish farming is perennially challenged by diseases that present a significant impediment to the profitability and sustainability of the industry. Preventive measures to improve and safeguard health have been the focal strategies in recent years, including heightened biosecurity [256], the use of balanced and functional diets [257], vaccines [258], and efficiently controlling the rearing environment [259]. However, there are instances where the use of therapeutics remains the only viable option. A significant portion of the chemotherapeutics being used in aquaculture target bacterial and parasitic infections, and for many of these, effective vaccines are not yet available. Though in several cases chemotherapeutics can address the health issue, there have been long-standing discussions on whether the application of these compounds fosters a sustainable industry [260, 261], especially given that resistance, imprudent usage and discharge, and unwanted ecological impacts are daunting challenges [229, 262]. Mitigating these risks will include implementing stricter rules, streamlined application backed up by research data, and the continuous exploration and development of more eco-friendly alternative therapeutics [117, 263].

Oxidative biocides are one of the widely known groups of chemical therapeutics being used in fish farming. In particular, hydrogen peroxide (H_2O_2) and peracetic acid (PAA) are considered ‘green’ therapeutics because they have been shown not to contribute harmful residues to the environment [263]. Unlike H_2O_2, which is readily available in a pure form, commercial PAA is an equilibrium mixture of PAA, H_2O_2, acetic acid, and water [117]. Both share a similar mechanism of action as biocides, which is the generation of free hydroxyl radicals that induce damage to DNA, enzymes, and proteins via oxidation, thus increasing the permeability of the cell walls [38]. Cell wall destruction may involve different targets, including the peroxidation and disruption of membrane layers, oxidation of oxygen scavengers and thiol groups, inhibition of enzymatic activity, oxidation of nucleosides, impaired energy production, disruption of protein synthesis, and eventually, cell death [116]. These mechanisms contribute to the strong biocidal properties of H_2O_2 and PAA, which have been identified to have broad-spectrum activity against aquaculture-relevant pathogens [27, 38, 80]. However, because of its fat solubility, PAA has far more potent antimicrobial properties than H_2O_2 [29]. In addition, concerns have been raised regarding the

excessive use of H_2O_2, as in Atlantic salmon (*Salmo salar*) farming, due to toxicity threats to other organisms, particularly shrimp [181]. PAA degrades relatively faster than H_2O_2, and the difference in the degradation kinetics is important for the PAA by-products not to persist for a more extended period in the environment and present risks to other organisms [117]. Hence, PAA offers some promising advantages over H_2O_2. Concentrations from 0.2 to 14 ppm have been evaluated in different fish species and responses to the PAA biocide are dictated by dose, application method and duration, stress status of fish and water chemistry [27, 83, 170].

The use of these oxidative biocides must account for the effects on the environment and target pathogens as well as the fish. It is crucial that the therapeutic doses being applied are effective against the target pathogen, and most importantly, present little to negligible effects on the host fish. This balance is often difficult to achieve because of our limited understanding of the physiological aspects of oxidative biocide application in fish. Nonetheless, several reports in recent years have offered insights into the mechanisms by which PAA interferes with the different physiological processes of salmonids. The biological data have raised several questions regarding the extent of its influence on fish health and welfare [80, 81, 83, 170, 229, 264]. Transient and chronic oxidative stress has been shown to be induced following PAA treatment, where the enormity of impact is dependent on dose and exposure duration. Therefore, despite its biocidal effectivity, PAA can be regarded as a potential oxidative stressor too. Understanding the magnitude of and how PAA induces oxidative stress, particularly the underlying processes and mechanisms, will be vital for its evidence-driven use in fish farming.

Here we report the physiological consequences of repeated exposures to the oxidative biocide PAA in Atlantic salmon (*Salmo salar*) smolts. Most treatment studies in fish have dealt with a single exposure to the therapeutics, but in a real-world scenario, fish are treated several times during a production cycle. The simulation performed in this study mimicked a treatment for a gill ectoparasite in salmon using a therapeutic being explored as a treatment option [170]. In this trial, we addressed the impacts on uninfected naïve salmon to profile the baseline physiologic response to repeated therapeutic interventions.

Materials and Methods

Ethical use of animals for research

All procedures involving fish described in this paper followed the Directive 2010/63/EU as amended by Regulation (EU) 2019/1010. The trial received approval from the Norwegian Food Safety Authority under FOTS ID 19321. Key personnel in the trial have FELASA C certification.

Oxidative biocide exposure trial and secondary stress test

The fish trial was conducted at the Tromsø Aquaculture Station (HiT; Tromsø, Norway) using Atlantic salmon smolts produced at the research station. Three hundred sixty smolts with a starting weight of approximately 80–90 g was stocked into nine 500 L circular tanks in a flow-through system at a density of 40 fish per tank (Fig. 1). Fish were allowed to acclimatise for a week under the following parameters, which were also maintained throughout the trial: water flow rate set at 6–7 $L \cdot min^{-1}$, salinity at 35 ‰, temperature at 13.0 ± 1 °C, dissolved oxygen >90 % saturation, photoperiod set at 24 L: 0 D, and a continuous feeding regime (Nutra Olympic 3 mm, Skretting, Averøy, Norway). There were three experimental groups – control, short exposure (SE), and long exposure (LE). Three tanks were dedicated for each treatment, which were randomly allocated inside the experimental hall.

Oxidant exposure was performed as follows: Water flow in the tank was stopped, and the oxidative biocide (Divosan Forte™, Lilleborg AS, Norway) was added to the water column to achieve a concentration of 10 mg L^{-1}. This concentration is twice the dose previously used for salmon [170].

Aeration was supplied to allow mixing and maintain oxygen levels >90 %. For the SE group, the exposure duration was 15 min, while for the LE group, the exposure lasted 30 min. After the exposure period, the water flow was opened, and at least 90% of the water was replaced within 8–10 mins to flush out the residuals. The control group was unexposed. This exposure protocol was repeated every 15 days, with 3 exposures in total over a 45-day trial period.

A week after the last sampling, the remaining fish were subjected to an acute stress by lowering the water volume in the tank to achieve a density 10× higher than the initial density. During the exposure period, the oxygen level in the tank was routinely followed and maintained at above 90 % saturation. After exposing the fish to this condition for 1 hr, the water level was returned to the initial level, and the fish were allowed to recover for post-stress sampling.

Sampling protocols

For the main exposure experiment, sampling was performed 24 h after each exposure. Briefly, 3 fish were taken from each tank and humanely euthanised with an overdose of Benzocaine (Benzoak vet, 200 mg/mL, EuroPharma, Leknes, Norway). The length and weight were measured, and the external welfare status was assessed [92]. Plasma samples were collected from blood drawn from the caudal artery using a heparinised vacutainer (BD Vacutainer™, Fisher Scientific UK Ltd, UK). Skin mucus samples from both sides of the fish below the lateral line were collected using FLOQSwab® (COPAN Diagnostics, CA, USA) and snap-frozen in dry ice. Sections of the skin below the dorsal fin and the second gill arch were collected for microarray and mucosal mapping analyses. Samples for the microarray were suspended in RNAlater® (Merck Life Science AS, Oslo, Norway) and stored at −70 °C until analysis, whereas tissues for mucosal mapping were preserved in neutral buffered formalin (BiopSafe ApS, Hellerup, Denmark). A portion of the liver was also collected and stored in RNAlater®. For the post-exposure stress trial, plasma samples were taken from 3 fish per tank before the stress and 2 and 4 hours after the stress following the protocol described above.

Plasma clinical biochemistry

The lactate, glucose, aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT), and creatinine plasma levels were measured with a Pentra C400 Clinical Chemistry Analyzer (HORIBA ABX SAS, Montpellier, France), while the cortisol (Demeditic Diagnostics GmbH, Kiel, Germany) and reactive oxygen species (ROS)/reactive nitrogen species (CellBiolabs, Inc., California, USA) were measured using commercially available kits. All analyses were run in duplicate.

Plasma metabolomics

Proteins in the plasma (ca. 200 µL) were initially precipitated using methanol followed by a chloroform and water liquid-liquid extraction and collection of the aqueous phase. The extracts were transferred to a liquid chromatography (LC) vial and dried under nitrogen flow. The LC/mass spectroscopy (MS) analysis was performed by MS Omics ApS (Vedbæk, Denmark) in an ultra-performance LC system (Vanquish, Thermo Fisher Scientific) coupled with a high-resolution quadrupole-orbitrap mass spectrometer (Q Exactive™ HF Hybrid Quadrupole-Orbitrap, ThermoFisher Scientific) using a slightly modified version of an earlier protocol [265] used for salmon plasma [170, 229]. Data were processed using Compound Discoverer 3.0 (ThermoFisher Scientific), and the metabolites were identified with four levels of annotation as described in detail in an earlier publication [170].

Microarray analysis – mucosal organs and liver

Total RNA was isolated from the RNAlater®-preserved samples using the Agencourt RNAdvance™ Tissue Total RNA Purification Kit (Beckman Coulter Inc., CA, USA). All samples had an RNA Integrity

Number (RIN) above 9.0 as evaluated by the Agilent® 2100 Bioanalyzer™ RNA 6000 Nano Kit (Agilent Technology Inc., Santa Clara, CA, USA). The microarray analysis was performed using a custom-designed 15K Atlantic salmon DNA oligonucleotide microarray SIQ-6 (Agilent Array, ICSASG_v2), and all reagents used were from Agilent Technologies. The One-Color Quick Amp Labelling Kit was used for RNA amplification and Cy3 labelling using 110 ng of RNA template per reaction. Gene Expression Hybridization Kits were used for the fragmentation of labelled RNA. This was followed by a 15 h hybridisation in a 65 °C oven with a constant rotational speed of 10 rpm. Thereafter, the arrays were successively washed with Gene Expression Wash Buffers 1 and 2 and scanned using the Agilent SureScan Microarray Scanner. Pre-processing was performed in Nofima's bioinformatics package STARS (Salmon and Trout Annotated Reference Sequences) [88].

Skin mucus proteomics

Skin mucus peptides were prepared using a double-digestion protocol (Pierce™ Mass Spec, ThermoFischer, USA) that was slightly modified for mucus samples. Skin mucus lysates were reduced for 45 mins in 10 mM dithiothreitol at 50 °C, alkylated for 20 mins in 50 mM iodoacetamide at room temperature, and the proteins were acetone-precipitated overnight at 4 °C. The protein pellet was then digested for 2 h in 1 µg Lys-C endoproteinase at 37 °C followed by a trypsin treatment at 37 °C overnight. The samples were frozen at −80 °C to stop digestion and concentrated in a vacuum evaporator. The protein digests were subjected to an LC-MS analysis, and the resulting spectral data were analysed using Mascot (Matrix Science, London, UK; version 2.6.1). The MS/MS-based peptide and protein identifications were validated in Scaffold (version Scaffold_4.8.9, Proteome Software Inc.) following the pipeline detailed in an earlier publication [266].

Mucosal mapping

The gill and skin samples were processed for Mucosal Mapping following Quantidoc's standard protocol [267]. Tissue sections stained by Periodic Acid Schiff – Alcian Blue were digitised, scanned, and processed through an automated software developed by Quantidoc AS for the stereological image analysis of mucosa. The analysed mucosal features include mucous cell density (D, % epithelium filled with mucous cells), mean mucous cell area (A, µm²), and barrier status (1/[A:D] × 1000) [122, 123, 138, 267].

Data handling and statistics

Statistics were performed in Sigmaplot 14.0 Statistical Software (Systat Software Inc., London, UK). A Shapiro-Wilk test was used to evaluate the normal distribution, and a Brown-Forsyth test was used to check for equal variance in the data set. Plasma parameters were subjected to a two-way analysis of variance (ANOVA) followed by multiple pairwise comparisons with the Holm-Sidak test to identify differences between treatment groups at a particular time point, differences within a treatment group over time, and the interaction of treatment and time. All tests for statistical significance were set at $P < 0.05$.

For the microarray, data normalised by the Lowess normalisation of Log2-expression ratios in STARS [88] were subjected to statistical comparisons using linear modelling as implemented in the Bioconductor package limma [268]. Significance values (P values) were adjusted for multiple testing using the Benjamini-Hochberg procedure. The differential gene expression significance cut-off was an adjusted p-value < 0.01. For each comparison, positive and negative fold changes indicated the up- and down-regulation, respectively, of gene expression in the oxidant-exposed group relative to the control group. A hypergeometric test was used to identify the gene ontology (GO) terms in which significant genes (defined using the adjusted p-value < 0.01) were over-represented. This was performed for all three ontologies: biological process, molecular function, and cellular compartment. Zebrafish orthologs for the

Atlantic salmon genes were retrieved from the Ensembl Compara database (https://www.ensembl.org/info/genome/compara/). The zebrafish orthologs of the significant genes from each comparison were then analysed for enrichment in the Reactome pathways (www.reactome.org) using a hypergeometric test. Each GO term or Reactome pathway enrichment ($p < 0.05$) was assessed for up- and down-regulated genes separately, as well as for the union of up- and down-regulated genes.

For the plasma metabolomics data, a multivariate principle component analysis (PCA) model was used to identify the effects influenced by different treatments. A Benjamini-Hochberg correction was then employed with the acceptable false-positive rate set at 0.1. The Benjamini-Hochberg critical value, (i/m)Q, where i = the individual p-value rank, m = total number of tests, and Q = the false discovery rate, was calculated for each compound. A compound was considered significantly affected by the treatment when the p-value from a *t*-test was < (i/m)Q. Further, a Log2 ratio was calculated by comparing the level in the PAA-treated group with the control.

For the proteomics analysis, data were processed and analysed in R (version 3.5.2, https://www.r-project.org/). Low abundant results were removed by deleting all proteins with mean counts smaller than or equal to 1. The remaining proteins were normalised by dividing the counts by the individual mean counts. To identify differences between groups, ANOVAs were calculated for each protein. A p-value of <0.05 was used as the cut-off for filtering. These proteins were prepared for a cluster analysis by calculating the group means, centred by dividing by row means, and Log2 transformations. The data were clustered by the function *hclust()* (*stats* package, for Euclidean distance and with complete linkage) and plotted with *heatmap.2()* (*gplots* package). Five sub-clusters with distinctive expression patterns were identified, and the mean values of these were plotted as bar plots with error bars showing the standard error of the mean (SEM).

Results

Behaviour, production performance, external welfare, and survival

There were three prominent behavioural changes observed during the exposure period: erratic swimming, increased opercular ventilation, and a loss of balance. These changes could be arbitrarily divided into three periods: *first 10 min* – active and erratic swimming activity with some fish attempting to jump out of the water; *the following 10 min* – slightly diminished swimming activity, some fish attempting to burrow at the base or side of the tank, and at least 60% of the fish demonstrating increased opercular ventilation; and *the last 10 minutes* – rapid opercular ventilation with at most, 10% of the fish exhibiting a loss of balance. These behavioural changes were documented for all exposure events.

Production parameters, including length and weight at termination, did not significantly change among the treatment groups, although there were some stochastic changes in different welfare indicators after the 3rd exposure (Fig. 1B). Nonetheless, the overall external welfare status of the experimental fish remained favourable, as the average score was below 1 (Fig. 1C). We also observed that the fish resumed feeding immediately after treatment for all exposure events.

There was one dead fish 24 h after the 1st and 2nd exposures in the LE group. A total of 6 dead fish were recorded in the SE group, while 8 were recorded in the LE group before conducting the 3rd exposure. After the 3rd exposure, 6 and 5 dead fish were recorded in the SE and LE groups, respectively. The survival rates of the oxidant-exposed fish were relatively lower than that of the control group at the termination of the experiment.

Plasma ROS levels

The plasma ROS levels significantly increased through time in the SE and LE groups, but not in the control group (Fig. 1D). However, it was only after the 3rd exposure that the ROS levels in the plasma of the oxidant-exposed groups significantly differed from the control. In addition, no significant difference was observed between the two oxidant-exposed groups.

Indicators of systemic stress and organ health

Of the 3 physiological stress indicators, namely cortisol (Fig. 2A), lactate (Fig. 2B), and glucose (Fig. 2C), only glucose showed significant changes following exposure to the oxidant. The glucose level in the LE group was significantly lower than those in the SE and control groups after the 2nd exposure. However, this change was not identified after the 1st and 3rd exposures. Moreover, the glucose level in the LE group was significantly lower after the 2nd exposure than after the 1st and the 3rd exposures.

The ASAT levels (for liver health) showed significant temporal changes in all experimental groups (Fig. 2D), with the level in the SE group significantly higher than that in the control after the 1st and 3rd exposures. However, the ASAT levels in both oxidant-exposed groups were significantly lower than that in the control group after the 2nd exposure. Alanine aminotransferase, another liver health indicator, significantly changed after the 3rd exposure, where an increase was identified in the SE group relative to the control (Fig. 2E). Both oxidant-treated groups had higher ALAT levels after the 3rd exposure than after the 1st exposure. Creatinine (for renal health) significantly increased over time in all groups (Fig. 2F), with a 40-fold increase between the 1st and 2nd exposures, particularly in the control group. There was at least a 13-fold increase in the creatinine level in all groups after the 3rd exposure compared with the 1st exposure. A significant inter-treatment difference was also identified after the 2nd exposure, where the levels in the SE and LE groups were lower than that in the control.

Subjecting the oxidant-exposed fish as well as the control group to a secondary stressor (i.e., crowding stress) resulted in a significant increase in plasma cortisol post-stress (Fig. 2G), where in terms of increment, SE group showed the most pronounced response after stress. The plasma cortisol levels in the SE group were significantly higher those in the control and LE groups at 2- and 4-hours post-stress. The lactate (Fig. 2H) and glucose (Fig. 2I) levels remained unchanged following the crowding stress.

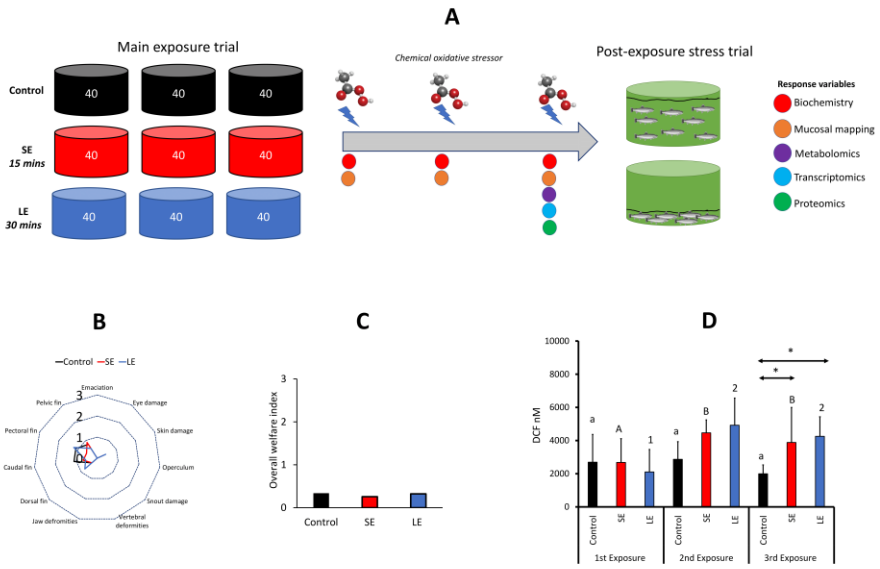

Figure 1. Experimental set-up, external welfare status, and plasma reactive oxygen species levels. (A) Fish were exposed to the oxidant every 15 days over a period of 45 days. The remaining fish were then subjected to the secondary stress of a high-density environment. Different response variables were analysed at 3 time points. (B–C) The external welfare status of the fish after 3 periodic exposures was assessed on a 0-to-3 scale system, where 0 means in good condition and 3 indicates a severely compromised state [92]. (B) Radial chart of the 11 indicators and (C) the overall welfare index based on the average score of all indicators (n=9 fish per treatment group). Oxidative stress was triggered as shown in (D) by the level of plasma reactive oxygen species (ROS). The ROS levels were analysed 24 h after each exposure using 9 fish per treatment group. Values are presented as mean ± standard deviation. Different lower-case letters, upper case letters, and numbers indicate significant differences over time in the control, SE, and LE groups, respectively. An asterisk (*) indicates a significant difference between two groups at a particular sampling point. SE, short exposure (15 min); LE, long exposure (30 min).

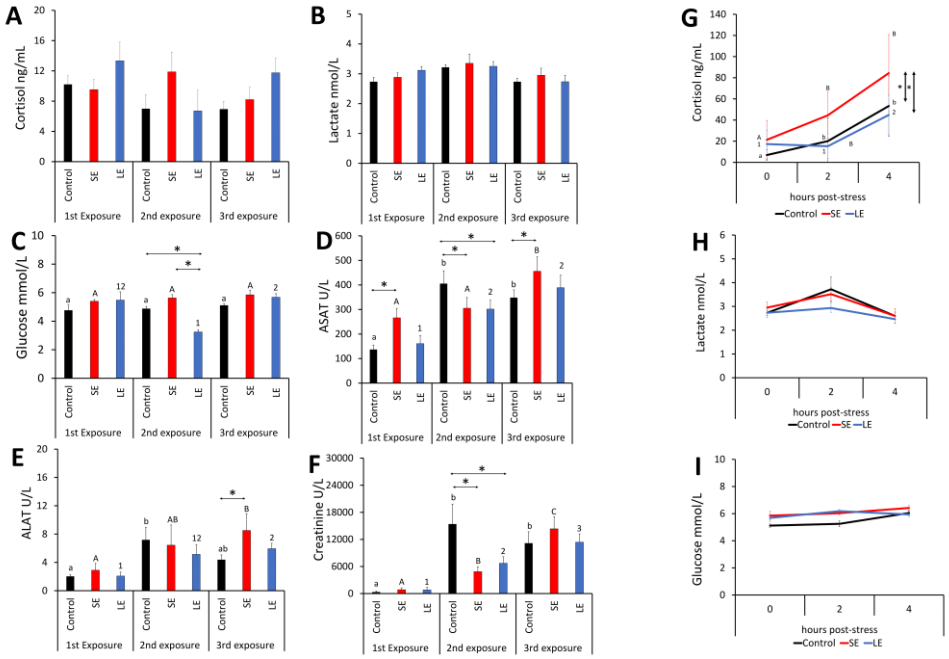

Figure 2. Plasma indicators for stress and organ health. The levels of (A) cortisol, (B) lactate, (C) glucose, (D) aspartate transaminase (ASAT), (E) alanine transaminase (ALAT), and (F) creatinine were measured in plasma samples (N = 9 fish per treatment group) taken 24 h after each exposure. Plasma levels of (G) cortisol, (H) lactate, and (I) glucose were assessed before and after the secondary stress induction. Different lower-case letters, upper case letters, and numbers indicate significant differences over time in the control, SE, and LE groups, respectively. An asterisk (*) indicates a significant difference between two groups at a particular sampling point. For the purpose of clarity, this was only indicated at 4 h post-stress in (G) but a similar trend of significant inter-treatment differences was likewise identified at 2 h post-stress. SE, short exposure (15 min); LE, long exposure (30 min). Values are presented as mean ± standard deviation of 9 individual fish per treatment group.

Alterations in the circulating metabolites

A total of 944 compounds were detected in the plasma samples collected after the 3rd exposure, of which 197 were annotated at Level 3, 75 at Level 2b, 23 at Level 2a, and 38 at Level 1 (Supplementary File 1). The score plot from a PCA model calculated on the compounds annotated at Level 1, 2a, or 2b in the reduced dataset is presented in Fig. 3A, and the loading plot (Fig. 3B) shows which variables are responsible for the patterns observed in the score plot. The grouping of the samples shows a clustering of the two oxidant-exposed groups in the upper part, separated from the control samples, which are all located in the lower part of the plot. Comparing the SE group with the control group, there were 6 differentially affected metabolites: inosine, 7-methyladenine, biotinsulfoxide, 4-acetamidobenzoic acid, hypoxanthine, and guanosine. Similarly, 5 differentially affected metabolites were identified in the LE

group, namely 4-acetamidobenzoic acid, inosine, valpromide, 7-methyladenine, and guanosine. Inosine (Fig. 3C, Level 1) and guanosine (Fig. 3D, Level 2a), two metabolites with higher annotation confidence, were commonly affected in the oxidant-exposed groups, with an up-regulation observed in the former and a down-regulation identified in the latter.

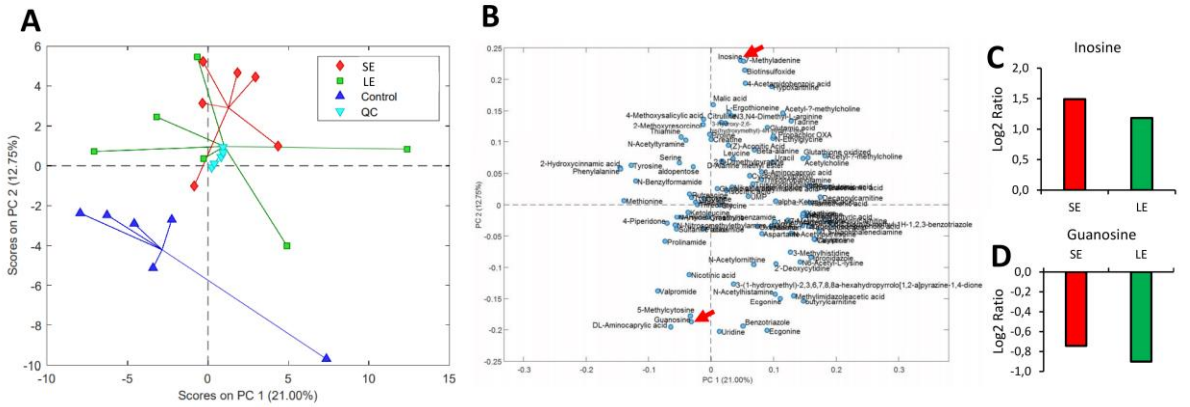

Figure 3. Plasma metabolomes of oxidant-exposed salmon smolts after the 3rd exposure. (A) Score plot from a principal component analysis (PCA) model calculated on the relative concentrations of the compounds annotated at Level 1, 2a, or 2b in the reduced dataset (see Supplementary File 1). (B) Loading plot from the PCA model calculated on the relative concentrations of the compounds annotated at Level 1, 2a, or 2b in the reduced dataset. Data presented in A and B have been auto scaled. (C) Inosine and (D) guanosine were the two metabolites significantly affected in both the SE and LE groups. Values are given as the Log2 ratio relative to the control (N = 6 fish per treatment group). SE, short exposure (15 min); LE, long exposure (30 min), QC, quality control.

Oxidant-induced transcriptomic changes in the gills, skin, and liver

The number of differentially expressed genes (DEGs) identified in the SE group was comparable across all three tissues (15 DEGs in gills, 11 in skin, and 23 in liver; Fig. 4A). Notably, none of these DEGs was significantly changed in more than one tissue after 15 min of exposure (Fig. 4B). Increasing the duration of oxidant exposure from 15 to 30 mins led to a marked increase in the numbers of DEGs identified in the gills (i.e., 693 DEGs). Twelve DEGs significantly overlapped in the gills of the SE and LE groups, including *mucin-7-like*, *IL15 receptor alpha chain isoform 3,* and C-*C motif chemokine 4-like* (Fig. 4B). The increase in DEGs in the LE group was also true for the liver, but to a lesser extent, with the number of DEGs increasing from 23 to 100. There were 13 hepatic DEGs that significantly overlapped between the SE and LE groups. Of the three tissues, the skin was least responsive to the oxidant exposure in terms of the number of DEGs identified, with only 13 DEGs identified in the LE group. The transcriptomic changes in the gills and liver were further reflected in the magnitude of responses as shown in volcano plots (Fig. 4C–F). The majority of the DEGs in the gills of the SE group were up-regulated (Fig. 4C), while a pronounced down-regulation profile was identified in the liver (Fig. 4E). For the LE group, the distribution of up- and down-regulated genes was almost equal in both the liver and gills (Fig. 4D, F).

The functional annotations by Gene Ontology (Fig. 5) and Reactome Pathway (Fig. 6) were focused on the gills and liver. The analysis for the skin yielded no significant results due to the low number of DEGs. No significantly enriched GO terms were identified in the gills of the SE group. In the gills of the LE group, however, the DEGs identified were enriched in pathways related to immunity such as ‘chemokine activity’ and ‘immune response’, and pathways related to ribosomal function such as ‘ribosome biogenesis’, ‘rRNA processing’, and ‘RNA binding’ (Fig. 5B). Enriched pathways in the livers of the SE group included ‘nucleotide binding’ and ‘ATP binding’ (Fig. 5A). Extending the exposure duration, the DEGs in the livers of the LE group were enriched in pathways such as ‘oxidoreductase activity’ and ‘oxidation-reduction process’ (Fig. 5C).

The Reactome analysis revealed that the zebrafish orthologs of the DEGs identified in the gills of the LE group were enriched in pathways related to the immune system and the ribosome (Fig. 6A, B). This mirrored the findings of the GO term enrichment (Fig. 5). In detail, the pathways enriched in zebrafish orthologs included ‘rRNA processing’, ‘mitochondrial translation’, ‘interleukin-20 family signalling’, and ‘deubiquitination’.

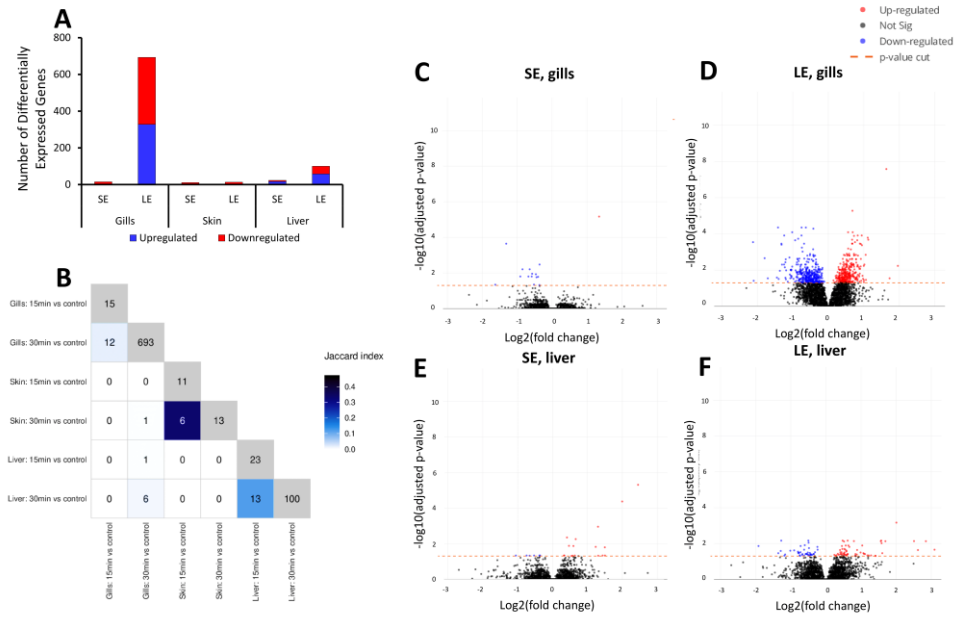

Figure 4. Transcriptomic changes in the gills, skin, and liver of oxidant-exposed salmon smolts. Tissues were collected after the 3rd exposure and subjected to a microarray analysis. (A) Differentially expressed genes, distinctively identified as either up- or down-regulated relative to the control group. (B) Heatmap demonstrating the overlap of different comparisons. Note that the numbers on the diagonal represent the total number of selected features found for each contrast. The colours of the squares represent the Jaccard index (the intersection over the union) for the contrasts on the x-axis with those on the y-axis. (C–F) Representative volcano plots of the (C, D) gill and (E, F) liver transcriptomes showing significance (as −log10 transformed p-values) against magnitude (Log2[fold change]). Features identified as having different levels between samples are represented as red (up-regulated) or blue (down-regulated) dots. SE, short exposure (15 min); LE, long exposure (30 min). Six individual fish per tissue were used.

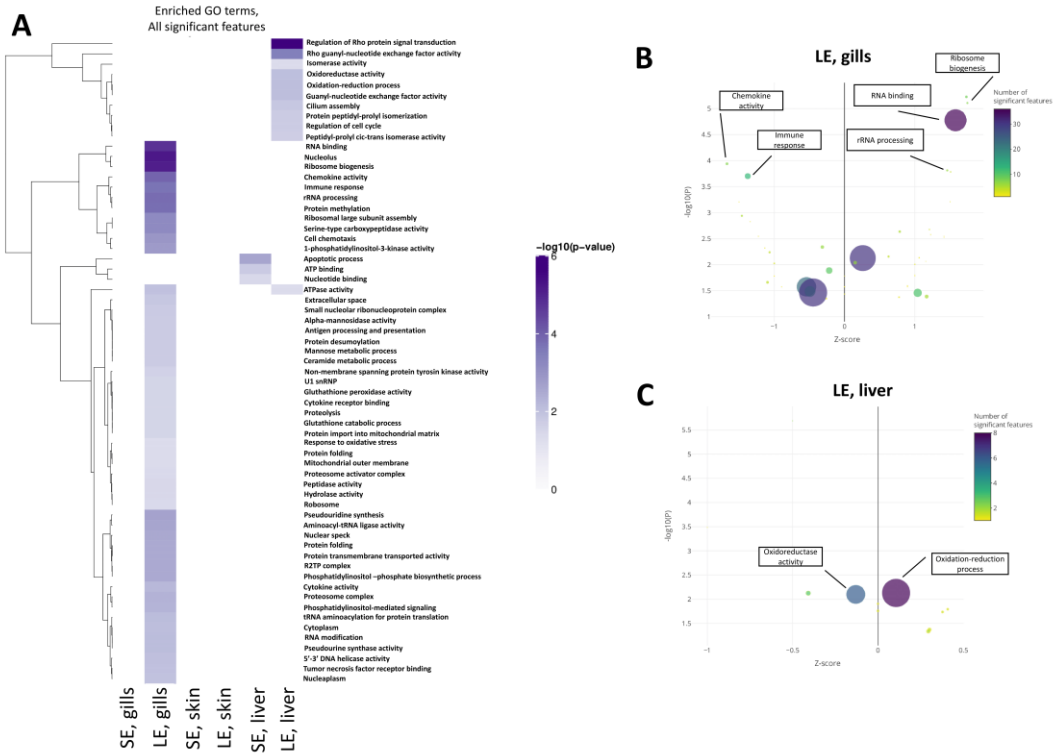

Figure 5. Gene ontology (GO) analysis of differentially expressed genes in the gills, skin, and liver of oxidant-exposed salmon smolts. (A) Heatmap of significantly enriched GO terms. Comparisons are shown on the X axis with GO terms on the Y axis. Colour is assigned based on the −log10(enrichment p-value), with lighter colours implying less significant enrichment. Hierarchical clustering was applied to the terms (rows). The most significant terms were clustered according to Euclidean distance using the complete linkage method. (B, C) Representative bubble plots of enriched terms showing all significant features. Only the transcriptomes of the (B) gills and (C) liver from the LE group are shown here. Enrichment analyses with the enrichment Z-score on the X axis and −log10(p-value) on the y-axis. Point size represents term size, and point colour represents the calculated Z-score. Some GO terms have been highlighted. SE, short exposure (15 min); LE, long exposure (30 min).

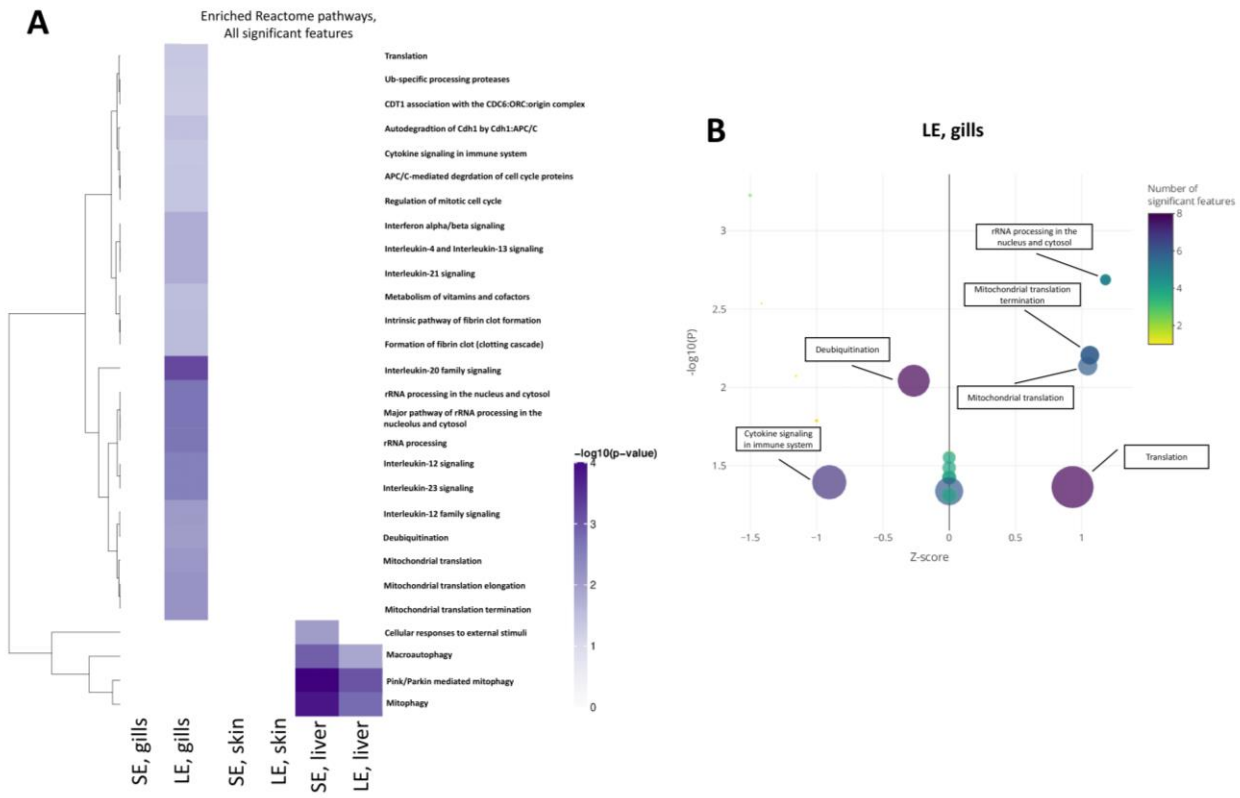

Figure 6. Reactome pathways of differentially expressed genes induced by oxidant exposure. (A) Heatmap of significantly enriched Reactome pathways. Significant features (at adjusted p-value < 0.05) from each contrast were analysed for an enrichment of Reactome pathway membership using a hypergeometric test by mapping features to genes (if appropriate). Enrichment (p-value < 0.05) was assessed for the combination of selected features. (B) Bubble plot of enriched pathways in the gills of fish from the LE group. SE, short exposure (15 min); LE, long exposure (30 min).

Alterations in the skin mucus proteome

There were 1333 proteins identified in the samples, and downstream analyses focused on the 189 differentially expressed proteins. A cluster analysis based on how the skin mucus proteins were affected by the oxidant revealed 5 major clusters (Fig. 7A). Cluster 1 includes proteins that were significantly up-regulated in the SE group but down-regulated in the LE group. Clusters 2 and 4 include proteins that were differentially up regulated by oxidant exposure. In Cluster 2, the effects were more substantial in the SE group than in the LE group. Cluster 3 includes proteins that were significantly down-regulated following oxidant exposure, with the magnitude of regulation higher in the LE group than in the SE group.

There were 69 differentially expressed proteins exclusively found in the LE group and 46 in the SE group (Fig. 7B). The LE and SE groups shared 14 differentially expressed proteins. We further investigated these to identify overlaps according to the direction of change. There were a relatively higher number of shared up-regulated than down-regulated proteins between the two oxidant-exposed groups (Fig. 8C). We performed a STRING analysis to understand the protein-protein interactions and functional groupings of the proteins (Fig. 8D). The protein interaction network created 115 nodes and 143 edges, with an average node degree of 2.49. We found that proteasome (yellow), ribosome (red), and spliceosome (brown) KEGG pathways were significantly enriched. Looking into the interactions, the network highlights the interaction of ribosomal proteins (rpl38, eif3m, rps23, rpl30, rpl14, rpl39, rps14, rpl26, rpl10a, rps11, rpl35a, rpl27, rpl36), ubiquitins (psmc2, psmd8, psmd3, psmc4, psmb2, psmc1b), proteasomal proteins (psmd10, psmc2, psmd8, psmd3, psmc4, psmb2, psmc1b), fibrin clot formation

(fgb, fgg), growth factors (fgb, f2, fgg, zgc:161979, zgc:113828), and proteins with a role in ROS detoxification (zgc:56493, prdx5).

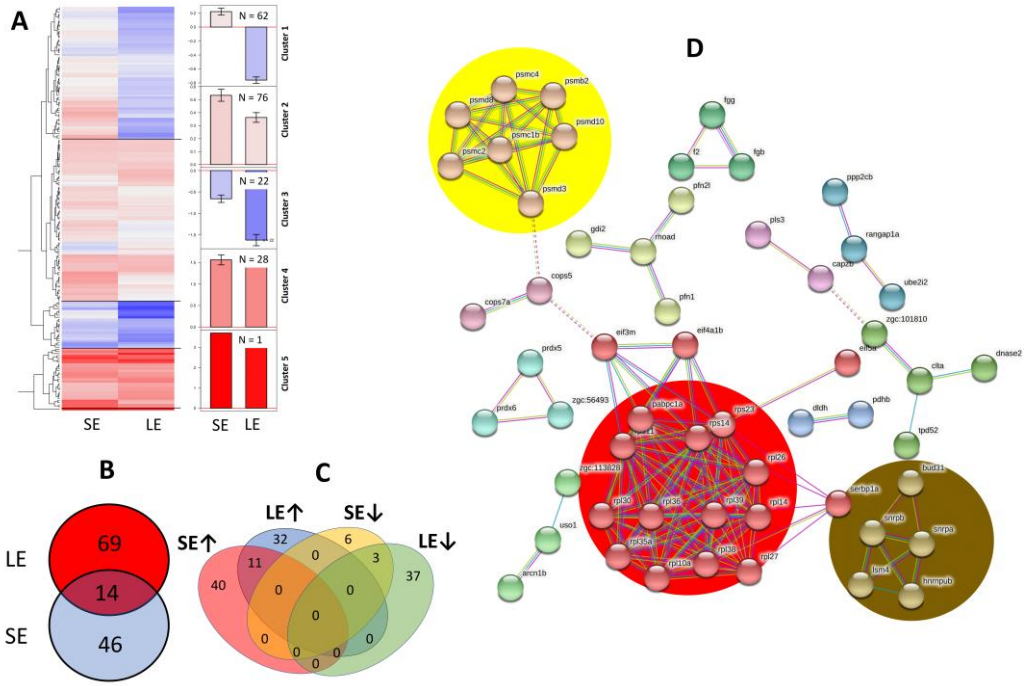

Figure 7. Skin mucus proteome of salmon smolts after the 3rd oxidant exposure. (A) Heatmap showing the relative down- and up-regulation of identified proteins (colours from blue to red) that were differentially regulated in the SE and LE groups relative to the control (N = 6 per group). The heatmap was divided into 5 sub-clusters, and the means of the respective expression values with SEM-error bars are shown as bar plots in the middle (n: number of proteins in a cluster). (B, C) Venn diagrams showing the associations of differentially expressed proteins in the different treatment groups. In (B), overlap did not account for the direction of change, while in (C), overlaps were clearly classified according to the direction of change. (D) Protein interaction map of identified skin mucus proteins. A possible protein-protein interaction map with high edge confidence was generated by string v.11. Joining lines represent a confidence of 0.700/1. The protein interaction network was created using zebrafish orthologs of the proteins identified in the salmon skin mucus. Accession numbers of the zebrafish proteins used in the construction of the interaction map are provided in Supplementary File 2. SE, short exposure (15 min); LE, long exposure (30 min).

Mucous cell morphometry

Mucous cell morphometries, including area, volumetric density, and barrier status, were measured in the gill lamellae after each exposure, but only after the 3rd exposure in the dorsal skin. There was a significant temporal change in the mucous cell area in the gill lamellae. The highest measurement was recorded after the 2nd exposure, though no significant inter-treatment differences were found. After the 3rd exposure, the mucous cells were significantly larger in the LE group compared to the control and SE groups. For the other two parameters, no significant temporal or inter-treatment differences were identified in the gill lamellae. None of the measured mucous cell parameters were changed in the dorsal skin after the 3rd exposure.

Discussion

When not managed properly, chemical oxidative stressors (often via chemotherapeutics) can be detrimental to fish health and welfare. Therefore, a holistic understanding of how fish respond to these stressors is important to developing an evidence-driven approach for their application in fish farming. This paper explored the molecules and processes involved in how Atlantic salmon respond and adapt to chemical therapeutics. Through a multi-platform approach, we demonstrated that repeated exposures

at longer durations impact both the mucosal and systemic responses in salmon, with the gills identified as highly susceptible to chemotherapeutics.

The behavioural changes observed during the exposure trial revealed that fish recognise danger and exhibit avoidance activities to escape contact with the oxidant, which is consistent with earlier observations [229]. Prolonging the contact time with PAA highlighted some of the classic behavioural changes when fish encounter chemical stimuli [269], including strong respiratory pressure as clearly demonstrated by opercular hyperventilation and eventually a loss of equilibrium. These changes indicate some of the welfare risks of using this chemical oxidant in salmon, including the mortality observed after the treatments. However, the oxidant-exposed fish recovered quickly, and the overall production performance and external welfare index were not significantly affected after 3 exposure events, suggesting that though it posed a mild risk, application optimisation could perhaps mitigate untoward consequences. In addition, the classical plasma stress indicators after each exposure were not significantly affected, implying the fish either adapted quickly or the treatment did not incite a strong stress response, which is slightly contradictory to our earlier observations using a lower PAA dose [81]. The lower glucose level in the LE group after the 2nd exposure could indicate interference in energy metabolism; however, this was likely a transitory response as the profile was not consistent throughout the trial.

We have previously shown that the oxidant used in this experiment (i.e., PAA) could trigger transient oxidative stress in salmonids, as shown either by the activation of antioxidant defences or by elevated or dysregulated levels of internal ROS [80, 81, 229, 264]. This response is predominantly attributed to the dissociation by-products of PAA when it comes into contact with water, which includes the generation of free hydroxyl radicals [117, 169]. The production of free radicals makes PAA a therapeutic with potent and broad microbicidal functions [30]. We found that exposure to the oxidant triggered the endogenous generation of ROS, with both treated groups exhibiting elevated levels, but only after the 3rd exposure. At present, we cannot conclusively account for whether the exogenous ROS from PAA degradation directly contributes to this increase. The increase in internal ROS indicates that the biocide triggered oxidative stress and points to the possibility that this consequence was likely cumulative, because the change relative to the control was pronounced only after 3 exposure events. Nonetheless, signs of the increasing tendency were already evident after the 2nd exposure. The neurobiology behind salmon's responses to PAA is an exciting avenue for future studies given the fact that the classical stress indicators of the hypothalamic-pituitary-adrenal axis (Fig. 2) and the oxidative stress marker (Fig. 1) did not show an apparent agreement. The interaction of these systems in fish is not yet elucidated, but in mammals, a link has been demonstrated [270]. Nevertheless, the plasma ROS profiles after the first two exposure events suggest the fish may be able to effectively regulate internal ROS homeostasis following treatment with an oxidative biocide, though such an ability was compromised after successive exposures. This internal ROS homeostasis regulation is likely mediated through the activation of molecules that participate in antioxidant mobilisation and/or excess ROS scavenging, which are discussed in detail in the next sections.

When an organism is under stress, several physiological adaptations are being initiated to counteract the threats, including modifications to the thousands of metabolites necessary for homeostasis and adaptation [271]. These metabolites will engage in different roles such as directly interacting with the stressors (e.g., ROS) by neutralising them thereby making them less detrimental to the organism and/or they will ensure that the different physiological systems are able to withstand the pressure of the stressful episode (e.g., immunity, energy metabolism). The systemic impact of the chemical oxidant was further clarified by the metabolomic changes in the plasma. Oxidant exposure altered the salmon's plasma metabolome, though these changes were not dependent on exposure duration. These metabolomic modifications may be an essential systemic buffering mechanism responding to the physiological dysregulation from a ROS level imbalance [272]. Compared with earlier studies in which

a lower PAA dose was employed (Lazado et al., 2020b; Lazado et al., 2021), the number of altered metabolites was relatively lower, indicating either the impact on the salmon metabolome may not be aggravated by a higher dose or that the treatment desensitised the ability of salmon to mount countermeasures to the physiological pressures from PAA.

Inosine and guanosine were the two metabolites identified to be significantly affected in both the SE and LE groups, suggesting their key role in salmon's adaptive response to the chemical oxidant. We earlier identified that plasma guanosine is altered when salmon are exposed to a similar oxidant but at a lower dose [170], thus underlining the fundamental function of this molecule in response to chemically induced oxidative stress in this species. These molecules have known distinct roles in mitigating oxidative damage caused by ROS by protecting cells from DNA damage [273]. In particular, in fish, dietary inosine may confer oxidative stress resistance [274]. Therefore, the regulation of these two metabolites likely played a role in ameliorating the effects of the imbalanced internal ROS that resulted in oxidative stress likely in a similar mechanism described in higher animal models. To explore the potential of these metabolites as biomarkers for stress in salmon, it is important to identify the baseline levels. The metabolomic profile for fish is often highly influenced by the handling associated with sampling [275], and hence, a standard framework for salmon plasma metabolite collection and interpretation would be beneficial.

The gills are far more sensitive to the oxidant than the skin, as demonstrated by both transcriptomics and, to some extent, by mucosal mapping, where the changes in the gills provided a clear exposure duration-dependent response profile. This study supports the striking difference between the responses of salmon gills and skin to a chemical oxidant. Previous studies have indicated that oxidative agents induce more pronounced morphomolecular changes in salmon gills relative to the skin, and this difference has been attributed to the inherent large branchial surface area exposed to the environment and the greater structural complexity of the gills [138, 143, 170, 267]. Mucosal surfaces such as the gill and skin mucosa provide the first line of defence by providing both structural protection and an array of molecules vital for defensive and adaptive responses under challenging conditions. One of its cellular components, the mucous cells, produce the slimy mucous layer and exhibit phenotypic plasticity under stress [267, 276]. Though not as evident as observed in earlier studies [138, 267], the mucous cells in the gills increased in size, especially in the LE group, following exposure to the oxidative stressor, which indicates remodelling that may be crucial for mucosal barrier integrity. However, it is possible that the marginal response of the mucous cells points to impeded normal physiological function under the stressful conditions induced by the periodic application of the chemotherapeutics.

There is a tight relationship between immunity and oxidative stress in fish, particularly in ensuring that ROS remain below the detrimental level. Antioxidants are mobilised for neutralisation and scavenging, and other defence factors are activated to provide the fish with protection from oxidative stress-related damage [80, 81, 93, 264]. The functional annotations through the GO and Reactome analyses both highlighted that the oxidative chemotherapeutics impacted gill mucosal immunity, especially molecules involved in immune cell-cell communications. Some of the DEGs that significantly overlapped between the SE and LE groups, notably in the gills, have key functions in mucosal defence, including *il15rα*, the putative receptor for IL-15. The roles of IL-15L and IL-15Rα in Type 2 immunity have recently been reported in fish [277], and this study points to the possibility that it may also be important in immunity to oxidative stress. In mammalian systems, the IL-15 myokine has been implicated to have a protective effect against H_2O_2-mediated oxidative stress [278]. Mucins are the major glycopolymeric components of mucus and represent a large class of proteins in vertebrates, and *Muc7* was found to be commonly regulated in both the SE and LE groups. It is a secreted non-oligomerising mucin that can self-aggregate, but it is not thought to contribute to the mucus properties in mammalian models [279]. Its functional role remains elusive in fish, but its strong regulation in the present study provides a potential connection to its role during chemically induced stress.

The ribosome is a ribonucleoprotein-based molecular machine that orchestrates protein biosynthesis, and this complex process presents numerous control points for stress response regulation [280]. We found that pathways related to ribosomes were significantly enriched in the gills of the LE group. Ribosomal proteins were similarly overly represented in the skin mucus proteome, where a pronounced hypothetical protein-protein interaction was observed. The significant changes in the number of affected ribosomal genes and proteins, including the magnitude of their alterations, demonstrate their role in the adaptive cellular responses to the oxidative chemotherapeutics, which may impact ribosome dynamics and function. Though the interplay of oxidative stress and ribosome function has not been fully elucidated in fish, it has been shown in other organisms that oxidative stress affects protein translation such as during translational errors [280]. Ribosomal proteins repair the damage, thereby ensuring ribosome homeostasis and stability as the organism adapts to the condition. We believe that such a mechanism may be at play in the gill and skin mucosa to counteract the pressure from the chemotherapeutics. The effects of the oxidant on the skin mucosa were clearer in the skin mucus proteome than in the skin tissue transcriptome. Though the difference in the platforms and bioinformatics strategies used could explain this disparity, it is also likely that post-translational modifications may have a significant impact on skin mucosal responses to PAA, which is an area of interest for future studies.

The liver has a major function in the detoxification and maintenance of the body's metabolic homeostasis. We found that repeated exposures to the chemical oxidant presented a risk to liver function, as elucidated by both the clinical biochemistry and transcriptomics. The ASAT and ALAT levels were affected by the chemical oxidant, with the changes more pronounced in the SE group, suggesting that the interference in liver function was likely not dose dependent. These plasma indicators are used as tools to detect liver disturbances in salmonids, and increased levels may indicate impaired liver function, liver tissue damage, and necrosis [281]. The levels are considerably influenced by pre-analytical causes for variation such as diet, stress, and production site, which could become a confounding factor in the interpretation of the data. The values documented in this study were lower than the proposed baseline levels for adult salmon (>3.5 kg) [282]. The specific clinical significance of these analytes in salmon health monitoring is yet to be fully substantiated, as clinical biochemistry is an approach that is not widespread in aquaculture. We hope that the values identified here will be beneficial to establishing the standards and biological relevance of these analytes in fish. The hepatic transcriptome provides the insight that the redox balance in the liver was altered by the chemical oxidant, as pathways related to oxidoreductase activity and the oxidation-reduction process had been altered. This further indicates that repeated exposures to the chemical oxidant not only trigger mucosal oxidative stress but may also be impacting internal organ oxidative status and a predisposing factor for the increased ROS levels in the plasma. There was also an indication that the oxidant exposure may influence renal function, with lower plasma creatinine levels after the 2nd exposure. Nonetheless, it is difficult to draw an implication because the control group also had a substantially higher creatinine level, and the effects were only observed at one time-point. We checked all parameters before the 2nd exposure that could explain the elevated creatinine in the control group but could not pinpoint a significant deviation.

We had previously found that crowding stress prior to treatment modifies the responses of salmon to chemotherapeutics [170]. Here, we tested whether exposure to the oxidant could alter the responses of salmon to a secondary stress such as crowding, which is a common stressor in fish farming. Repeated exposures to the oxidant did not significantly impact the salmon's responses to a secondary stressor, as they were able to regulate plasma cortisol levels similarly to the unexposed fish. However, it was interesting to observe that the SE group had higher cortisol levels than the two other groups, indicating that short-term exposure to the oxidant may influence the kinetics after a stressful episode. Overall, the post-stress responses suggest that repeated exposure did not pose a considerable risk to the ability of the salmon to respond to a secondary stressor.

Conclusions

This study demonstrated that periodic exposures to oxidative chemotherapeutics affect salmon physiology at different magnitudes. The chemical oxidant altered the balance of internal ROS and consequently, triggered systemic oxidative stress. Localised oxidative stress was likewise induced in the mucosal organs, particularly in the gills. A similar impact was also displayed in the hepatic transcriptome. Gills were found to be more highly sensitive to the chemical oxidant than the skin and may be the organ that plays a crucial role in the adaptive mucosal response to a chemical stressor. Transcriptomic changes in the gills highlighted the different countermeasures the salmon mounted to address the threat of the chemotherapeutics. The molecules identified both at the mucosal and systemic levels, especially those with known functions in antioxidant defence, and ROS scavenging and detoxification underscored those responses were orchestrated and these critical molecules initiated a complex signaling in a broad variety of cellular processes. The chemical oxidant may also interfere with liver function, and thus its use must be considered with caution, though minimal risks were documented in terms of performance and ability to respond to a secondary stressor. Our results offer new mechanistic insights into salmon's physiological responses and adaptations to chemotherapeutics-induced oxidative stress. This information will be beneficial for designing optimised treatment strategies for using oxidative biocides such as PAA in fish farming.

Publication 9*

Recurrent oxidant treatment induces dysregulation in the brain transcriptome of Atlantic salmon (*Salmo salar*) smolts

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ABSTRACT

Peracetic acid (PAA) is an organic peroxide that produces free radicals, which contribute to its potent disinfection power. At therapeutic doses, PAA is considered a mild stressor that can trigger transient local and systemic oxidative stress in fish, but the resulting consequences in the brain have yet to be identified. Therefore, we report the brain transcriptome of Atlantic salmon (*Salmo salar*) smolts that have been periodically exposed to PAA. Fish were treated 3 times with PAA with either short (15 min) or long (30 min) exposure periods. The whole brain was collected after the third treatment and subjected to biochemical and transcriptomic analyses. The level of reactive oxygen species in the brain was not significantly affected by recurrent PAA treatments. Microarray analysis was performed on the whole brain and revealed 205 differentially expressed genes (DEGs), regardless of the duration of the treatment. The short exposure duration had a more considerable impact on the brain transcriptome, correlating with 70 % more DEGs than the long exposure. Strikingly, the brain transcriptome was characterised by downregulation of gene expression, especially in the short exposure group, and around 82% of the identified DEGs were downregulated. Some of the genes that were highly affected were key molecules of the vasotocinergic and isotocinergic systems, as well as the corticotropin-releasing factor signalling system, indicating that PAA treatment interfered with the stress axis. In addition, there were alterations in genes involved in cellular metabolism and processing, signalling and trafficking, and innate immunity, which underscores the physiological dysregulation in the brain following recurrent PAA treatment. Overall, the transcriptomic data reveal that recurrent oxidant treatment could influence brain functions, and although the magnitude was marginal, the alterations suggested neurological adaptations of fish to PAA as a chemical stressor. The results identify the risks of PAA, which would be valuable in drafting a framework for its empirically driven use in fish farming.

Introduction

Modern fish farming uses strategies that improve robustness through preventive measures, which is mainly achieved by enhanced biosecurity in farms, balanced and fortified nutrition, and the use of effective vaccines [283]. However, many of these strategies are still not effective in addressing standing bottlenecks, and the only viable alternative has been to resort to chemotherapeutics to treat bacterial, viral, fungal, and parasitic infections. Unlike a few decades ago, when chemotherapeutics were used imprudently, modern aquaculture strives to use these treatment options cautiously, especially since resistance poses a higher risk [284].

Oxidative biocides such as chlorine, hydrogen peroxide (H_2O_2), and peracetic acid (PAA) are a group of oxidising agents that target many relevant fish pathogens. As oxidants, they remove electrons from susceptible chemical groups, oxidise them, and become reduced in the process [116]. PAA is one of the oxidative chemotherapeutics that has received considerable attention in the last years because of its innate features that set it apart from other commonly used therapeutics, particularly regarding safety, effectiveness, and environmental impact. Commercial PAA products are available as acidified mixtures of acetate and hydrogen peroxide, which degrade into inert and harmless residuals [117] and are found to be potent against several fish pathogens, even at very low concentrations [169].

As an oxidising agent, PAA functions through the denaturation of protein, disruption of cell-wall permeability, and oxidation of sulfhydryl and sulphur bonds in proteins, enzymes, and other metabolites [285]. The oxidative action can be a highly reversible process, and organisms have evolved many defences against the effects at lower concentrations. Nevertheless, at higher levels, these defence mechanisms can be exhausted, which results in significant surface, cell-wall, and intracellular damage [116, 255]. Hence, its use in aquaculture must find a balance between effectiveness against pathogens and minimising the impact on the health and welfare of host fish.

In recent years, we have progressively established the health impacts of using PAA on fish, which has revealed that salmonids (i.e., Atlantic salmon and rainbow trout) can mount strong physiological adaptive responses to PAA [80, 170, 229, 286, 287]. The series of studies on Atlantic salmon revealed that PAA application could be a mild stressor and that it could trigger transient mucosal and systemic oxidative stress. This striking consequence is associated with the formation of radicals upon its decay, which directly interact with the fish, or it could also be due to an indirect effect of dysregulating the internal redox homeostasis [117, 288]. The gills and the olfactory organ are the main organs that are sensitive to PAA in salmon. Strikingly, these mucosal organs can orchestrate a cascade of counteractive responses to the physiological threats of PAA, especially by activating the antioxidant systems.

In general, PAA is considered a welfare-friendly antimicrobial [37]. This is exemplified by the stress responses during and following PAA treatments, indicating that fish can mobilise an adaptive response, habituate to single and repeated exposures, and demonstrate unaltered responses to a secondary stressor. However, most of our understanding of the stress physiology concerning PAA treatment in fish is focused on circulating molecules, such as the traditional indicators cortisol, glucose, and lactate [37, 287]. There has not been explorations of how the response to PAA in the brain, a central organ of the central nervous system that regulates an array of vital processes, such as endocrine function and the stress response [289]. Environmental pollutants in the aquatic environment trigger oxidative stress and induce brain damage or dysfunction in fish [290, 291]. The brain and neurons are highly sensitive to reactive oxygen species (ROS), and oxidative stress has been considered as a critical factor in neurotoxicity and brain injury [292]. Given the oxidative stress-inducing potential of PAA, we expect that the brain could be a target organ that influences the stress responses to the oxidant.

We hypothesise that PAA regulates brain functions, but the neurotoxicity of the therapeutic dose is low. Using microarray analysis, we report the first transcriptome of the brain of Atlantic salmon exposed to therapeutic doses of PAA. Atlantic salmon smolts were exposed periodically to PAA to simulate a husbandry scenario where fish are subjected to several rounds of oxidant treatment to prevent parasitic infection during a production cycle [286].

Materials and methods

Ethical use of animals for research

All procedures involving fish in this study adhered to the guidelines of the Norwegian Animal Welfare Act (Dyrevelferdsloven 2009) and Directive 2010/63/EU of the European Union (amended 2019/1010). The trial was approved by the Norwegian Food Safety Authority under FOTS ID 19321. Key personnel in the trial have FELASA C certification.

Recurrent exposure to peracetic acid (PAA) trial

Commercially available PAA product (Divosan Forte™ VT6) was provided by Lilleborg AS, Norway. The product is a stabilised PAA solution (15%) that is non-foaming. To ensure correct PAA dosing, the actual concentration of PAA in the product was analysed by an external laboratory (DTU Aqua, Denmark through Dr. Lars Flemming Pedersen). The samples used were collected from an exposure trial that was reported in a sister study [286]. We confirm that no data have been duplicated in this study since a different perspective is reported regarding the large-scale *in vivo* exposure trial.

Briefly, the fish trial was performed at the Tromsø Aquaculture Station (HiT; Tromsø, Norway). Atlantic salmon smolts (approximately 80–90 g) produced at the station were distributed into nine 500-L circular tanks in a flow-through system at a density of 40 fish per tank. The system had the following parameters:

a water flow rate of 6–7 L·min^{-1}, salinity of 35 ‰, temperature of 13.0 ± 1 °C, dissolved oxygen > 90 %, and saturation and photoperiod of 24 L: 0 D. A continuous feeding regime was applied (Nutra Olympic 3 mm, Skretting, Averøy, Norway). The fish were allowed to acclimatise for one week before the first PAA exposure was performed.

There were three treatment groups, and each group had 3 replicate tanks that were randomly distributed in the experimental hall. The two PAA treatment groups were exposed to 10 mg L^{-1} of PAA for either 15 min (short exposure) or 30 min (long exposure). Exposure was performed as follows: the water inlet was closed, and PAA was added to the water column to achieve the target concentration. Aeration was supplied to allow mixing and maintain oxygen levels > 90 %. After the exposure period (15 or 30 min), the water flow was opened, and at least 90 % of the water was replaced within 8–10 min. The control group was not exposed to PAA. The experimental fish were exposed to PAA every 15 days over 45 days, and there were 3 exposures in total.

Sample collection

Brain samples were collected 24 h after the last PAA exposure. Sampled fish were humanely euthanised with an overdose of benzocaine (Benzoak vet, 200 mg/mL, EuroPharma, Leknes, Norway). Five fish were collected from each tank (15 fish for each experimental group). The whole brain was dissected by making an incision on the posterior region of the skull, immediately placed in dry ice, and eventually stored at -70 °C until analysis. Prior to ROS determination and RNA extraction, the brain samples were homogenised using a micro pestle to ensure that different regions were analysed en masse.

Determination of reactive oxygen species (ROS) in the brain

Brain lysate was prepared by suspending the tissue in 10 times its volume of sterile chilled 1X phosphate-buffered saline. Samples were sonicated in ice and centrifuged at 13,000 *g* for 10 min at 4 °C. The supernatant was transferred to a new tube and immediately used for the assay. The level of ROS/RNS was determined using a commercially available kit (OxiSelect™ In Vitro ROS/RNS, CellBiolabs, Inc., USA). The assay utilises a quenched fluorogenic probe, dichlorodihydrofluorescin DiOxyQ (DCFH-DiOxyQ), which is a specific ROS/RNS probe. Hydrogen peroxide (H_2O_2) was used as the standard. All 15 fish per treatment group were used, and analyses were run in duplicate.

RNA isolation and microarray analysis

Automated total RNA extraction from whole brains (9 fish per treatment group) was carried out in a Biomek 4000 Benchtop Workstation using the Agencourt RNAdvance™ Tissue Total RNA Purification Kit (Beckman Coulter Inc., CA, USA). The quantity and quality of purified RNA were determined by a NanoDrop 8000 spectrophotometer (Thermo Scientific, USA). RNA quality was further assessed by an Agilent® 2100 Bioanalyzer™ RNA 6000 Nano Kit (Agilent Technology Inc., Santa Clara, CA, USA). All samples used for microarray had an RNA integrity number of 8.4 or higher. A custom-designed 15K Atlantic salmon DNA oligonucleotide SIQ-6 microarray (Agilent Array, ICSASG_v2) was used.

RNA amplification was carried out by the One-Color Quick Amp Labelling Kit followed by Cy3 labelling using 110 ng of RNA template per reaction. Gene expression hybridization kits were used for the fragmentation of labelled RNA, and the arrays were hybridised for 15 h in an oven at 65 °C with a constant rotational speed of 10 rpm. Next, the arrays were successively washed with Gene Expression Wash Buffers 1 and 2 and scanned using an Agilent SureScan Microarray Scanner. Pre-processing was performed in Nofima's bioinformatics package STARS (Salmon and Trout Annotated Reference Sequences) [88]. All reagents were purchased from Agilent Technologies.

Data analysis

Sigmaplot 14.0 Statistical Software (Systat Software Inc., London, UK) was used to analyse the ROS level. A student t-test was used to compare the change in ROS level in the brain and statistical significance was set at $P < 0.05$.

The microarray results were exported from STARS as log2 transformed expression ratios (ER) and further processed in R (version 4.0.2, https://www.r-project.org/). ERs of the treatment groups were normalized by subtracting the respective ER values of the control group. Significant differential expressed genes (DEGs) were defined by a p-value cut-off of <0.05 (one-way ANOVA, aov() function, *stats* package) between the controls and the two treatment groups and a minimum mean ER difference of 0.5 between the highest and the lowest group. This resulted in 205 DEGs, which were represented in a heatmap (heatmap.2() function, *gplots* package, Figure 3). Distances between genes were calculated, using the Euclidean distance method and the dendrogram was calculated by the complete linkage algorithm. The dendrogram was split into four clusters with distinctive expression patterns. The functional annotation terms, as they are used in STARS, where tested for significant enrichment within these clusters (fisher.test() with alternative hypothesis set to “greater” only, *stats* package). Terms with p-values <0.05 are shown next to the heatmap with indication, in which cluster they were identified.

Results and Discussion

PAA is one of the greener chemotherapeutic alternatives in aquaculture because its chemical behaviour is characterised by superior potency against diverse pathogens, rapid degradation, and inert residuals and by-products. Despite the evidence that application of PAA could be a mild stressor for the fish, acute stress responses are not significantly affected, and robust adaptive responses are mounted, which underscores its applicability as a welfare-friendly antimicrobial agent in aquaculture [37, 286, 287]. We have made significant advancements in understanding the biology of PAA in fish, especially in regard to how it affects health and welfare, but its neurological effects remain elusive. To the best of our knowledge, this is the first report describing the brain responses at a molecular level in fish exposed to PAA. We found that salmon brains responded to recurrent PAA treatment. Moreover, the transcriptomic changes reveal that the short exposure duration had a more substantial impact on the brain than the long exposure duration.

Recurrent PAA exposure does not alter the ROS level in the brain

Reactive oxygen species (ROS) are reactive molecules and free radicals derived from molecular oxygen that are the key molecular actors in oxidative stress [293]. In particular, exogenously and endogenously generated peroxides are ROS that are powerful activators of cellular oxidative stress [294]. Chemotherapeutic interventions may cause oxidative stress, which is associated with cognitive impairment [295]. Brain tissue is considered to be highly sensitive to oxidative stress due to its limited antioxidant capacity [296]. There is evidence indicating that PAA application could alter the mucosal and systemic ROS balance in fish [80, 229, 286], which provides evidence that it is a strong regulator of oxidative stress.

We have shown previously that intermittent administration of PAA with either short or long exposure durations resulted in the increase of ROS/RNS in plasma, which was indicative of perturbed redox homeostasis [286]. In the present study, we did not find inter-treatment differences in the level of ROS in the brains of salmon (Figure 1). Hence, with the concentration and administration strategies tested, PAA administration does not trigger neurological oxidative stress via increased ROS in the brain. Xenobiotics such as drugs and pollutants are often observed to alter the redox balance in the brain, and

this neurotoxicological effect is often used to evaluate safety [297]. Even although there were behavioural changes in response to single and recurrent exposure to PAA, which suggest neurological interference [286, 298], the present study clarifies that these may not be related to the elevation of ROS levels in the brain.

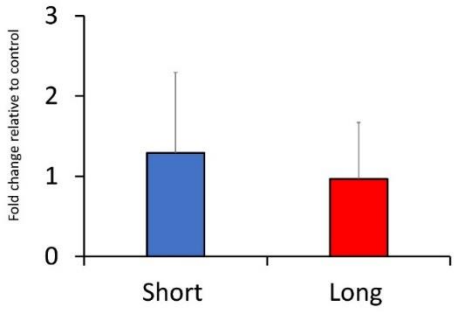


Figure 1. Changes in the level of ROS in the brain of Atlantic salmon smolts subjected to recurrent PAA treatment. Fold change was expressed relative to the level of ROS arbitrarily as H_2O_2 in the control group. There two exposure durations were short (15 min) and long (30 min). No significant change was identified. N = 15 fish.

More genes are differentially expressed in the brains of fish with short PAA exposure than long exposure

We attempted the first molecular elucidation of the consequences of PAA on brain functions in fish. There were 205 differentially expressed genes (DEGs) identified regardless of comparisons (Figure 2). We found 109 DEGs when short exposure was compared to the control, of which 90 were downregulated while 19 were upregulated. In contrast, only 32 DEGs were identified when long exposure was compared with the control. Around 70 % (22 genes) of the DEGs identified were upregulated, demonstrating a different response profile from the short exposure versus the control. Comparing the two PAA-exposed groups, 120 DEGs were identified, and 105 of them were upregulated.

Peroxides are potentially neurotoxic and are known to alter the brain transcriptome across several organisms, including fish [296, 299]. It is apparent in the number of genes that short exposure to PAA resulted in a more substantial dysregulation in the brain than long exposure, which emphasises the regulatory influence of exposure duration on how PAA impacts brain functions. Previous reports have demonstrated that the duration of PAA exposure influences how salmonids mobilise physiological adaptive responses to PAA [37, 80]. In a sister study, we found that long exposure instead of short exposure duration to PAA had a more considerable impact on the gills and liver transcriptome [286]. Therefore, the present data provide new insight on how the salmon brain is more sensitive to a shorter duration of PAA exposure than the organs in terms of mucosal response and hepatic metabolism of the oxidant.

Interestingly, even a 15-min difference in the exposure duration could elicit a substantial contrast between the two treatments, which exemplifies the small window of neuroregulatory function of PAA. Such a toxicological profile was also revealed in earlier studies [169]. The apparent sensitivity of the brain to the short duration could be related to the abrupt response to PAA, which was somehow abated upon longer exposure.

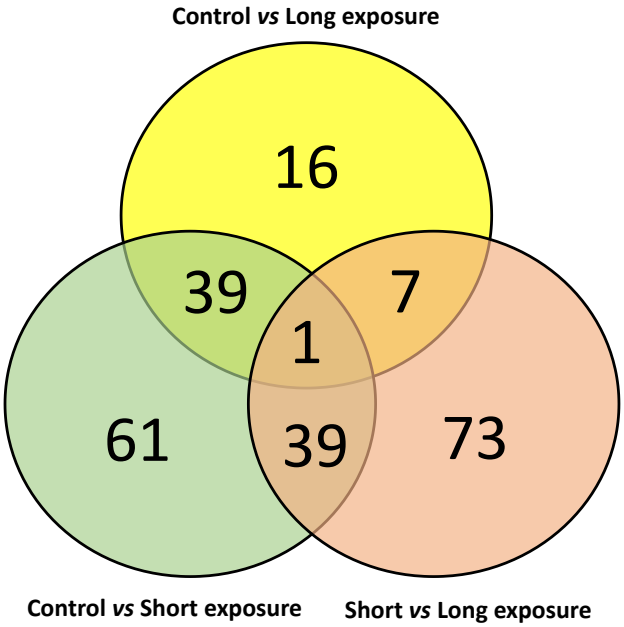


Figure 2. Differentially expressed genes (DEGs) in the brain of Atlantic salmon smolts subjected to recurrent PAA treatment presented as a Venn diagram showing the interactions of different group comparisons. Complete list of DEGs is supplied in Supplementary File 1.

Dysregulation in the brain is typified by a downregulation of gene expression following recurrent PAA administration

Next, we grouped the genes according to the signature of their transcriptional profile (Figure 3A, Tables 1-3). Cluster 1 is composed of 20 genes that are typified by downregulation relative to the unexposed control group. In terms of magnitude, downregulation was more substantial with short exposure than long exposure (Figure 3A, Table 1). With only 2 genes, Cluster 2 had the lowest number. Both PAA-exposed groups showed downregulation relative to the control group. As with Cluster 1, the magnitude of the change was higher in the short-exposure group.

Cluster 3 had the greatest number in the clustering with 177 genes (Figure 3A, Tables 1-3). This cluster is characterised by downregulation in the short-exposure group and upregulation in the long-exposure group. Lastly, Cluster 4 showed a similar tendency to Cluster 3 with downregulation was observed in the short-exposure group and upregulation in the long-exposure group (Figure 3A, Table 3). Functional enrichment of these DEGs showed no clear overall patterns (Figure 3B). Nonetheless, we have identified three major functional groups of genes that stood out: those exhibiting downregulation were genes involved in tissue differentiation and tissue endocrine, while upregulated genes were related to nucleotide metabolism.

PAA is considered a mild stressor, and as such, exposure to it necessitates an array of stress responses in fish, which are considered an evolutionary adaptation [300]. Some of the genes that were significantly downregulated and exhibited considerable change were *vasotocin-neurophysin VT 1* and *isotocin-neurophysin IT 2.* These molecules are biologically active nonapeptides in teleosts produced in separate neurosecretory neurons in the hypothalamic nuclei and are known to be involved in both osmotic and handling stress in fishes [301]. For example, in gilthead seabream, exposure to air for about 3 min alters the hypothalamic expression of vascotocin and isotocin precursors and receptors, which have been implicated in the activation of the stress system [302].

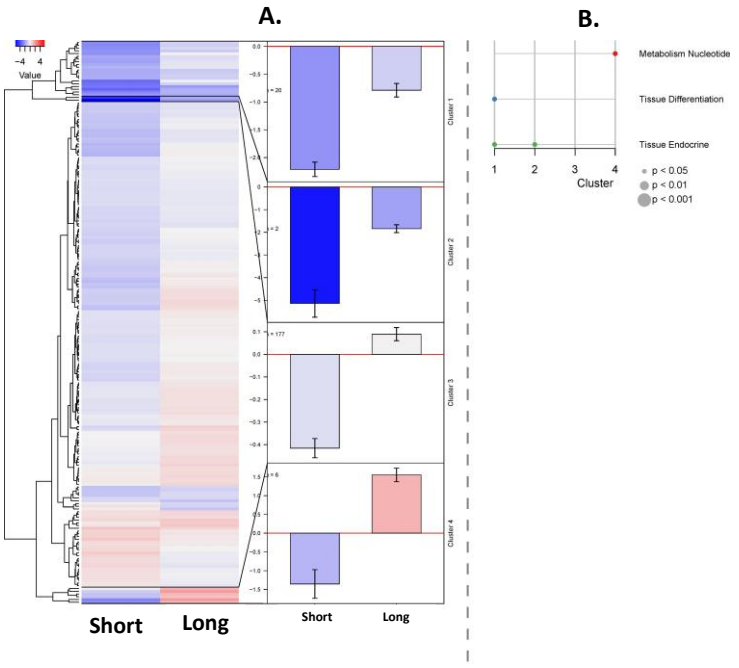


Figure 3. Brain transcriptome of Atlantic salmon smolts exposed to recurrent PAA treatment either short (15 min) or long (30 min) exposure duration. A) The heatmap on the left shows the down- and upregulation of DEGs in a colour gradient from blue to red. The dendrogram was split into 4 sub-clusters, and the mean values for genes within these clusters are represented in bar plots (error bars show +/- standard error of the mean) in the centre. B) Enrichment analyses of the 4 sub-clusters. The identified functional gene categories are shown along the Y-axis, and the six clusters are along the X-axis. Dots were coloured according to the categories, and the size indicates the p-value according to Fisher's exact test.

Table 1. List of differentially expressed genes in the brain from Clusters 1 and 2. Expression is given as Log2 change relative to the control group. Genes without annotated function were not included.

Cluster	Annotated function	Ids	Gene name	Short	Long
1	Tissue Differentiation	Ssa#TC69706	protein LBH-like	-2.43	-0.72
1	Tissue Differentiation	Ssa#S35548105	protein LBH-like	-2.48	-0.82
1	Metabolism Calcium	Ssa#GRASP209738279	Calcyphosin-like protein	-2.41	-0.60
1	Tissue ECM mucus	Omy#S15320733	SCO-spondin	-2.27	-0.94
1	Tissue Secretory	Ssa#S18862026	type-4 ice-structuring protein LS-12-like	-2.34	0.02
1	Unknown Uncertain	Ssa#S19188800	transmembrane 4 L6 family member 4-li	-1.72	-0.55
1	Tissue Differentiation	Ssa#S30269065	Sp9 transcription factor	-1.94	-0.30
1	Cell Transcription	Ssa#DW577494	SAM pointed domain-containing Ets trar	-1.66	-0.89
1	Tissue Neural	Ssa#TC93898	Prospero-related homeobox gene 1a	-1.61	-0.78
1	Tissue Differentiation	Ssa#GRASP209734699	Anterior gradient homolog 2	-1.61	-0.80
1	Tissue Endocrine	Ssa#S31999799	Somatostatin-1A	-3.20	-0.02
1	Cell Signaling	Ssa#S18886120	Arrestin-C	-2.87	-2.08
1	Tissue Endocrine	Ssa#GRASP209736901	isotocin-neurophysin IT 2-like	-3.36	-1.56
1	Tissue Endocrine	Omy#S15341086	AF106006_1 arylalkylamine N-acetyltran	-2.08	-1.60
2	Tissue Endocrine	Ssa#S32007779	vasotocin-neurophysin VT 1	-4.53	-1.66

Table 2. List of differentially expressed genes in the brain from Cluster 3. Expression is given as Log2 change relative to the control group. Genes without annotated function were not included.

Cluster	Annotated function	Ids	Gene name	Short	Long
3	Tissue Differentiation	Ssa#S35666309	Hepatocyte nuclear factor 3-beta	-0.90	-0.35
3	Unknown Uncertain	Ssa#S35664619	Glutaminyl-peptide cyclotransferase-	-0.95	-0.35
3	Immune Complement	Ssa#S18849877	Complement factor Bf-1	-0.94	-0.40
3	Cell GTP signaling	Ssa#GRASP2091548	RAB3C, member RAS oncogene family	-0.92	-0.28
3	Immune Lectin	Ssa#S18833713	ladderlectin-like (LOC106568836)	-1.02	-0.39
3	Tissue Endocrine	Ssa#GRASP2097369	catechol O-methyltransferase-like	-1.04	-0.31
3	Metabolism Amino acid	Ssa#EG819142	Glutaminyl-peptide cyclotransferase-	-1.03	-0.14
3	Tissue Differetiation-hox	Ssa#DW553112	Homeobox protein DLX-1	-1.07	-0.20
3	Metabolism Iron heme;Immun	Ssa#S18835321	Serotransferrin 2	-1.06	-0.06
3	Immune Eicosanoid	Ssa#S35546405	Prostaglandin endoperoxide synthase	-1.29	-0.37
3	Tissue plasma	Ssa#CB511693	Thrombospondin-3	-1.24	-0.38
3	Tissue plasma	Ssa#S19100148	Coagulation factor IX precursor	-1.30	-0.31
3	Unknown Uncertain	Ssa#KSS2742	Unknown	-1.12	-0.36
3	Immune Acute phase	Ssa#CK895788	uncharacterized LOC106605369	-1.17	-0.32
3	Tissue Neural	Ssa#S47729301	neuropeptide B-like	-1.43	-0.07
3	Unknown Uncertain	Ssa#GRASP2212202	Netrin-G1 ligand	-1.26	-0.04
3	Immune Lectin	Ssa#S50369596	Galectin-4	-1.36	0.12
3	Tissue Neural	Ssa#S35505983	neuropeptide B-like	-1.38	0.13
3	Tissue Differetiation-hox	Ssa#GRASP2097309	Distal-less homeobox gene 3b	-1.48	0.11
3	Cell transcription factor;Tissue	Ssa#S47729686	Hairy-related 6	-0.53	-0.03
3	Tissue Differentiation;Tissue N	Ssa#GRASP2236484	Protein tyrosine phosphatase, recept	-0.52	-0.03
3	Immune Complement	Ssa#S27548663	complement C2-like	-0.51	-0.07
3	Tissue Endocrine	Ssa#S34426295	follicle stimulating hormone receptor	-0.61	-0.06
3	Tissue Adhesion	Ssa#GRASP2236473	tetraspanin-8-like	-0.61	-0.10
3	Tissue Neural	Ssa#GRASP2097308	Secretogranin V	-0.55	-0.11
3	Metabolism Ion	Ssa#STIR43810	sodium channel subunit beta-3-like	-0.52	-0.22
3	Unknown Uncertain	Ssa#S35562993	CD209 antigen-like protein E (c209e)	-0.52	-0.22
3	Tissue Differentiation	Ssa#GRASP2097383	Nanos homolog 1	-0.54	-0.25
3	Cell Reticulum	Ssa#GRASP2212194	Transmembrane emp24 protein trans	-0.54	-0.28
3	Cell Cytoskeleton microtubule	Ssa#GRASP2212201	Tubulin polymerization-promoting pr	-0.56	-0.29
3	Tissue Endocrine	Ssa#S35685886	C-type natriuretic peptide 3-like	-0.67	-0.25
3	Immune Platelet	Ssa#GRASP2236480	Platelet-derived growth factor recept	-0.63	-0.27
3	Cell cytoskeleton	Ssa#GRASP2091486	PDZ and LIM domain protein 2	-0.61	-0.28
3	Tissue plasma	Ssa#S35025091	Plasminogen	-0.77	-0.32
3	Metabolism Protease inhibitor	Ssa#S46932341	serine (or cysteine) proteinase inhibi	-0.62	-0.40
3	Metabolism Protease inhibitor	Ssa#GRASP2091545	serine (or cysteine) proteinase inhibi	-0.69	-0.37
3	Tissue Neural	Ssa#TC73710	Adrenergic receptor, beta 3a	-0.73	-0.01
3	Cell endocytosis	Ssa#GRASP2091474	EH domain-containing protein 3	-0.79	0.04
3	Immune	Ssa#S18835494	C1q and TNF-like domains	-0.70	0.06
3	Tissue Secretory	Ssa#S18848149	Beta-2-glycoprotein 1	-0.84	-0.10
3	Tissue Differentiation	Ssa#GRASP2091546	hepatocyte growth factor a	-0.86	-0.09
3	Metabolism P450;Metabolism X	Ssa#S35596838	CYP2J2	-0.67	-0.20
3	Unknown Uncertain	Ssa#GRASP2236485	Transmembrane protein 184a	-0.69	-0.20
3	Cell Autophagy;Cell Apoptosis	Ssa#CK897976	cell death activator CIDE-A-like	-0.69	-0.13
3	Tissue Differentiation	Omy#S38753637	CCAAT/enhancer binding protein (C/E	-0.73	-0.20
3	Metabolism Protease;Immune	Ssa#S35025085	plasma protease C1 inhibitor-like	-0.77	-0.17
3	Tissue Growth factor	Omy#S23931884	Pappalysin 2	-0.90	0.13
3	Metabolism Protease inhibitor	Ssa#S48436303	trypsin inhibitor ClTI-1-like	-1.00	0.08
3	Metabolism sulfur	Ssa#GRASP2097363	Sulfotransferase family 1, cytosolic su	-0.88	0.33
3	Metabolism Ion	Ssa#S31987297	GTP-binding protein GEM	-1.14	0.10
3	Metabolism Lipid	Ssa#GRASP2091556	Lipolysis stimulated lipoprotein recep	-1.26	0.19
3	Metabolism Protease inhibitor	Ssa#S35527824	trypsin inhibitor ClTI-1-like	-1.04	0.22
3	Tissue Differentiation	Ssa#TC110577	Leukocyte cell derived chemotaxin 1	-0.80	0.48
3	Tissue ECM collagen	Ssa#GI226374736	Procollagen, type V, alpha 1	-0.80	0.52
3	Metabolism Protease inhibitor	Ssa#S35472944	Kunitz-type protease inhibitor 2 precu	-0.91	0.55
3	Cell Transcription	Ssa#GRASP2091544	V-ets erythroblastosis virus E26 oncog	-0.92	0.52
3	Tissue Secretory;Metabolism Ir	Ssa#S18835396	hemopexin-like	-0.82	0.63
3	Tissue Growth factor	Ssa#GRASP2212195	Mimecan precursor	-1.19	0.51

Table 3. List of differentially expressed genes in the brain from Clusters 3 (continuation) and 4. Expression is given as Log2 change relative to the control group. Genes without annotated function were not included.

Cluster	Annotated function	Ids	Gene name	Short	Long
3	Tissue Differentiation	Ssa#STIR36587	Protein Wnt-11	-0.39	0.26
3	Metabolism Protease	Ssa#S31973583	Aminopeptidase N	-0.39	0.24
3	Tissue Differentiation	Ssa#S35509837	Pancreatic progenitor cell differentiation a	-0.39	0.21
3	Metabolism Iron heme	Omy#S23924054	Ferritin, heavy subunit	-0.39	0.11
3	Metabolism P450;Metabolism X(	Ssa#S35700248	Cytochrome P450 27C1	-0.52	0.15
3	Metabolism Calcium	Omy#S48432679	s100 calcium binding protein	-0.54	0.17
3	Immune T cell;Immune Cytokine	Ssa#S35562605	Possible interleukin-16	-0.49	0.15
3	Immune Complement;Immune A	Ssa#KSS1868	C1q-like adipose protein	-0.46	0.18
3	Metabolism Calcium	Ssa#GRASP209736275	Protein S100-A11	-0.56	0.12
3	Unknown Uncertain	Ssa#GRASP223648051	Olfactomedin-like protein 2A	-0.58	0.03
3	Metabolism Xenobiotic	Ssa#S35675658	ATP-binding cassette, sub-family C	-0.45	0.05
3	Metabolism P450;Metabolism St	Ssa#GRASP209155257	Cytochrome P450 3A65	-0.45	0.05
3	Cell cytoskeleton	Ssa#TC106546	Desmoplakin	-0.46	0.04
3	Tissue Differentiation	Ssa#GRASP209736013	chibby homolog 1	-0.48	0.09
3	Cell GTP signaling	Ssa#GRASP209732025	Intercellular adhesion molecule 2	-0.47	0.09
3	Cell transcription factor	Ssa#S18876527	Zinc finger protein 385B - Ident 54	-0.53	0.02
3	Metabolism Retinoid	Ssa#GRASP209737503	Retinol binding protein 1a, cellular	-0.70	0.23
3	Cell cytoskeleton	Ssa#GRASP209155923	Desmin b	-0.68	0.27
3	Cell Ubiquitin	Ssa#S18888523	E3 ubiquitin-protein ligase RNF130	-0.67	0.14
3	Cell Transcription	Ssa#S18886064	Nuclear receptor subfamily 2, group F, me	-0.75	0.17
3	Cell cycle	Ssa#GRASP223647747	G1/S-specific cyclin-D1	-0.35	0.34
3	Unknown Uncertain	Ssa#GRASP209734581	nuclear protein 1-like	-0.29	0.38
3	Unknown Uncertain	Ssa#S48411712	Leucine-rich repeat and calponin homolog	-0.31	0.46
3	Tissue ECM	Ssa#GRASP209154383	Lumican	-0.32	0.44
3	Cell Myofiber	Ssa#DW541352	Ryanodine receptor 1b	-0.29	0.44
3	Immune Complement	Ssa#S23659844	Mannan binding lectin serine proteases	-0.41	0.42
3	Tissue Neural	Ssa#GRASP221221407	neurogranin-like	-0.40	0.43
3	Metabolism Transport	Ssa#S50694600	ATPase, Cu++ transporting, alpha polypept	-0.42	0.37
3	Tissue ECM collagen	Ssa#STIR40939	Col6a2 protein - Ident 96	-0.39	0.50
3	Immune T cell	Ssa#NP9934346	modified T cell receptor alpha	-0.43	0.50
3	Tissue Endothelium;Immune Eic	Ssa#GRASP223648725	vascular endothelial growth factor c	-0.35	0.53
3	Tissue Motility	Ssa#CA037592	Unconventional myosin-Ib	-0.24	0.30
3	Unknown Uncertain	Ssa#S47726096	methyltransferase DDB_G0268948	-0.28	0.24
3	Tissue ECM collagen	Ssa#GRASP223648099	Procollagen-proline, 2-oxoglutarate 4-dio	-0.26	0.24
3	Immune regulator	Ssa#S18889874	Dual specificity phosphatase 22b	-0.67	0.82
3	Tissue Endothelium	Ssa#S35592699	Angiopoietin-like 7	0.07	0.51
3	Metabolism	Ssa#GRASP209155279	Phosphoethanolamine/phosphocholine p	0.01	0.53
3	Metabolism P450;Immune Eicos	Ssa#S30239290	cytochrome P450 2J2-like	0.01	0.62
3	Cell GTP signaling	Ssa#TC108644	RasGEF domain family, member 1Ba	-0.04	0.58
3	Metabolism Steroid, bile	Ssa#TC108320	catechol O-methyltransferase domain-cor	-0.06	0.54
3	Metabolism sulfur	Ssa#S30289499	Sulfotransferase 6B1	-0.04	0.49
3	Unknown Uncertain	Ssa#KSS627	Leucine-rich repeat and calponin homolog	-0.15	0.42
3	Metabolism Nucleotide	Ssa#GRASP209736395	catechol O-methyltransferase domain-cor	-0.11	0.41
3	Cell transcription factor	Ssa#S35538660	Basic leucine zipper and W2 domain-conta	-0.16	0.49
3	Cell Ubiquitin	Ssa#S31996646	Ubiquitin-60S ribosomal protein L40	-0.14	0.67
3	Unknown Uncertain	Omy#S24636898	Musculoskeletal embryonic nuclear protei	-0.13	0.58
3	Cell Ubiquitin	Ssa#S50701189	Ubiquitin-60S ribosomal protein L40	-0.27	0.63
3	Cell Ubiquitin	Ssa#GRASP223649343	Ubiquitin-60S ribosomal protein L40	-0.16	0.78
3	Cell Transcription	Ssa#TC69925	FACT complex subunit SPT16	0.15	0.55
3	Cell Signaling	Ssa#GRASP221220449	Transmembrane protein 100	0.13	0.58
3	Cell cytoskeleton	Ssa#S31992041	beta-taxilin	0.18	0.61
3	Tissue Growth factor;Tissue ECM	Ssa#TC72388	Periostin, osteoblast specific factor b	0.20	0.56
3	Metabolism Amino acid	Ssa#S35589996	Glutamine synthetase	0.20	0.54
3	Cell Apoptosis	Ssa#GRASP209735205	Cell death activator CIDE-3	0.15	0.72
3	Immune Complement	Ssa#S34822137	Complement C3;	-1.14	-0.70
3	Immune T cell	Ssa#S31980404	Protein kinase C, delta a	-1.07	-0.80
3	Metabolism	Ssa#GRASP209735437	Prostaglandin-H2 D-isomerase	-1.04	-0.57
3	Tissue Differentiation	Ssa#DW571249	Nuclear receptor subfamily 4, group A, me	-1.07	-0.58
3	Metabolism RNA	Ssa#GRASP209153979	HEXIM protein	-0.59	-0.62
3	Metabolism Calcium	Ssa#GRASP209154751	Calsequestrin-1	0.12	-0.63
3	Cell Exocytosis	Ssa#EG850628	EH-domain containing 1a	0.40	-0.66
3	Metabolism Xenobiotic	Ssa#GRASP209737177	Epoxide hydrolase 1	0.14	-1.10
3	Cell Ubiquitin;Immune IFN-virus	Ssa#KSS3245	Ubiquitin-like protein-2	0.50	0.68
3	Immune Effector	Ssa#DY700748	SAM domain and HD domain-containing pr	0.60	0.52
3	Metabolism Sugar	Ssa#S35505408	6-phosphofructokinase type C	0.69	0.97
3	Tissue plasma	Ssa#S35681202	Coagulation factor VIII, procoagulant comp	0.19	0.91
3	Unknown Uncertain	Ssa#S31992042	beta-taxilin	0.31	1.05
3	Cell Myofiber	Ssa#S48416777	Alpha-tropomyosin	1.14	0.72
3	Tissue Differentiation	Ssa#S35570017	Mab-21-like 2	0.75	0.35
3	Metabolism Amine	Ssa#GRASP209736025	Arginase-1	0.75	0.21
3	Metabolism Amino acid	Ssa#S35523051	aspartyl/asparaginyl beta-hydroxylase-like	0.57	0.17
3	Tissue Neural	Ssa#DY699176	Glycine receptor, beta b	0.62	0.05
3	Tissue Neural;Metabolism Lipid	Ssa#S23659857	Sphingomyelin phosphodiesterase	0.72	-0.18
3	Tissue Neural	Ssa#GRASP223649159	Neuronal calcium sensor 1a	0.54	-0.19
3	Tissue ECM mucus	Ssa#S35547501	Mucin-5B	0.62	-0.34
3	Tissue Adhesion	Ssa#S35522677	basal cell adhesion molecule-like	0.62	-0.34
3	Tissue Differentiation	Ssa#GRASP209155331	Mab-21-like 1	0.48	-0.08
3	Cell Folding, protein modificatio	Ssa#S31981996	Protein arginine methyltransferase 1	0.49	-0.05
3	Immune Cytokine receptor	Ssa#S48403715	interferon gamma receptor 1	0.40	-0.10
3	Metabolism Mitochondria	Ssa#S30285335	NADH dehydrogenase [ubiquinone] iron-s	0.38	-0.12
3	Cell Folding, protein modificatio	Ssa#STIR38053	heat shock protein beta-1-like	0.32	-0.20
4	Metabolism Nucleotide	Ssa#S35590245	High affinity cGMP-specific 3 ,5 -cyclic pho	-0.13	1.52
4	Unknown Uncertain	Ssa#S18883768	cGMP-dependent protein kinase 1-like	-0.86	2.08

We have shown that PAA exposure interferes with the vasotocinergic and isotocinergic systems in the brains of salmon, which is indicative of its involvement in the stress response to PAA. Repetitive exposure to PAA in salmonids has not been shown to substantially alter the ability of fish to respond to the stressor [37, 287]. Hence, the striking changes in these genes in the present study imply moderate interference, which may be interpreted as compensatory response given that other variables like plasma-stress parameters and behaviour following PAA exposure did not change dramatically [286]. A number of chemical pollutants known to have neurotoxic activity in fish have been identified to disrupt the vasotocin/isotocin system [303]. This is the first report in fish showing that PAA affects this system, regardless of whether it is delivered with short or long exposure durations, and it should be cautiously considered in terms of the risk assessment concerning its use.

Stressors elicit endocrine, autonomic, visceral, and behavioural responses from an organism, which are largely coordinated by the activation of the corticotropin releasing factor signalling system in the brain [304]. Two genes with key involvement in this mechanism, *corticoliberin-1-like* and *somatostatin-1A*, were downregulated following PAA exposure. This lends further support to the observation that while the general impact of PAA might marginal, there is interference to varying degrees with several molecules involved in the stress response. *Corticoliberin* (also known as *corticotropin-releasing hormone*) is the hallmark brain peptide that triggers the response to stress and mediates the stimulation of the hypothalamus-pituitary-adrenal (HPA) axis during stressful episodes. This response includes other hormonal, behavioural, autonomic, and visceral components [305]. Moreover, it has been shown to exert neuronal protection against oxidative stress [306].

Somatostatin comprises a relatively large class of genes that are well distributed in the brain and respond to acute stress by counteracting the various components of the stress response, such as the associated dampening of hypothalamic CRF release or its actions [304]. The transcriptomic response suggests that the tight regulation of these two molecules can be altered by recurrent PAA administration. The changes in the genes involved vasotocinergic and isotocinergic systems and the corticotropin releasing factor signalling system in the brain suggest that PAA is an oxidant that can interfere with the stress response of fish. The long-term cost of this interference remains to be investigated, but previous studies have shown that it could be revealed in altered kinetics of the response to a secondary stressor [287].

Like any other cells, brain cells respond to stress in a number of ways, which range from the activation of pathways that promote survival to the elicitation of programmed cell death to eliminate damaged cells [307]. There are clear differences in how various molecules that regulate cellular activity respond to PAA administration: downregulation in the short-exposure group and upregulation in the long-exposure group, as shown by Cluster 3 in Figure 3. Cell signalling is a complex process that is orchestrated when an organism is prompted with a stressful stimulus [308].

The homeobox genes constitute a special group of highly conserved transcription factors characterised by a common DNA binding motif [309] and tissue regeneration and repair [310]. We observed two homeobox genes that were differentially expressed in particular in the short-exposure group: *homeobox protein DLX-1* and *distal-less homeobox gene 3b*. Their regulation might be connected to tissue repair following recurrent oxidant exposure. On the other hand, the knockdown of homeobox protein HOXB13 in HEK293 cells reduces the toxicity of oxidative stress [311]. Although this has yet to be functionally verified, the downregulation observed in the current study points to a potential role of the homeobox genes identified in resolving potential neurotoxic damage.

GTPases and related molecules play an important role in various aspects of neuronal development and functions. The Ras homolog family of guanosine triphosphate hydrolases (Rho GTPases), Ras homolog family member A (RhoA), Ras-related C3 botulinum toxin substrate 1 (Rac1), and cell division cycle 42

(Cdc42) are important regulators in somatosensory neurons, where they elicit changes in the cellular cytoskeleton. Furthermore, they are involved in diverse biological processes, including transduction of signals that contribute to fundamental cell-dynamic and survival events [312, 313]. Several genes have been identified to be differentially expressed, including *GTP-binding protein GEM*, *Ras-dva-2 small GTPase*, *Intercellular adhesion molecule 2*, *RasGEF domain family*, *member 1Ba, RAB3C, member RAS oncogene family,* and *Rho GTPase activating protein 5*. In most of these cases, downregulation was observed. Since these molecules are important for spatiotemporal fine-tuning of physiological processes, their significant regulation during oxidant exposure indicates a crucial control of cellular turnover that dictates cellular survival following intermittent exposure to a chemical stressor.

The ubiquitin-proteasome system (UPS) is the major pathway for the regulation of protein homeostasis in the eukaryotic cells [314]. This process is governed by ubiquitin, a highly conserved 76-amino-acid protein that is conjugated to substrate proteins through linkage via its C-terminal glycine residue. Ubiquitin plays a vital role in degradation, DNA repair, endocytosis, and inflammation [314, 315]. Neurons rely on ubiquitin-mediated quality-control mechanisms for misfolded proteins or damaged organelles [316]. The regulation of several ubiquitin-related genes in the study, including *E3 ubiquitin-protein ligase RNF130*, *Ubiquitin-60S ribosomal protein L40* (3 transcripts), and *Ubiquitin-like protein-2,* indicates that ubiquitin-mediated processing was activated, especially for damaged proteins.

Upregulation was explicitly exhibited in the long-exposure group, indicating that quality control via ubiquitin targets PAA-induced brain changes with a longer exposure. Mild oxidative stress has been shown to upregulate the ubiquitination system and proteasome activity in cells and tissues and transiently enhances intracellular proteolysis [317]. Although it was not convincingly established that oxidative stress was triggered locally in the brain, earlier evidence demonstrated that the PAA administration protocol in this study triggered systemic elevation of ROS [286]. A direct relationship has yet to be established, but given the known interaction among these systems, we believe that such a mechanism occurs in the brain.

Neuronal metabolic processes in the brain ensure that nutrients and oxygen are supplied to neurons and astrocytes [318], especially when physiological demands are high, such as during exposure to a chemical stressor. Different aspects of tissue metabolism were affected following recurrent PAA treatment. For instance, the long-exposure group showed significant upregulation of genes responsible for nucleotide metabolism, such as *catechol O-methyltransferase domain-containing protein 1-like* and *High affinity cGMP-specific 3,5-cyclic phosphodiesterase*, but these were negatively and marginally affected in the short-exposure group. On the other hand, the short-exposure group showed downregulation in genes involved in calcium metabolism, such as *Calcyphosin-like protein*, *s100 calcium-binding protein*, and *Protein S100-A11*, but the opposite was observed in the long-exposure group.

Dysregulation of neuronal intracellular Ca2+ homeostasis can play a crucial role in many neurotoxic effects, including impaired brain functions and behaviour [319]. It could be possible that recurrent PAA treatment alters the Ca2+ balance in the brain, which increases the risk of neurotoxicity of PAA with a shorter exposure. It has been reported that the metabolic function of calcium is crucial during oxidative stress through activation of the membrane permeability transition, release of cytochrome c, and respiratory inhibition, among others [320]. We believe that this is also involved in salmon exposed to PAA.

Proteolytical processing of membrane-bound molecules is a fundamental mechanism for the degradation of these proteins and controlling cell-to-cell communication [321]. Gene encoding for proteases was also represented in the set of DEGs, including *serine (or cysteine) proteinase inhibitor clade E, nexin, plasminogen activator inhibitor type 1-like*, *plasma protease C1 inhibitor-like*,

Aminopeptidase N, *trypsin inhibitor CITI-1-like,* and *Kunitz-type protease inhibitor 2 precursor*. In most cases, downregulation was observed in the short-exposure group, while upregulation was demonstrated in the long-exposure group. This differing response suggests that proteasomal and lysosomal proteolytic pathways that continually maintain protein turnover are inhibited by short exposure duration. The relevance of such inhibition to the neurological risk of PAA remains to be functionally elucidated, but this observation warrants consideration in assessing the health risk of PAA to salmon.

Fish have an established neuroimmune interaction [322], but this interplay is not often explored in the context of chemotherapeutics administration. Studies have demonstrated that PAA is a potent modulator of immune functions in salmon, particularly at the mucosal surfaces [170, 229]. We have identified some immune genes that are affected by PAA treatment, including those involved in T cells (*modified T cell receptor alpha*), cytokines (*interferon gamma receptor 1*, *interleukin-16*), and the complement system (*Complement C3*, *Mannan binding lectin serine proteases*, *C1q-like adipose protein*, *complement C2-like*, *Complement factor Bf-1*). Microglial cells are the main innate immune cells of the complex cellular structure of the brain and they respond quickly to pathogens, stress, and injury by activating a cascade of pro-inflammatory responses [323]. The complement system is crucial for microglial cells [324]. It consists of over 30 independent proteins and provides rapid recognition and response to danger to the host [325]. Aside from their key roles in defence, complement proteins in the brain exert non-inflammatory functions in regulating structural plasticity and functional homeostasis of synapses [324]. Their considerable regulation of several complement genes following recurrent exposure to PAA is perhaps related to ensuring brain homeostasis, which is crucial for the adaptive response to the oxidant.

In summary, this study has presented the first brain transcriptome data from fish subjected to PAA treatment. Overall, the transcriptomic changes indicate that recurrent exposure to PAA alters brain functions, but the magnitude seems marginal given the number of differentially expressed genes compared with previous transcriptomics studies on salmon smolts exposed to PAA [170, 229, 286]. Although it was not quite clear whether PAA triggered oxidative stress in the brain, genes involved in stress responses were affected, especially those involved in the hypothalamic-pituitary-interrenal (HPI) axis.

Differentially expressed genes indicate that the short exposure had a substantially greater impact on the brain than the long exposure. The results offer new insight that even a 15-min window of exposure has consequential impacts on brain functions. These transcriptomic alterations present another perspective on how PAA could produce interference and possibly pose a threat if treatment protocols are not executed properly. This in spite of PAA generally being considered as a welfare-friendly antimicrobial for fish [37, 80]. In addition, these results should be valuable in guiding evidence-driven use of PAA in aquaculture, particularly as a chemotherapeutic.

5.3 PAA as chemotherapeutics

5.3.1 Amoebicidal activity of PAA

N. perurans are susceptible to PAA, even at low concentrations (Figure 1). The amoebae were exposed to different concentrations of PAA either for 15, 30 or 60 mins. The concentrations tested were the same concentrations used for salmon exposure. The percentage viability after exposure was quantified using the WST-1 vital dye.

Exposure dose rather than exposure duration appeared to have more influence on the toxicity of PAA towards the amoeba. At 0,6 ppm and regardless of the exposure time, the viability of amoeba in culture was at least 60 %. The viability was reduced significantly to at least 25 % when exposed to 2,4 and 4,8 ppm. The viability was almost negligible when exposed to 9,6 ppm. Microplate-based viability assay was complemented with the conventional neutral red staining of individual amoeba exposed to PAA

(Figure 2). The membrane integrity of amoeba is compromised by PAA, as shown by the inability of PAA-exposed amoeba to uptake the vital dye.

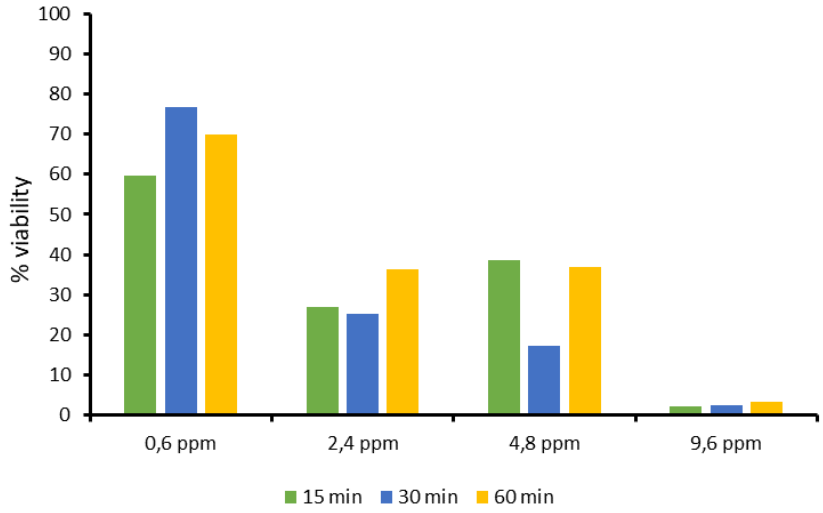


Figure 1. Percentage viability of N. perurans exposed to different concentrations of PAA and under varying exposure duration.

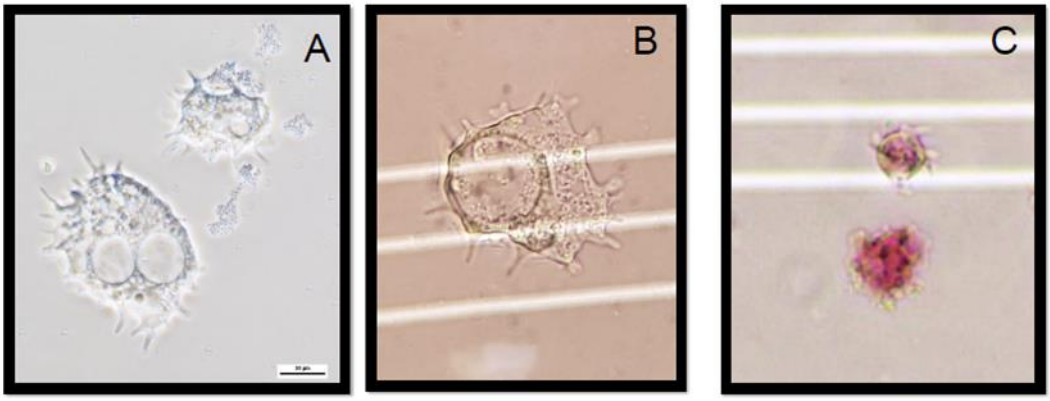


Figure 2. Neutral red staining of amoeba. ***A)*** *Unstained amoebas.* ***B)*** *Dead amoeba after exposure to 2,4 ppm PAA for 30 mins. Note the change in morphology.* ***C)*** *Live amoeba from the unexposed group.*

Factors affecting PAA toxicity towards *P. perurans*

We evaluated several factors affecting the susceptibility of P. perurans to PAA (Figures 3). PAA at 0.6 ppm is more toxic towards a two-week-old amoeba culture compared with a 1-week old culture, particularly at 200 amoebae/well seeding concentration (Figure 3). It appeared that amoeba density has more influence on PAA toxicity in 1-week old than 2-week old culture. Under standard PAA toxicity assay condition (200 amoebae/well, 15oC) it was shown that viability was reduced significantly at higher amoeba density in the well, especially at concentration > 4.8 ppm (Figure 3). One might expect that the denser colony might be less susceptible to a chemical, but such a case was not observed here. This might be related to metabolic competition during oxidative stress – more cells, higher metabolic requirements during stress, the thereby higher competition for limited resources.

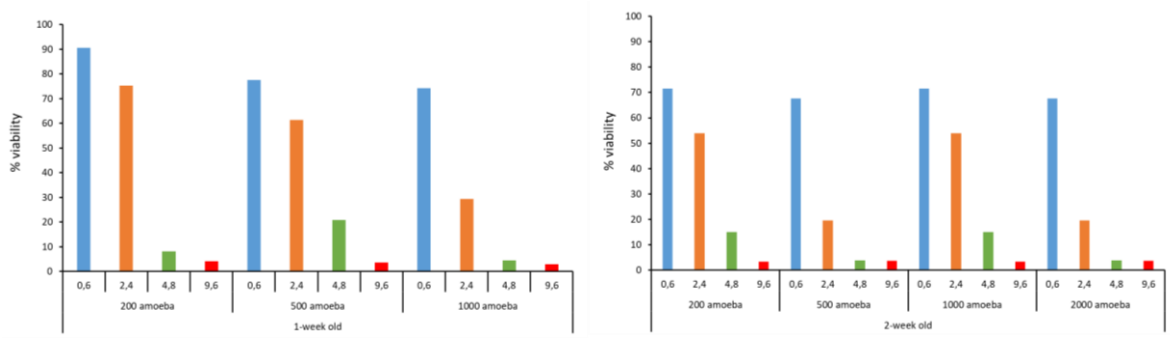


Figure 3 Effects of culture age on the toxicity of PAA towards N. perurans.

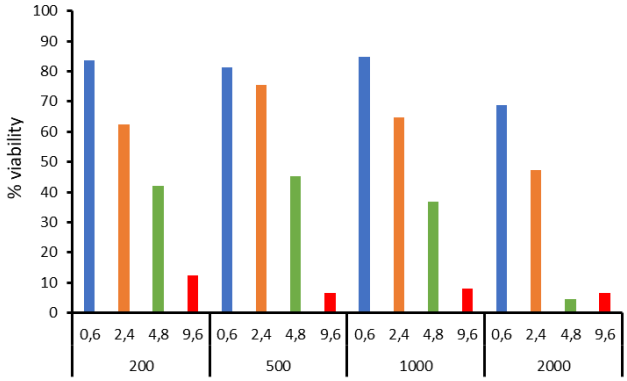


Figure 4 Effects of amoeba density on PAA toxicity.

Since light is crucial in the kinetics of decay of PAA, it was hypothesised that they might play an important function as well in its toxicity (Figure 4). One striking observation was noted in amoebae exposed to 2,4 ppm under light, where the viability was at least 15 % lower compared with the same PAA-exposed group without light. We could not establish whether exposure in the presence of light increased the toxicity since, at higher doses, the viability of amoebae either with light or without light during exposure were almost identical.

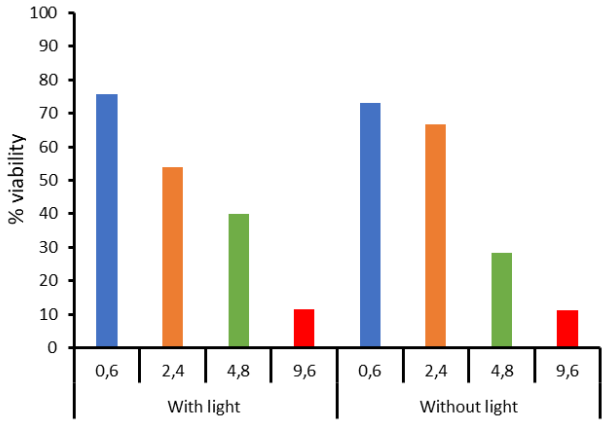


Figure 5. Effects of amoeba density on PAA toxicity.

Exposure temperature did not reveal a clear tendency, whether it had a negative or positive impact on the toxicity of PAA (Figure 37). The profile at 15 ºC was consistent with the other *in vitro* trials that the increase in dose resulted in reduced viability. It appeared that a relatively higher exposure temperature altered this profile, where amoeba viability at 4.8 and 9.6 ppm at room temperature was almost identical.

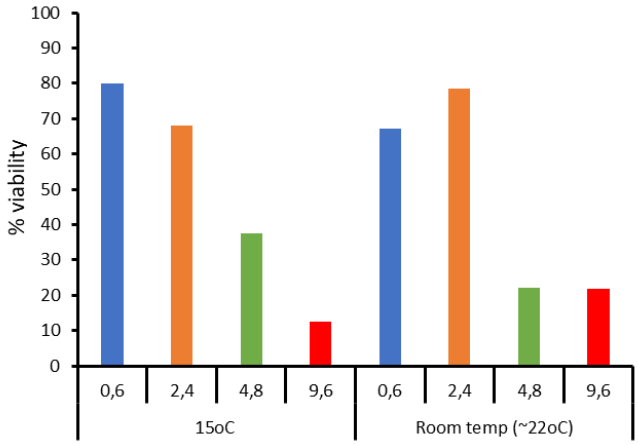


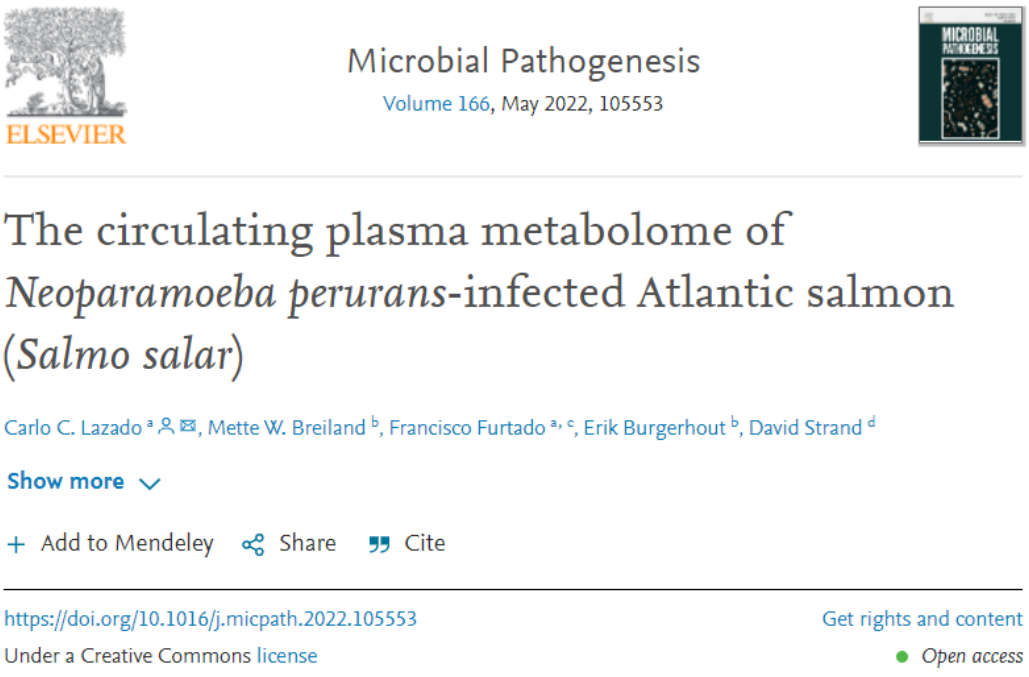
Figure 6. Effects of exposure temperature on PAA toxicity

5.3.2 New insights into the pathophysiology of AGD

The final version of the results appeared on the following publication:

Lazado, C.C., Breiland, MW., Furtado, F., Burgerhout, E., Strand, D. 2022. The circulating metabolome of *Neoparamoeba perurans*-affected Atlantic salmon (*Salmo salar*). Microbial Pathogenesis. 166, 105553.

Publications 10



ABSTRACT

Metabolomics can provide insights into the dynamic small-molecule fluctuations occurring in response to infection and has become a valuable tool in studying the pathophysiology of diseases in recent years. However, its application in fish disease research is limited. Here, we report the circulating plasma metabolome of Atlantic salmon (Salmo salar) experimentally infected with *Neoparamoeba perurans*—the causative agent of amoebic gill disease (AGD). Plasma samples were collected from fish with varying degrees of infection inferred from an external gross morphological score of gill pathology (i.e., gill score [GS] 1 -- GS3), where a higher GS indicates advanced infection stage. Uninfected fish (GS0) served as the control. Typical pathologies associated with AGD infection, such as hyperplastic lesions and lamellar fusion, were evident in infected gill samples. Plasma metabolites were identified by ultra-performance liquid chromatography coupled with a high-resolution quadrupole-orbitrap mass spectrometer. Identification of compounds were performed at four levels of certainty, where level 1 provided the most accurate compound identity. A total of 900 compounds were detected in the samples of which 143 were annotated at level 3, 68 on level 2b, 74 on level 2a, and 66 on level 1. Versus GS0, GS1 showed the highest number of significantly affected metabolites (104), which decreased with a higher GS. Adrenaline and adenosine were the two Level 1 compounds significantly affected by AGD regardless of GS, with the former increasing and the latter decreasing in infected fish. Hippuric acid significantly increased in GS1 and GS2, while the tryptophan metabolite indole-3-lactic acid decreased in response to the initial stage of infection but returned to basal levels at a higher GS. There were ten significantly affected metabolic pathways: Eight of which were significantly downregulated while two were downregulated in GS1 relative to GS0. The super-pathway of purine nucleotide salvage was enriched both within the upregulated metabolites in GS1vsGS0 and the down-regulated metabolites in GS3vsGS1. This is the first report on the circulating plasma metabolome of AGD infected salmon, and the results show that low infection levels resulted in a more dramatic metabolomic dysregulation than advanced infection stages. The metabolites identified are potential biological markers for the systemic physiological impact of AGD.

Introduction

Parasitic infestations have severe economic, animal welfare, and ecological impacts on Atlantic salmon (*Salmo salar*) aquaculture. Although sea lice (*Lepeophtheirus salmonis* and members of the *Caligus* genus) remain the major parasitic issue, other parasitic infestations pose serious concerns in salmon farming. Amoebic gill disease (AGD) is a proliferative gill condition primarily affecting the sea cage phase of salmon. It was first described in Tasmania, Australia in 1986, and since then, cases have been documented in the United States, Chile, Ireland, Spain, France, Japan, and Norway [9]. The causative agent, *Neoparamoeba perurans,* is a free-living and opportunistically parasitic amoeba species that attach to the gill lamellae [1]. The severity of AGD in the farms are assessed through histopathological and gill gross evaluations. Infected fish exhibit epithelial multifocal gill hyperplasia, hypertrophy, oedema, and interlamellar vesicle formation [11]. These can be grossly detected by increased mucus production and formation of white mucoid spots and plaques on the gill surface [10]. Behavioural manifestations of AGD include lethargy, anorexia, congregation at the water surface, and increased ventilation rate [7] leading to respiratory distress that can result in mortality between 50-80% if left untreated [8, 9]. On-farm assessment of AGD is often performed through a systematic scoring of the white mucoid patches and gross lesions on the gills where infestation severity is rated from 0 to 5 [326]. Gill score is a gross measure of the degree of the host response to the presence of *N. perurans,* and the degree of lesion development is known to be in direct proportion to the parasite load and severity of infection [327].

The mucosal pathophysiology of AGD has been elucidated by transcriptomics, proteomics, and targeted biochemical analyses in the gills and mucus. Through microarray analysis, Young and colleagues

reported a coordinated down-regulation of the genes involved in the major histocompatibility complex class I (MHC I) pathway in addition to the downregulation of cytokines, particularly interferons, in AGD-affected gills[328]. Upregulation of *mucin 5* and Th2 cytokines (*il4/13a* and *il4/13b2*) were observed in experimental and natural *N. perurans* infections supporting the hallmark responses of salmon to AGD, which include increased hyperplasia and mucus production [329]. It has been further demonstrated that gill transcriptomic responses, particularly the molecular regulators of inflammation (i.e. cytokines) and the different immune cell markers (i.e. antigen presenting cells, B cells and T cells), are highly influenced by regional differences of the lesions [330]. The protein–protein interaction networks generated from gill mucus proteomics revealed that affected proteins formed part of cell to cell signalling and inflammation pathways [331]. Profiling of some key immune markers in the gill mucus of AGD-affected fish showed that IgM levels and the activities of peroxidases, lysozymes, esterases, and proteases, decreased in fish with high disease severity; nonetheless, a sequential recovery was observed after treatment [332]. A similar tendency of immune-suppression associated with AGD was identified in the gills proteomes especially during the initial phase of infection, where inhibition of protein expression with immune signalling, phagocytosis, and T-cell proliferation was documented [333]. A recently published novel model for host-parasite interaction during AGD pathogenesis integrated host and parasite functional response profiles to reveal how the different players for invasion of host tissue, evasion of host defence mechanisms and formation of the mucoid lesion are orchestrated during infection [334].

Despite the advances made in understanding the molecules and processes involved in the mucosal responses of salmon to *N. perurans*, the systemic physiological impacts of AGD infection remains barely understood. Nonetheless, some available studies offered a fragmentary insight into the systemic host response. While, a panel of selected humoral immune parameters was found to be unaffected in serum relative to the gill mucus of AGD-affected salmon [332], a large scale serum proteomic profiling revealed an increase in the expression of immune-related molecules at the early phase of infection followed by a strong inhibition at later stages [333]. Studying the systemic responses of salmon to *N. perurans* will be pivotal to our understanding of the extent of the impact of the parasitic infection and will facilitate the identification of new markers of disease status.

Metabolomics has become a powerful tool in physiological studies in aquaculture, although its application in understanding the onset and development of a disease is limited [335]. In humans, the identification of metabolic biomarkers in diseased individuals has novel potential advantageous features such as more accurate diagnosis, dynamic disease evaluation, non-invasive sampling, or personalised treatment assessment [336]. There has been an increasing regard to metabolomics in salmonid research, where it is widely applied in ecotoxicological and nutritional studies [337]. Its application to understand host-pathogen interaction is still limited though its potential to resolve physiological alterations during host-pathogen interaction is immense. Here, we applied large scale metabolomics in the plasma of salmon exhibiting different AGD infection levels inferred by the gross gill pathology. The profiled circulating metabolomes provide insight into the panel of metabolites altered by parasitic infestation and their role in the adaptive physiological response of salmon to AGD.

Materials and methods

Ethical use of animal for research

The specimens used here were collected from an associated infection trial (Norwegian Food Safety Authority FOTS ID 20/23121) performed at Aquaculture Research Station (Tromsø, Norway). This strategy supports the 3Rs (reduce, replace, refine) in aquaculture research by collecting samples from another trial thus reducing the number of fish used for research.

Neoparamoeba perurans

The isolate of *Neoparamoeba perurans* used in the trial was retrieved from a natural outbreak in 2019 by the Norwegian Veterinary Institute (facilitated through Sigurd Hytterød). A polyclonal culture (less than 1 year old) was sent to the Fish Health Department of Nofima AS in Tromsø, Norway where its pathogenicity and virulence were established. Thereafter, an infection model was developed for this isolate in another project (Basis Funding-Norwegian Research Council, *AGD-modell*), which was applied in this trial. In this infection model, fish infected with the parasite developed GS 0.5 – 1 after 8-10 days, GS 1-2 after 14-16 days, and GS 2-3 around 3 weeks. The double gill scoring system developed by Fomas - Fiskehelse og Miljø AS is a modified version of the widely known Taylor system [326] and was used to evaluate the severity of AGD infection. The isolate was routinely cultured in Malt Yeast Broth (with filtered seawater, 35 ppt) at 15 °C.

Description of the infection trial

Smolts produced at HiT with a starting weight of 83±7.8 g (n=720) was evenly stocked in two 1,000-L octagonal tanks in a flow-through. The fish had been acclimated to seawater for at least 2 weeks before they were transferred to the experimental units. One tank served as the uninfected control group (Tank C), and the other tank was used for the infection trial (Tank I). In this study, we were only interested in comparing the infected and uninfected fish and considered the individual fish as a biological replicate. Fish were allowed to acclimatise experimental conditions for a week in the before they were exposed to the parasite under the following parameters: water flow rate set at 6-7 L min^{-1}, salinity at 35 ‰, temperature at 14.0±0.5 °C, dissolved oxygen >90 % saturation, photoperiod set at 24 L:0 D, and a continuous feeding regime (Nutra Olympic 3 mm, Skretting, Averøy, Norway). Experimental infection (Tank I) was performed by closing the water flow in the tank, and the fish were exposed to *N. perurans* at an exposure dose of 1,500 amoeba/L of water for 1 hr. Oxygen was supplied during the experimental infection to maintain a DO >90 % saturation and facilitate continuous mixing. After the challenge period, water was flushed and replaced, and the system was operated following the conditions during acclimation. The control-uninfected group (Tank C) was exposed to the same manipulation except no parasite was added. The production protocol employed during the acclimation period was similarly followed during the disease development period.

After three weeks, a group of fish were humanely euthanised with an overdose of benzocaine (Benzoak vet, 200 mg/ml, EuroPharma, Norway), and the gills were scored by an experienced researcher according to the method described above. Five fish were chosen per each GS group (e.g., 1, 2, and 3) and control group, and blood was extracted from the caudal vessels using a heparinised vacutainer (BD Vacutainer™, Fisher Scientific UK Ltd, UK). Plasma was obtained by centrifugation for 10 mins at 5,000 rpm maintained at 4 °C, and thereafter stored at -70 °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 subsequent detection of the parasite by qPCR. The second gill arch from the right side was collected and stored in neutral buffered formalin (BiopSafe ApS, Hellerup, Denmark) for consequent histological processing. Gills from randomly infected fish were excised and sent to the laboratory for isolation and re-culture of the parasite.

Detection of the parasite in the gill swabs by qPCR

DNA was extracted from the gill swabs using the DNeasy blood and tissue kit (Qiagen). A *N. perurans* specific qPCR assay (David A. Strand, unpublished), with 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', was used to analyse the extracted DNA for the presence of *N. perurans*. The samples were analysed on the CFX96 Touch System (Biorad, CA, USA) with 25 µl reactions consisting of 12.5 µl TaqPath qPCR Mastermix, 500 nM of each primer and

250 nM of probe, PCR grade water and 5 µl DNA sample. The following qPCR cycling condition were used: an initial denaturation at 95 °C for 20 sec, followed by 50 cycles of denaturation at 95 °C for 3 sec and annealing at 60 °C for 30 sec. The specificity of the designed assay was analysed *in silico* against closely related organisms by performing a BLAST search at National Center for Biotechnology Information database. The assay was further tested *in vitro* against several in-house (Norwegian Veterinary Institute) *N. perurans* strains and against the closely related organisms N. pemaquidensis, N. branchiphila and N. aestuarina. All of the N. perurans strains were amplified with the assay, while *N. pemaquidensis*, *N. branchiphila* and *N. aestuarina* did not amplify.

Histology

The formalin-preserved gills were embedded in paraffin following a series of ethanol dehydration, xylene clearing, and paraffin infiltration in a benchtop histoprocessor (Leica TP1020, Germany). Paraffin-embedded tissues were cut into 5-µm-thick sections (Leica RM2165, Germany) and stained with haematoxylin-eosin and digitised using a slide scanner (Aperio CS2, USA).

Plasma metabolomics

Plasma samples were sent to MS-Omics ApS (Vedbæk, Denmark) for metabolite profiling. The analysis was conducted using a Thermo Scientific Vanquish LC coupled to Thermo Q Exactive HF MS. An electrospray ionisation interface was used as an ionisation source and analysis was performed in negative and positive ionisation modes. An ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) was used with a slightly modified protocol first described by Doneanu et al. [89], which had been applied earlier to salmon plasma samples [170]. Peak areas were extracted using Compound Discoverer 3.1 (Thermo Scientific). Identification of compounds were performed at four levels: Level 1 offered identification by retention times (compared against in-house authentic standards), accurate mass (with an accepted deviation of 3 ppm), and MS/MS spectra. Level 2a used identification by retention times (compared against in-house authentic standards) and accurate mass (with an accepted deviation of 3ppm). Level 2b used identification by accurate mass (with an accepted deviation of 3 ppm) and MS/MS spectra. Level 3 used identification via accurate mass alone (with an accepted deviation of 3 ppm). For unidentified compounds, the elemental composition was determined if there was a good match between the accurate mass obtained and the isotope pattern.

Data handling and statistics

Metabolomic data (also referred to as samples in this section) are scored (outliers identified) based on objective scoring methods: commonly used scoring methods are Hoeffding's D-statistic, mean Pearson correlation with other samples, sum of Euclidean distance to other samples, and the Kolmogorov-Smirnov test statistic. Samples were sometimes "blacklisted" (manually failed) or "whitelisted" (manually passed despite failing automatic checks) based on detailed manual inspection and consideration of the experimental design. A sample was classified as an outlier if it failed two or more of these objective parameters. Normalised data provided the input for statistical hypothesis testing in which metabolites that were significantly different between sample groups were identified. Statistical comparisons were performed using linear modelling as implemented in the Bioconductor package limma.

Significance values (p-values) were adjusted for multiple testing, by controlling the false discovery rate. For each comparison (e.g., GS0*vs*GS1), a positive log2 (fold change) indicates up-regulation in GS1 relative to GS0. Significant metabolites (defined using $P < 0.05$) from each comparison were analysed for enrichment of MetaCyc pathway membership (https://metacyc.org/) using a hypergeometric test. Enrichment ($P < 0.05$) was assessed for up-, down-, and bidirectional-regulated metabolites separately.

Results and Discussion

Alterations in endogenous and environmental factors profoundly impact the organism's metabolome, which acts as an essential chemical bridge connecting the environment with the different levels of a biological system [338]. In particular, infection changes the physiological state of an organism [339] and these modifications are reflected in the identity and nature of metabolites involved in the host responses [340]. Although some studies have shed some insights into these interactions in fish [335, 341], the systemic metabolomic consequences of parasitic infestation have been barely explored. To the best of our knowledge, this study is the first to evaluate the plasma metabolome of AGD-affected salmon and one of the few studies in farmed fish that has applied high-throughput metabolomics to unravel host-parasite interactions. We found that AGD altered the circulating metabolome of salmon, and that the changes were more pronounced in fish exhibiting the lowest infection level than in the groups with severely compromised gill status.

The samples were collected at the same time point post-infection; therefore the confounding effect of age [335] has been minimised and the changes observed were predominantly attributed to the disease state. The amoebae were detected by qPCR in all gill swab samples from infected fish, and its absence in the control, uninfected group was likewise verified. In addition, the parasite was re-isolated from the gills of the infected fish and managed to be re-cultured under laboratory conditions. This confirms that the biological samples used in this analysis represents both AGD-free and AGD-affected fish. Further, histopathology evaluation of infected gill samples indicated epithelial hyperplasia and microscopic lesions consistent with AGD (i.e., multifocal hyperplasia and fusion of the lamellar epithelium (Figure 1), where attached amoebae were observed [342]. Although cases of lifting and lamellar clubbing were also detected in both uninfected and AGD-affected fish, no considerable difference was detected between the groups. The frequency of occurrence is similar to previously reported healthy, AGD-free salmon smolts [170, 267]. These changes may likely be non-specific environmental responses and not primarily associated with the disease.

Traditional AGD research focuses mainly on histopathological alterations and molecular response profiling through gene and protein expression in the gills, and recent developments have made significant contributions to unravel the systemic physiological alterations associated with the disease [329, 331, 332]. AGD has systemic metabolic consequences, and these have been documented by traditional metabolic assays including respirometry [343] or targeted plasma biochemical analyses, such as quantification of the levels of glucose, lactate, and cortisol [344]. These analytical tools have provided insights into how varying degrees of AGD infection resulted in physiological dysregulation, however, they only slightly captured the extent of systemic changes in infected fish. Metabolomics allowed high-throughput analysis of several hundreds of metabolites associated with different biological pathways, thereby offering a promising tool to understand the overall disease state. Here we showed that AGD altered the plasma metabolome of salmon (Figure 2) supporting earlier evidence from targeted analysis [332] that the gill parasitic infection triggers not only a local response but also results in systemic physiological dysregulation. Using plasma as a biological matrix for this analysis provides an appropriate snapshot of the disease state, because the analysis of biofluids or tissues from infected hosts represents the most accurate methodology to describe the metabolic changes associated with disease [338]. Future studies must also account tissue-specific metabolomic response, given that the disease state of the gills have profound impact on distant organs such as head kidney and spleen [334]. This will facilitate the understanding of the metabolomic regulators of different organs with known key involvement in orchestrating host responses to AGD, thereby, establishing a global snapshot of inter-tissue communication during infection.

We have 900 compounds in the samples. Of these, 143 compounds were annotated on level 3, 68 on level 2b, 74 on level 2a, and 66 on level 1. Only these compounds were included for further analysis, and the unannotated compounds were provided in the for future reference.

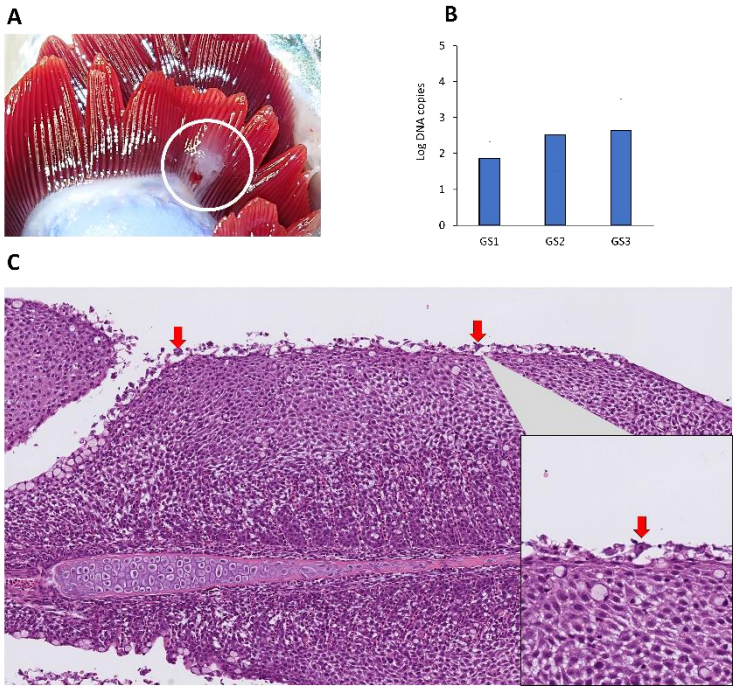


Figure 1. Gross and microscopic pathologies of AGD-affected salmon. (A) Macroscopic AGD lesions in experimentally infected fish showing the typical mucoid patches from the base to the mid-section of the filaments (inside the white circle). (B) qPCR detection of N. perurans. Log DNA copies are provided. C) The gills of AGD-affected fish showing the classical pathologies for AGD including multifocal hyperplasia and fusion of lamellar epithelium, where amoeboid bodies are likewise observed (inset).

The effects of AGD infection level (assessed by gross gill score) on the plasma metabolome were more pronounced when the level of infection was low (Figure 2). This was clearly shown with the decreasing number of differentially affected metabolites (DAM) relative to GS0 (uninfected fish) in fish with higher GS (Figure 2A-C). There were 104, 39, and 20 DAM in GS1, GS2, and GS3, respectively (Figure 2A-C). Despite this clear tendency of decreasing number of DAM at higher GS, the distribution of upregulated and downregulated metabolites remained consistent across different comparisons with no clear distinction suggesting that the pattern of dysregulation is not dependent on GS at least within the range used here. It would be interesting to explore in the future whether such a profile is still evident at GS > 3, where the gills are already severely compromised. Likewise, the correlation of the metabolomic responses between laboratory and natural infections is an area that should be explored further to expand our knowledge on how metabolome is altered during AGD.

We further compared the DAM amongst GS within infected fish (Figure 2D-F). The profiles further suggested that fish with GS1 demonstrated marked metabolomic dysregulation compared with GS2 (Figure 2D) and GS3 (Figure 2F). The lowest number of identified DAM was between GS2 and GS3 (Figure 2E) indicating that metabolomic changes between moderate and severe cases of infection were minimal. The fish were challenged at the same time, and the period of disease development is similar for all infected fish used in the analysis. Therefore, the plasma metabolome captured the disease state and offered insights into how some fish were more susceptible while others could ward off infection in the same environment and with a similar infection history. The pronounced alterations in the plasma metabolome with lower GS may be related to the continuous active physiological adaptations to fight off

the infection and would not allow it to develop further. As the severity of infection progressed, the fish became less able to mobilise the required metabolites to combat the pressure of infection, which could indicate an impairment of adaptive physiological response. It was earlier reported that immunity is impaired when the severity of AGD infection progressed as demonstrated by gene expression studies [332, 345]. This similar tendency of physiological response is reflected in the plasma metabolome. Metabolites can shape the function of immune cells and thus playing an important function in the outcome of immune responses [346]. The metabolomic profile in the present study and the immune response identified earlier for AGD establish a potential link between metabolites and immune regulators in resolving the host response to AGD. These profiles likewise reveal that the application of metabolomics for AGD is perhaps more appropriate during the early stage of infection because we observed striking changes on this level.

To investigate the overlap between selected metabolites from the multiple contrasts performed (Figure 2), we counted the number of overlapping DAM (defined using p-value<0.05) between all pairwise combinations of the comparisons performed, and the amount of overlap is represented in a heatmap (Figure 3A). Most of the significant overlap was found when contrast was made against the DAM of GS1*vs*GS0. In particular, the highest number of overlaps were found in the following comparisons: GS1*vs*GS0 versus GS3*vs*GS1, GS1*vs*GS0 versus GS2*vs*GS1, and GS2*vs*GS1 versus GS3*vs*GS1. Notably, the fold changes of the metabolites from the contrasts GS1*vs*GS0 versus GS3*vs*GS1 (Figure 3B) and GS1*vs*GS0 versus GS2*vs*GS1 (Figure 3C) were significantly negatively correlated. This may indicate that some changes observed in the low infection level are subsequently reversed as the infection progresses/regresses; or normal physiological functions may be inhibited in advanced infection stage.

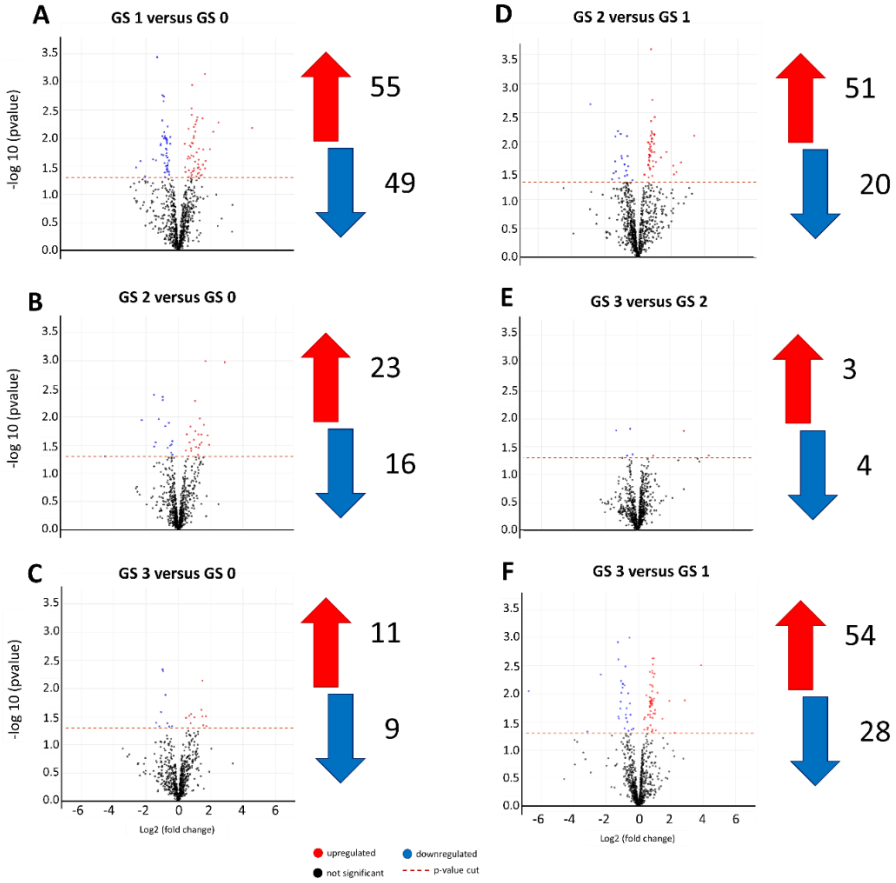


Figure 2. Volcano plots showing significance (as -log10 transformed p-values) against magnitude (log2(fold change)) of differentially affected metabolites. Metabolites identified as having different levels between samples are represented as red (up-regulated) or blue (down-regulated) dots/arrows. Numbers appearing beside the arrow indicates the actual number of metabolites in that specific group. To improve performance when there are tens or hundreds of metabolites, the non-significant metabolites displayed in black are a representative subsample of the entire dataset. For this set of comparisons, significantly differentially expressed metabolites were defined as those with a p-value < 0.05 represented by the horizontal orange line.

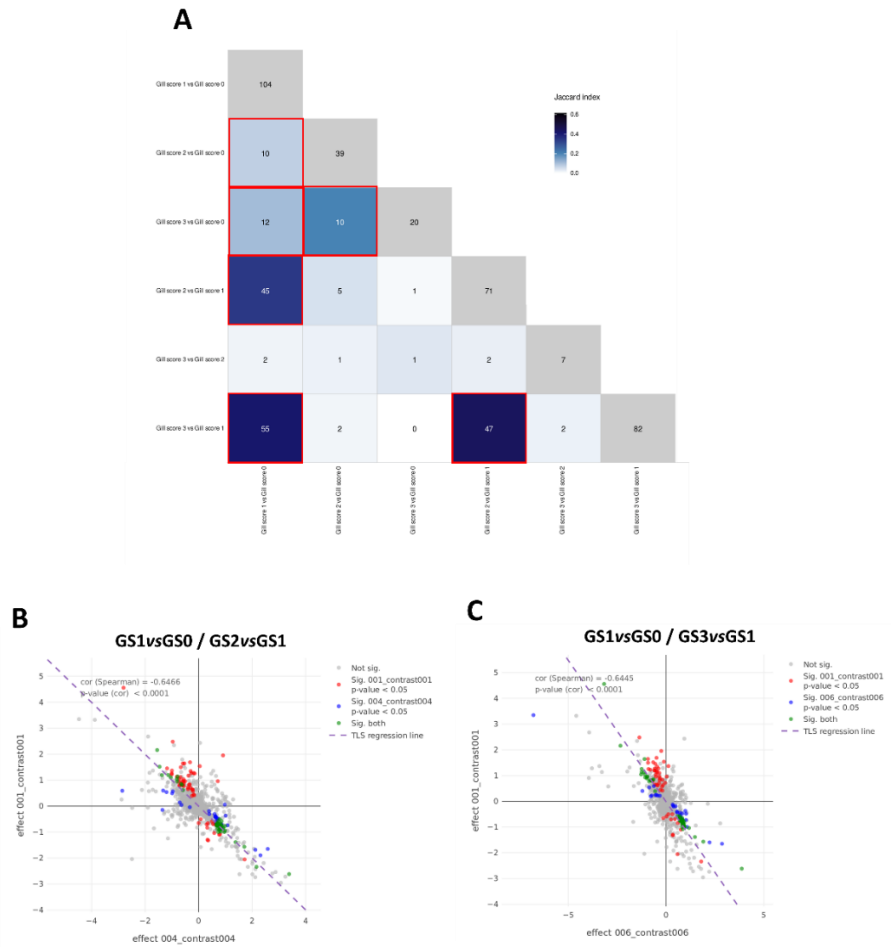


Figure 3. A) Heatmap showing the number of overlapping selected metabolites between the contrasts performed. Note that the numbers on the diagonal represent the total number of selected metabolites found for each contrast. For each comparison, the value in the plot represents the number of intersecting selected metabolites, and the colour represents the Jaccard index (the intersection over the union) for the two types of contrast under consideration. The box with a red border indicates that the overlap is statistically significant (defined using p-value < 0.05). B-C) Scatter plot comparing significant metabolites in B) GS1vsGS0 versus GS3vsGS1. Metabolites are represented by points and C) shows GS1vsGS0 versus GS2vsGS1. The colour of the point indicates which set the metabolite is assigned to. For each metabolite, the log2(fold change) in the GS1vsGS0 contrast (y-axis) and the log2(fold change) in the GS2vsGS1 (B)/ GS3vsGS1 (C) contrast (x-axis) are shown.

Regarding the DAM, adrenaline and adenosine were the two Level 1 DAM found in all AGD-affected fish (GS1-3) suggesting the important role of these molecules in the systemic responses of salmon to AGD (Figure 4). Notably, adrenaline showed a larger than 4-fold increase in abundance when comparing infected (GS1 to GS3) to uninfected (GS0) fish (Figure 4A). A similar statistically significant increase in adrenaline was observed upon comparing individual infected groups to uninfected samples. Adrenaline is a steroid hormone that participates in the stress response of fish and is produced from the hydroxylation of phenylalanine to tyrosine [347]. One of the adaptive physiological responses of salmon to AGD is the mobilisation of stress response such as the induction of plasma cortisol that is linked to the mediation of inflammation during infection [348, 349]. On the other hand, immunosuppression has

been documented in AGD-affected salmon [329, 332, 344]. Neuroendocrine hormones have important role in immunity where bidirectional communication between the endocrine and immune systems via hormones and cytokines have been established [350]. Hence, it could be possible that the increased plasma adrenaline level likely mediated immunosuppression because it has been shown to have an immunosuppressive role in fish [351]. In humans, adrenaline has been identified to regulate T cells and the regulatory function seems to indicate that it directly inhibit the T cells but not for their precursors [352]. Though such a relationship remains to be fully elucidated in fish, we speculate that the pronounced changes in adrenaline in the study present a potential cellular trafficking mechanism of T cells during AGD infection. The previous study that identified T-cells increase within the AGD-affected gills, where CD8+ cells and not CD4+ T-cells were prominent [330], lends support to this interplay and hence, an area for further exploration.

Adenosine was 1.6-fold less abundant in infected samples than healthy samples (Figure 4B). This trend was observed in GS1 to GS3 when compared to GS0. Adenosine is a naturally occurring nucleoside present in various cell types. It is an essential molecule for energy production and utilisation, and it exerts profound immune regulatory functions in many organisms [353]. In particular, it has anti-inflammatory properties [354]; therefore, its downregulation in infected fish suggest interference of its putative role in inflammation, especially in the recruitment of immune cells. Earlier, AGD was shown to have higher severity and an impaired local inflammatory response [329]; whether this is related to the downregulation of circulating adenosine remains an open question. On the other hand, hypoxia is a possible consequence of compromised gill structures due to AGD. The production of adenosine under hypoxic condition is critical for adaptation, maintenance of cellular function, and protection of hypoxia-induced tissue injury [355]. Therefore, the reduced level of adenosine in infected fish suggests that this crucial mechanism had been likely inhibited, which could result in other pathophysiological alterations if the infection progresses further.

Hippuric acid (Level 1 annotation) increased in response to infection showing a significant 3-fold increase in salmon with a GS1 compared to those with a gill score of 0. Similar trends were observed in GS2 compared to GS0. Hippuric acid is a metabolic derivative of benzoic acid, but its role in fish is still largely unknown [356]. In mammals, it is a biomarker for high dose exposure to certain toxic compounds such as toluene and is also commonly used as an indicator of renal health [357]. It remains to be explored whether such association is likewise present in salmon though the strikingly elevated levels in salmon with low AGD infection warrants further studies.

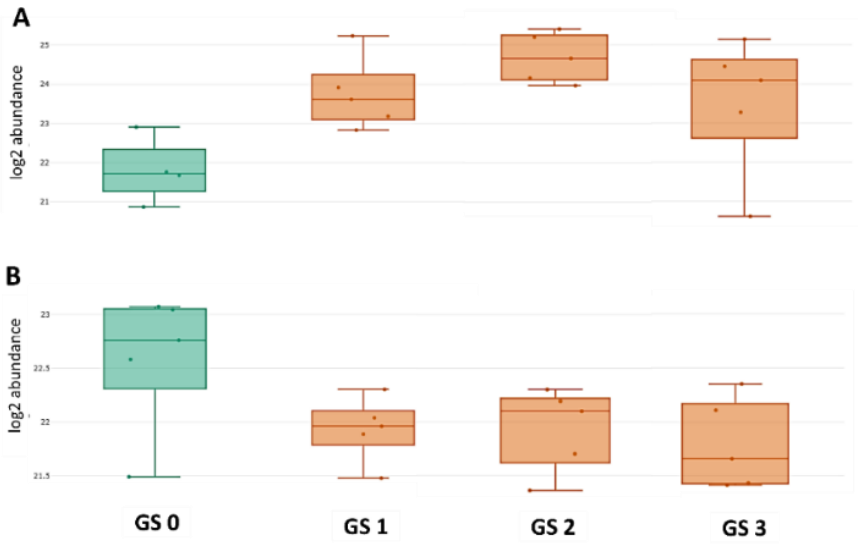


Figure 4. Plasma level (Log2 abundance) of A) adrenaline and B) adenosine in healthy and AGD-affected salmon. Both of these metabolites were found in all AGD-affected fish, and their level was significantly different from GS0 (p-value < 0.05). Values given are from five individual fish.

The tryptophan metabolite indole-3-lactic acid (ILA) decreased in response to the initial stage of infection with a significant 1.8-fold decrease in salmon with a GS1 compared to GS0. ILA abundance appears to return to baseline at the later stages of infection showing no significant reduction in salmon with GS2 or 3 compared to GS0. Note that the annotation level 2a was assigned to ILA in the data set indicating some uncertainty in the annotation of compound TF00162 as ILA.

We performed MetaCyc pathway analysis to identify pathways enriched in DAM. Enrichment analysis yielded significant results mainly when GS0 and GS1 were compared (Figure 5A); the same comparison that showed the highest number of DAM (Figure 2). Of the 208 compounds with annotation levels 2b or higher, only 101 were included in the MetaCyc database limiting the functional enrichment analysis. This also reflects the status of metabolomics in fish research, which is still in its infancy. There were ten enriched pathways (p-value<0.05) regardless of the direction of change (Figure 5A). Most were represented by pathways with upregulated metabolites (Figure 5C, D). Looking closer into the affected pathways in GS0*vs*GS1, the super pathway of purine nucleotide salvage showed the most well-represented DAM including adenosine, guanosine, hypoxanthine, inosine, and L-glutamate (Figure 5B, Figure 6). This was also the only pathway enriched in GS3*vs*GS1. Figure 6 shows a reconstructed MetaCyc pathway of the purine nucleotide salvage super pathway showing key metabolites significantly affected by the early stage of AGD infection. The upregulation of metabolites associated with purine nucleotide salvage—especially inosine, hypoxanthine, and L-glutamate in the low infection level before returning to pre-infection levels in the later stages of infection—might indicate a role for the pathway in the early infection response. Nucleotide balance is critically important in maintaining cellular functions and integrity. In eukaryotes, this is maintained by *de novo* synthesis and salvage of nucleosides formed during degradation of RNA and DNA. Imbalance or deficiencies in nucleotide salvage and synthesis have been implicated in neurological disorders and DNA damage [358]. Previous studies have indicated that AGD may result in DNA damage either by differential modulation of growth arrest and DNA damage-inducible gene-45beta gene [359] or oxidative damage through induction of oxidative stress [360]. It is likely that the substantial changes in this pathway in GS1 may provide a compensatory response to ensure that this physiological threat is mitigated. The superpathway role of purine nucleotide salvage in salmon remains to be functionally elucidated. This study provided insight into its role during a parasitic infestation.

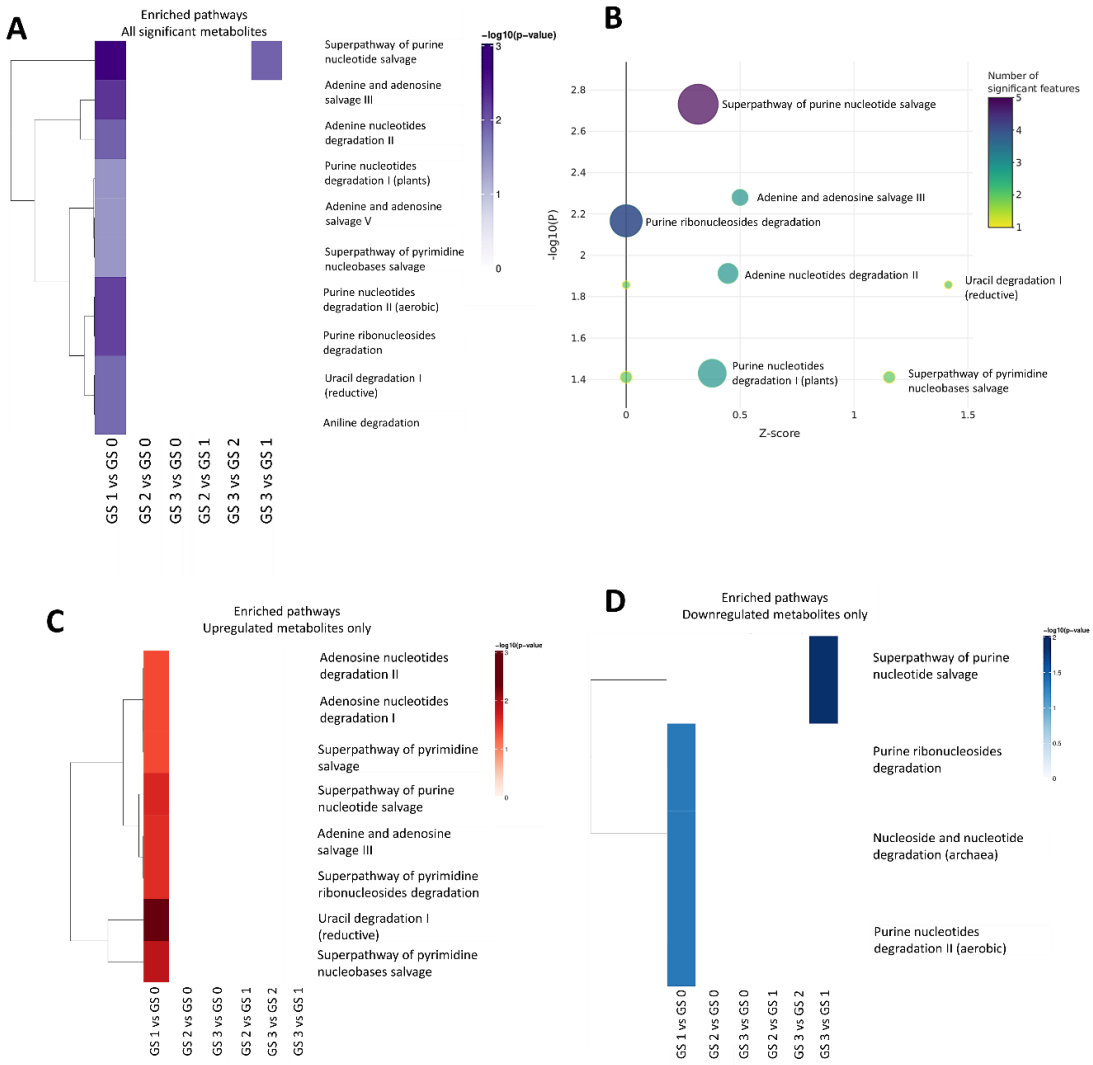


Figure 5. Comparisons are shown on the X axis with MetaCyc pathways on the Y axis. When performing tests for enrichment, pathways were restricted to include only those with two or more compounds. Note that for the purposes of display, only MetaCyc pathways with enrichment p-values less than 0.05 were included. The results show the (A) union of significant up- and down-regulated metabolites as well as for significant (C) up-regulated and significant (D) down-regulated metabolites separately. Note that different compounds of a given MetaCyc pathway may be both up- and down-regulated within a single comparison. Red, blue, and purple indicate up-regulated, down-regulated, and the union of up- and down-regulated compounds, respectively. These colors were assigned based on the -log10(enrichment p-value) with lighter colours implying less significant enrichment. Hierarchical clustering was applied to pathways (rows). The most significant pathways were clustered according to Euclidean distance using the complete linkage method. (B) Significant up- and down-regulated metabolites (at p-value < 0.05) in the GS1vsGS0 comparison were mapped to 16 compounds and assessed for MetaCyc pathway enrichment. The pathways reported were limited to those with two or more compounds. These significantly enriched pathways are indicated by purple boxes in the MetaCyc pathway heatmap in the overview section. Enrichment analyses with enrichment Z-score on the X axis and -log10(p-value) on the y-axis. Point size represents pathway size and point colour represents Z-score calculated as $Z=(Su-Sd)/N--\sqrt{Z=(Su-Sd)/N}$ where Su and Sd are the number of significant up-regulated and down-regulated compounds in the pathway, respectively, and N is the total number of compounds in the pathway.

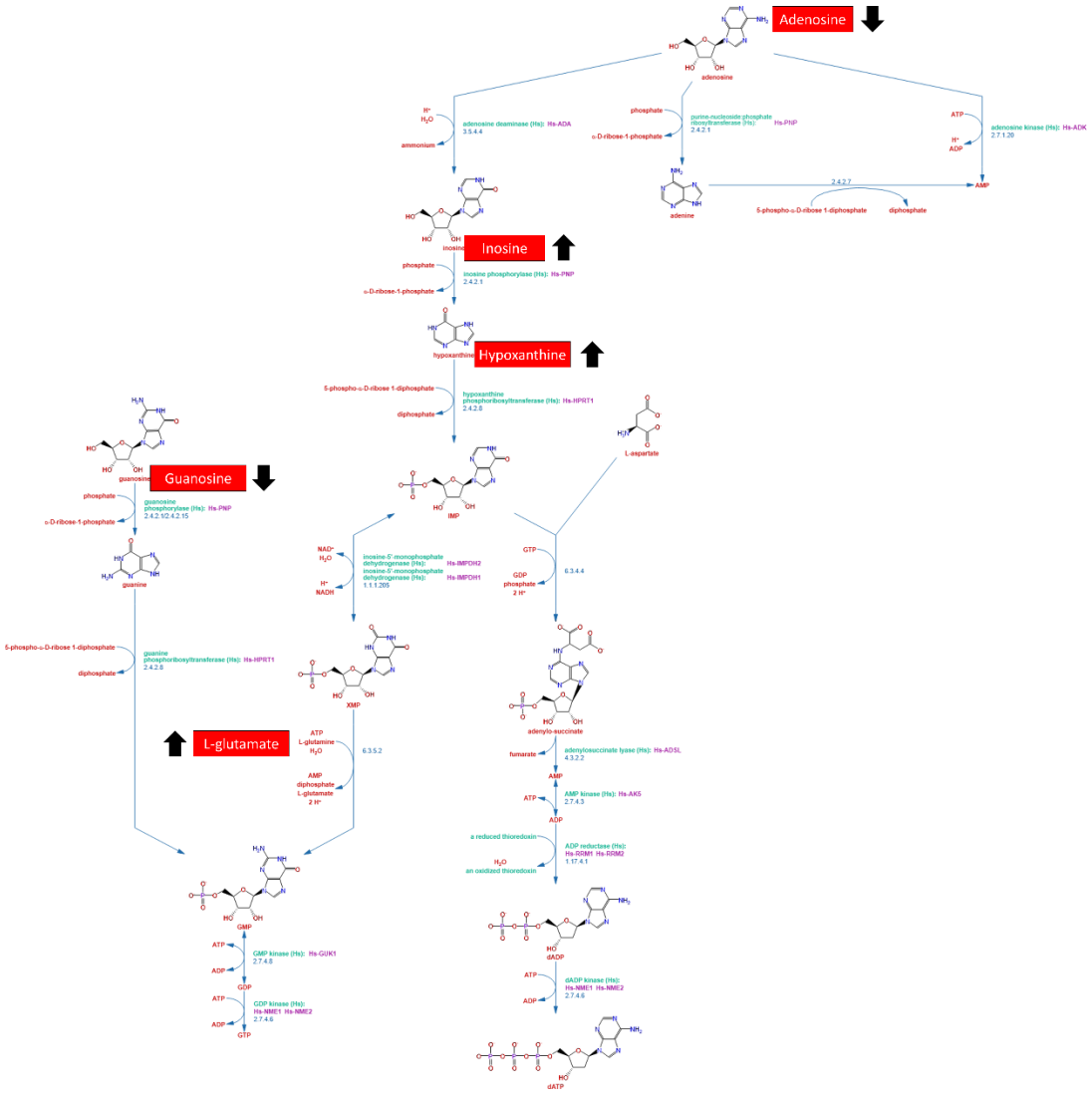


Figure 6. MetaCyc superpathway of purine nucleotide salvage. Metabolites found to be significantly affected within the pathway are highlighted in red boxes. The upward/downward arrow indicates the direction of change in the comparison GS1vsGS0; such comparisons yielded a significant enrichment. The pathway diagram was generated from MetaCyc database [361].

In summary, the modifications in the circulating plasma metabolome revealed the systemic impacts of AGD in salmon. It offered insights into the molecules salmon mobilised as physiological countermeasures to infection pressure. The metabolomic changes were more evident in fish with lower GS, and the infection level was not severe. We speculate that such a profile is related to the extensive metabolomic interventions so as the infection will not progress and be severe. Further, the metabolites with neuroendocrine functions showing pronounced changes during infection are likely involved in cellular trafficking, especially in the host immune response, thus offering insight into the neuro-immune axis associated with AGD. One limitation of the present study was that the infected fish were taken from a common garden trial, thereby, the confounding impact of tank environment was not accounted. We acknowledge that the challenge concentration is higher than the concentration of amoebae in farm environment, which is often an issue in lab-based AGD trials. In addition, the farm environment is complex and there are issues that present as confounding factors to AGD. In particular, gill health-related issues such as complex gill disease, proliferative gill disease, proliferative gill inflammation, to name a few, are often detected together with AGD [362]. How these diseases/disorders affect the host metabolome remains to be unravelled, though we believe that they may have varying degrees of impacts, which may interfere with the AGD-related metabolomic dysregulation reported in this study.

Therefore, further studies are needed to verify the suitability of the identified metabolomic signatures of infection under different laboratory and field AGD scenarios. Moreover, a temporal map of the plasma metabolome during disease development is necessary to provide another depth of understanding of how infection alters the salmon metabolome and link these changes to host immunity using a multi-platform response profiling.

5.3.3 Effects of PAA on disease resolution - Influence of different PAA treatment protocols

The results presented here is now a manuscript draft and will soon be submitted for peer-review.

Publication 11*

Mucosal immune and stress responses of *Neoparamoeba perurans*-infected Atlantic salmon (*Salmo salar*) treated with peracetic acid shed light on the host-parasite-oxidant interactions

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(*This paper is soon to be submitted for peer-review)

ABSTRACT

Treatment development for parasitic infestation is often limited to disease resolution as an endpoint response and physiological consequences are not thoroughly considered. Here we report the impact of exposing Atlantic salmon (*Salmo salar*) affected with amoebic gill disease (AGD) to peracetic acid (PAA), an oxidative chemotherapeutic. AGD was experimentally induced in salmon and 25 days after infection, uninfected control and AGD-affected fish were treated with PAA either by exposing them to 5 ppm for 30 mins or 10 ppm for 15 mins. Unexposed fish from both infected and uninfected groups were also included. Samples for molecular, biochemical and histological evaluations were collected at 24 h, 2 weeks and 4 weeks post treatment. Erratic swimming, increased opercular ventilation and loss of balance were manifested during PAA exposure. Post-treatment mortality was higher in the infected and PAA treated groups, especially in 10ppm-15mins. Plasma indicators showed that organ health (i.e.,

liver) was affected by AGD, though PAA treatment did not exacerbate the infection-related changes. Transcriptome profiling in the gills showed significant changes triggered by AGD and PAA treatments, and the effects of PAA were more notable 24 h after treatment. In particular, genes related to immune pathways of B- and T- cells and protein synthesis and metabolism were downregulated, where the magnitude was greater in 10ppm-15mins group. Even though treatment did not fully resolve the gross and microscopic pathologies associated with AGD, 5ppm-30mins group showed lower parasite load at 4 weeks post treatment. Mucous cell parameters (i.e., size and density) were higher at termination especially in AGD-affected fish, though not heavily influenced by PAA. AGD-affected untreated fish had significantly larger mucous cells, higher densities and higher defence activities while those treated with 10ppm-15mins PAA had significantly higher densities and defence activities than the uninfected untreated control group. AGD and PAA treatments resulted in oxidative stress – at the early phase in the gill mucosa while systemic reactive oxygen species (ROS) dysregulation was evident at the later stage. Nonetheless, infected fish were able to respond to elevated circulating ROS by increasing antioxidant production. Exposing the fish to a secondary stressor (i.e., crowding) revealed interference in the post-stress responses. Lower cortisol response was displayed by AGD-affected groups. Collectively, the study established that PAA, within the evaluated treatment protocols, could not provide a convincing treatment resolution and thus require further optimisation. Nonetheless, PAA treatment altered the immune and stress responses of AGD-affected Atlantic salmon, shedding light on the host-parasite-treatment interactions.

Introduction

Fish gills are multifunctional organs and play a fundamental role in gas exchange, ion regulation, osmoregulation, acid-base balance, ammonia excretion, and hormone production [363]. With its close contact with the aquatic environment, they are portals and attachment sites for many pathogens and as such, exhibit a wide range of immune defence mechanisms [364]. In Atlantic salmon (*Salmo salar*) farming, gill health is presently considered one of the major production problems worldwide [365]. Numerous physical, chemical and biological factors can affect gill health on the farms, especially in sea cages where risks are higher compared with land-based closed systems; one of which, amoebic gill disease (AGD) is a long-standing challenge that dates back to the 80s [8, 366]. AGD is caused by the parasite *Neoparamoeba perurans*, initially identified in Tasmania, Australia but is now found in different parts of the globe where salmon is being farmed. In Norway, the first documented case of AGD in farmed salmon was in 2006 and the report suggested that the marine environment was a reservoir for the amoeba [9]. The colonisation of the gills by the parasite initiates a tri-phasic host response which includes a localised reaction to parasite attachment, non-specific immuno-regulatory cell infiltration and advanced hyperplasia with epithelial stratification [367]. Gross pathology is commonly assessed by gill scoring based on the severity and prevalence of white mucoid patches on the gill surface [326, 368], and histologically, lesions are characterised by hyperplasia, lamellar fusion, lamellar vesicles in addition to the presence of amoeba [342, 369]. Although mortality is often considered a moderate problem associated with the disease, it is a significant cause of compromised welfare and reduced growth. These consequences, if not addressed, will serve as contributing factors to other serious health problems [370, 371]. According to the Norwegian Fish Health Report [372], the number of AGD outbreaks and the degree of severity in Norwegian salmon farms varies from year to year, and this is related to climatic conditions.

With the unavailability of vaccines, AGD management is relying on therapeutic interventions. Freshwater bath treatment is the most common therapeutic method for AGD and by far, the only method that provides favourable treatment results [368, 373]. Even though this method is effective in controlling AGD to a greater extent, the strategy entails significant infrastructure costs and is labor expensive. One important consideration and remains a major challenge is the requirement for a nearby freshwater source [17]. Oxidative disinfectants are often used against many ectoparasites in fish [374], and likewise,

have been explored for the treatment of AGD. Hydrogen peroxide (H_2O_2) was identified to affect AGD and is regarded as a preferred method when freshwater accessibility poses a daunting logistical challenge [26]. Its effectiveness is found to be lesser than freshwater bathing, although both treatments are not fully effective because AGD was observed to reappear in treated fish both under natural and controlled experimental conditions [368, 373]. Moreover, issues on the H_2O_2 toxicity towards non-target organisms, such as shrimps, have been highlighted in recent years and thus raise a major concern about H_2O_2 use particularly in Norwegian aquaculture [76]. Therefore, new chemotherapies for AGD that are effective and pose minimal risk on fish health and environment must be explored.

In the present study, we evaluated peracetic acid (PAA), a strong oxidant commercially available as an acidified mixture of acetate and H_2O_2 [31], as a candidate treatment for AGD. PAA has been shown to have a broad spectrum of antimicrobial activity [36, 374], and its biocidal action is underscored by denaturation of protein, disruption of cell-wall permeability, and oxidation of sulfhydryl and sulphur bonds in proteins, enzymes, and other metabolites [285]. Toxicity is often a major issue in using PAA in fish, nonetheless, the effective dose for many aquaculture pathogens is low compared with H_2O_2 [169]. In a series of studies, we have identified the health and welfare consequences of using PAA in Atlantic salmon smolts and found the range of doses that carries minimal risks, at least for naïve, uninfected fish [81, 170, 286, 287]. PAA, as an oxidant, has been demonstrated to be a potent trigger of oxidative stress and induced structural alterations in the mucosa. Nonetheless, salmon smolts orchestrated a cascade of mucosal and systemic adaptive responses to counteract the physiological pressures from PAA exposure [286, 287]. Here we report the first study that employed PAA as a candidate treatment for a parasitic infection in Atlantic salmon. Using an array of response parameters – behaviour, visual evaluation, histology, biochemical analysis and gene expression profiling, we identified the effects of AGD on host physiology and elucidated how the oxidative chemotherapeutics regulated these responses, which could influence potential disease resolution.

Materials and methods

Ethical considerations

The study adhered to the Guidelines of the European Union (Directive 2010/63/EU) and the Norwegian Animal Welfare Act and was approved by the Norwegian Food Safety Authority under FOTS ID 20/37233. Key personnel of the trial hold a FELASA C Certificate.

Induction of amoebic gill disease in Atlantic salmon smolts

The fish experiment was performed at the Fish Health Laboratory of Tromsø Aquaculture Research Station (HiT), Norway. Atlantic salmon eggs were purchased from a commercial supplier and reared at the station under normal production protocol until they reached the experimental size of around 100 g. Fish were screened for relevant bacterial, viral and parasitic pathogens before commencing the trial. There were 720 fish at the start of the trial. They were divided into two groups - one group was the “uninfected” group while the other group was the “infected” group. Each group had 360 fish stocked in 1800-L tank. AGD was induced in the infected group by exposing the fish to *Neoparamoeba perurans:* the isolate was from an outbreak in South-West of Norway during Autumn 2019 [375]. The pathogenicity of the isolate was established earlier in a pre-trial. Experimental infection was induced as follows: water flow was closed, the amoeba culture was added directly to the tank achieving a concentration of 1500 parasites per L, and fish were exposed for 1 hr. 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 immediately flushed out and replaced with clean water. The fish from the uninfected group were handled similarly but without the addition of the parasite. The experimental fish were under the following conditions: water flow rate in the tanks was 6-7 L/min, water temperature at 14.5 ºC, oxygen

at >85 % saturation, salinity at 35 ppt, 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 adapted throughout the trial.

Oxidant treatment by peracetic acid (PAA)

Twenty days after infection, fish were distributed to 12 500-L exposure tanks, 6 tanks for untreated and 6 for treated groups. Each tank was stocked with 60 fish per tank. Fish were allowed to acclimate for 5 days before peracetic acid (PAA) treatment was performed. The exposure protocol was as follows: Water flow in the tank was closed. Divosan Forte VT6 (Lilleborg AS, Olso, Norway), a commercially available PAA product, was added to the tank at several locations to achieve the final concentration of either 5 or 10 ppm. To allow mixing, aeration was provided. Before treatment, the actual PAA of hydrogen peroxide concentration in the trade product was analytically verified by DTUAqua. The fish group treated with 5 ppm was exposed for 30 mins, while the 10 ppm group was exposed for 15 mins. These concentrations and exposure durations were earlier identified to pose moderate health risks to naïve Atlantic salmon smolts [170, 286]. During the exposure period, fish behaviour was documented. Oxygen was supplied during exposure to ensure that DO was >85 % saturation. After the exposure period, water flow was opened, and water was replaced to at least 75 % in the next 8-10 mins (Figure 1).

Sample collection

The sampling outlay is presented in Figure 1. There were 3 major tissue samplings – 24 h, 2 weeks and 4 weeks after the treatments. For each sampling occasion, five fish were randomly netted from each tank and humanely euthanised with a bath overdose of Benzoak Vet (ACD Pharmaceuticals AS, Leknes, Norway). The length and weight of the fish were recorded. Blood was collected using lithium heparinised vacutainer (BD, Plymouth, United Kingdom) from the caudal artery, centrifugated for 10 min at 5 200 rpm (Heraeus Labofuge 200, Thermo Scientific, Massachusetts, USA), plasma was separated and stored at -80 ºC until analysis. After the macroscopic gill score was assessed [326], gill swabs (Sarstedt, Germany) were taken from the left side of the gills and stored in ATL buffer (Qiagen, Hilden, Germany) for qPCR quantification of parasite load. The first gill arch of the right side of the fish was collected and suspended in 10 % neutral buffered formalin (BiopSafe®, Stenløse, Denmark) for Quantidoc's mucosal mapping analysis. The second gill arch was likewise dissected and divided into two - one fraction (i.e., non-lesion region) was placed in RNAlater (Ambion©, Connecticut, USA), kept at room temperature for 12 hours for penetration and afterwards stored at -80 ºC until further use while the other fraction was stored in 10 % neutral buffered formalin (BiopSafe®, Stenløse, Denmark) for histological use.

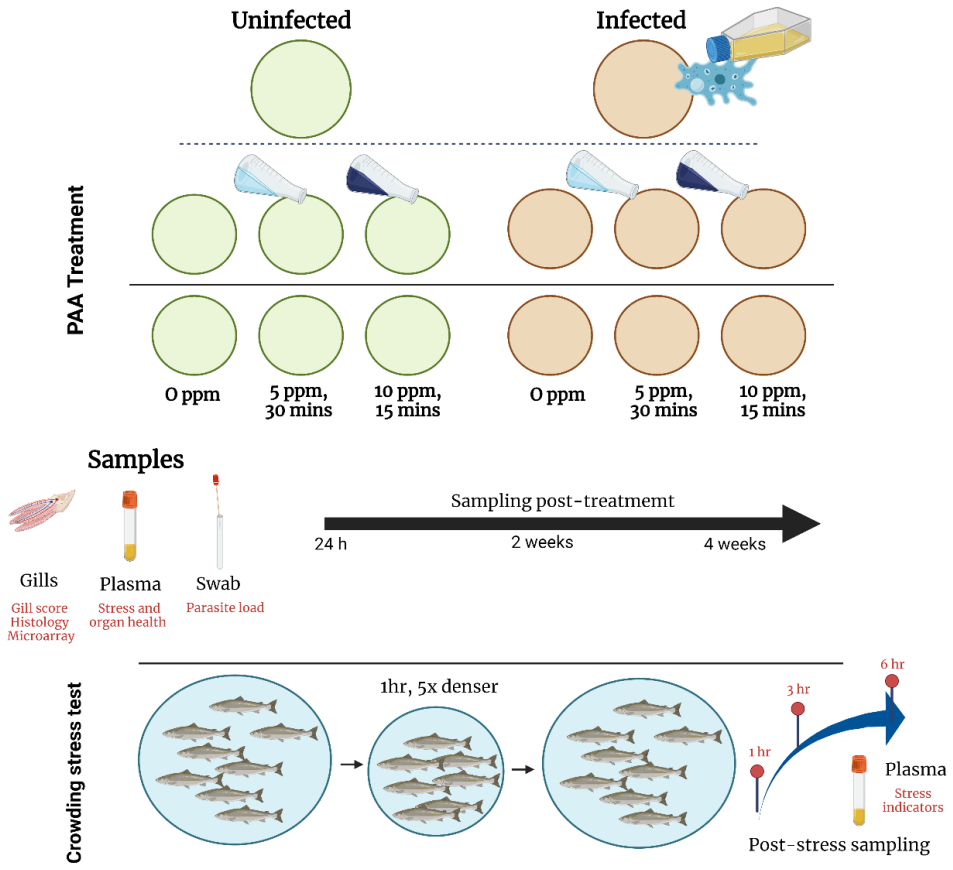

Figure 1. Distribution of treatment groups and sampling strategies. Created with BioRender.com.

Crowding stress

Twenty (24) hrs after the last tissue sampling, the remaining fish were subjected to crowding stress by lowering the water volume in the tank to achieve a density 5x higher than the initial density. Fish were under this condition for 1 hr, thereafter the water level was returned to the initial level. DO level was maintained at >85 % saturation and whenever necessary, oxygen was injected during the crowding. Five fish at 1, 3 and 6 hr were sampled from each tank after the stress challenge for plasma collection. Euthanasia and sample handling were similar as described in Section 2.4.

Plasma stress and organ health indicators

Pentra C400 Clinical Chemistry Analyzer (HORIBA ABX SAS, Montpellier, France) was used to determine the level of lactate, glucose, alkaline phosphatase (ALP), alanine aminotransferase (ALAT), lactate dehydrogenase (LDH) and creatinine in plasma. Commercially available kits were used to measure cortisol (Demeditic Diagnostics GmbH, Kiel, Germany), total antioxidant capacity (TAC, Sigma Aldrich, Missouri, USA) and reactive oxygen species (ROS)/reactive nitrogen species (CellBiolabs, Inc., California, USA) in plasma. All analyses were performed in duplicate.

RNA isolation and microarray analysis

Total RNA from the gills was isolated from the RNAlater®-preserved samples using the Agencourt RNAdvance™ Tissue Total RNA Purification Kit (Beckman Coulter Inc., CA, USA). All samples had an RNA Integrity Number (RIN) above 8.4 as evaluated by the Agilent® 2100 Bioanalyzer™ RNA 6000 Nano Kit (Agilent Technology Inc., Santa Clara, CA, USA). The microarray analysis was performed using a custom-designed 15K Atlantic salmon DNA oligonucleotide microarray SIQ-6 (Agilent Array,

ICSASG_v2), and all reagents used were from Agilent Technologies. The One-Color Quick Amp Labelling Kit was used for RNA amplification and Cy3 labelling using 110 ng of RNA template per reaction. Gene Expression Hybridization Kits were used for the fragmentation of labelled RNA. This was followed by a 15 h hybridisation in a 65 °C oven with a constant rotational speed of 10 rpm. Thereafter, the arrays were successively washed with Gene Expression Wash Buffers 1 and 2 and scanned using the Agilent SureScan Microarray Scanner. Pre-processing was performed in Nofima's bioinformatics package STARS (Salmon and Trout Annotated Reference Sequences) [88].

Parasite load quantification

DNA was extracted from the gill swabs using the DNeasy blood and tissue kit (Qiagen, Hilden, Germany). The DNA samples was analysed using a *N. perurans* specific qPCR to confirm the presence of *N. perurans* and estimate DNA copies, as a measure for parasite load. The samples were analysed 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 sec, followed by 50 cycles of denaturation at 95 °C for 3 sec and annealing at 60 °C for 30 sec. In each qPCR run, a 10-fold standard dilution series were included in order to estimate DNA copies per sample reaction. The standard dilution consisted of synthesised dsDNA (gBlocks™ gene fragment, Integrated DNA Technologies, Iowa, USA) of the qPCR target region with known DNA concentrations.

Histological evaluation

Histological assessment of the gills was carried out with semiquantitative scoring (0-5) of the following parameters: epithelial hyperplasia as seen in AGD (segmental hyperplasia dominated by epithelial cells), other epithelial hyperplasia, lesions in lamellar vessels. Presence of amoeba, epitheliocysts or were noted as recorded or not recorded.

Mucosal mapping

The formalin-preserved gill samples were subjected to mucosal mapping following Quantidoc's standard protocol [267]. Tissue sections stained by Periodic Acid Schiff – Alcian Blue were digitised, scanned, and processed through an automated software developed by Quantidoc AS for the stereological image analysis of the gill mucosa. The analysed mucosal features include mucous cell density (D, % epithelium filled with mucous cells) and mean mucous cell area (A, µm²) [122, 123, 267].

Data handling

All statistical tests were performed in SigmaPlot (Systat Software Inc., London, UK), except for microarray data. The normal distribution was evaluated using a Shapiro-Wilk test, while equal variance was analysed by a Brown-Forsythe test before a two-way analysis of variance (ANOVA) was employed to evaluate differences within treatment groups and time points. When at least one of the ANOVA requirements was not met, the data set was log transformed before performing the analysis. The statistical level of variance was set at $p < 0.05$. All data are presented as mean ± standard deviation (S.D.).

For mucous cell parameters, a Linear mixed effect model (lme) was used to test differences among treatments and sampling points (R studio, Massachusetts, USA). Statistical significance was set at $P \leq 0.05$.

The microarray results were exported from STARS as log2 transformed expression ratios (ER) and further processed in R (version 4.0.2, https://www.r-project.org/). Expression levels were normalized to the mean expression after 24h, untreated and uninfected group. Significant differential expressed genes (DEGs) were defined by (1) p-value cut-off of <0.05 (ANOVA, aov() function, *stats* package) for any of the three factors, time point (three levels), treatment (two levels) and AGD infection (two levels). (2) Group means were calculated for twelve subgroups and a minimum difference of >1 between the lowest and the highest mean was used to exclude genes with low effect-sizes. Genes that fulfilled both requirements (1) and (2) were defined as DEGs. This resulted in 1054 DEGs, which were represented in a heatmap (heatmap.2() function, *gplots* package, Figure 3). Distances between genes were calculated, using the Euclidean distance method and the dendrogram was calculated by a complete linkage algorithm. The dendrogram was split into twelve clusters with distinctive expression patterns. Four clusters with similar expression pattern contained only one or two genes and were combined into a single cluster for presentation. The functional annotation terms, as they are used in STARS, where tested for significant enrichment within these clusters (fisher.test() with alternative hypothesis set to "greater" only, *stats* package). Terms with p-values <0.05 are shown next to the heatmap with indication, in which cluster they were identified.

Results

Behavioural changes and performance indicators

Behavioural manifestations in the 5 ppm, 30 mins groups during treatment were as follows: 1) the first 5-10 minutes showed abrupt swimming patterns, typified by avoidance, jumping out of the water; 2) the next 5-10 minutes were exemplified by rapid opercular ventilation; 3) the last 10 minutes showed clustering at the bottom of the tank, close to water inlet; opercular ventilation was magnified and not more than 10 % of the fish showed loss of balance. For the groups treated with 10 ppm for 15 mins, there were two main periods of behavioural changes: 1) The first half of the exposure period was characterised by rapid erratic swimming and increased opercular ventilation by at least 30 % of the population; and 2) the second half showed reduced swimming speed with some fish still showing abrupt swimming, clustering near the water inlet, increased opercular ventilation and loss of balance in at least 15 % of the population. In addition, there was a clear difference between infected and uninfected groups, with the former displaying heightened responses. Mortality was observed a few hours after in the "10 ppm-15mins" group, especially in the groups previously infected. After 24 hrs, the survival was as follows: "uninfected-5 ppm, 30 mins" = 100 %; "infected-5 ppm, 30 mins" = 95 %; "uninfected-10 ppm, 30 mins" = 92 %; "infected-10 ppm, 15 mins" = 85 %. No recorded mortality thereafter.

There were no significant differences in weight (average weight 136±12.5 g) and length (23.4±6.2 cm) among the groups at termination.

Organ health indicators in the plasma

Two plasma indicators of liver health (i.e., alanine transaminase = ALT; alkaline phosphatase = ALP) showed significant inter-treatment and temporal differences (Table 1). ALT levels in "infected-0 ppm" and "infected-5ppm,30 mins" groups showed significant variations – for the former, a significant decrease in ALT level at 2 weeks post-treatment, while in the latter, the highest level was identified at 4 weeks post-treatment. The level of ALT in the "infected-0ppm" group was significantly higher than "uninfected-0ppm" at 24 h and 4 weeks post-treatment. ALP level demonstrated significant temporal variations in all groups, where an increasing tendency over time was predominantly manifested. At 4 weeks post-treatment, the ALP level in all infected groups was significantly higher than the "uninfected-0ppm" group. For creatinine (CR), no significant inter-treatment differences were identified. However, significant temporal changes were observed in the "infected-5 ppm,30 mins" and "infected-10 ppm,15 mins" groups where the level significantly decreased through time, demonstrating the lowest

at 4 weeks post-treatment. There were significant temporal changes in the plasma LDH level – “uninfected-0 ppm” and “infected-10ppm,15mins” groups demonstrated decreasing tendency; “infected-0ppm” was at highest at 2 weeks post-treatment; and “infected-5 ppm, 30 mins” displayed increasing tendency and peaked at 4 weeks post-treatment. In all time points, LDH level in “infected-0ppm” was significantly higher than the “uninfected-0ppm”. Such a significant difference from the “uninfected 0 ppm” was only observed at 24 h and 4 weeks post treatment in “infected-5ppm, 30 mins” and “infected-10ppm,15 mins”.

Table 1. Plasma indicators of organ health

Indicator	Treatment	Post treatment		
		24 h	2 weeks	4 weeks
ALT (U/L)	*Uninfected-0 ppm*	0.50 ± 0.22^{a}	0.79 ± 0.32^{a}	1.09 ± 0.19^{a}
	Infected-0 ppm	1.60 ± 0.31^{a*}	0.80 ± 0.63^{b}	1.69 ± 0.12^{a*}
	Infected-5 ppm, 30 mins	0.48 ± 0.23^{a}	0.66 ± 0.20^{a}	1.39 ± 0.21^{b}
	Infected-10 ppm, 15 mins	1.16 ± 0.43^{a}	0.95 ± 0.47^{a}	1.12 ± 0.29^{a}
ALP (U/L)	*UninfecteAtd-0 ppm*	49.1 ± 16.0^{a}	116.5 ± 19.5^{b}	103.7 ± 22.3^{b}
	Infected-0 ppm	110.1 ± 19.7^{a*}	119.5 ± 18.8^{a}	185.2 ± 19.9^{b*}
	Infected-5 ppm, 30 mins	70.2 ± 19.9^{a}	114.1 ± 17.4^{b}	162.2 ± 23.5^{b*}
	Infected-10 ppm, 15 mins	73.1 ± 14.8^{a}	84.9 ± 23.3^{a}	143.0 ± 11.5^{b*}
CR (U/L)	*Uninfected-0 ppm*	8.05 ± 1.1	6.26 ± 1.29	5.05 ± 2.78
	Infected-0 ppm	10.1 ± 2.2	5.63 ± 1.71	2.75 ± 2.32
	Infected-5 ppm, 30 mins	10.5 ± 1.24^{a}	5.91 ± 1.41^{a}	3.70 ± 2.71^{b}
	Infected-10 ppm, 15 mins	14.5 ± 4.01^{a}	5.91 ± 1.13^{b}	4.72 ± 2.53^{b}
LDH (U/L)	*Uninfected-0 ppm*	179.7 ± 60.9^{a}	345.1 ± 54.8^{b}	305.0 ± 45.7^{b}
	Infected-0 ppm	467.5 ± 93.8^{a*}	654.8 ± 43.6^{b*}	445.5 ± 50.0^{a*}
	Infected-5 ppm, 30 mins	317.4 ± 44.4^{a*}	310.9 ± 75.2^{a}	444.6 ± 62.2^{b*}
	Infected-10 ppm, 15 mins	390.8 ± 70.1^{a*}	231.7 ± 51.6^{b}	392.2 ± 50.4^{a*}

Note: Different letters denote significant difference over time within a treatment group. Asterisk (*) indicates that the infected group is significantly different from the uninfected group at a particular time point.

Level of ROS and TAC in plasma

Plasma ROS level displayed significant temporal changes in all groups where an increasing tendency was observed, except in “uninfected-0ppm” (Figure 2A). The highest plasma ROS level in “infected-0ppm”, “infected-5ppm,30 mins” and “infected-10ppm,15 mins” groups was observed at 4 weeks post treatment. At this point, the ROS level in these 3 groups was also significantly higher than the “uninfected-0ppm” group.

The plasma TAC level likewise showed significant temporal changes in all groups except in “uninfected-0ppm” (Figure 2B). For “infected-0ppm” and “infected-5ppm,30 mins” groups, the highest level was observed at 4 weeks post treatment; at this timepoint, the levels were significantly higher than the “uninfected-0ppm” group. On the other hand, “infected-10ppm,15 mins” group demonstrated significantly lower TAC level at 2 weeks post-treatment compared with the two other time points. In all timepoints, TAC level in “infected-10ppm,15 mins” group was significantly different from the “uninfected 0ppm” – it was significantly higher at 24 h and 4 weeks post-treatment, while significantly lower at 2 weeks post treatment. Moreover, the TAC level in “infected-10ppm,15mins” group was significantly lower compared with “infected-0ppm” group at 2 weeks post-treatment.

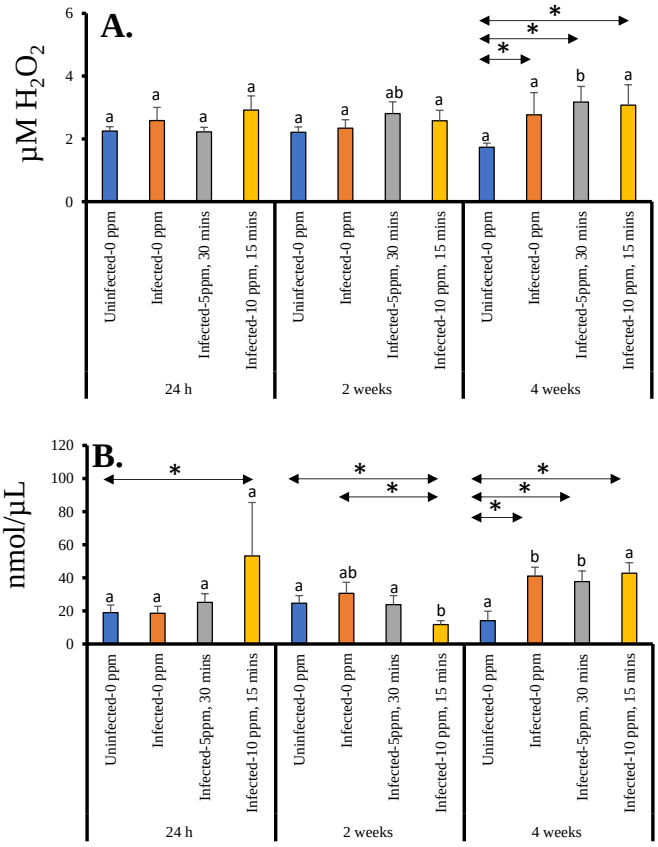

Figure 2. Plasma level of A) reactive oxygen species (ROS, expressed as µM H_2O_2) and B) total antioxidant capacity (TAC). Values represent mean ± SD of 10 individual fish per group at each sampling point. Different letters denote significant difference over time within a treatment group. Asterisk (*) indicates that the two groups at a particular time point exhibit significant difference.

Differentially expressed genes in the gills

Differentially expressed genes were identified based on the three key factors - timepoints, PAA treatment and AGD status (Figure 3A). Timepoint-wise (i.e., 24h, 2 weeks and 4 weeks after treatment), there were 893 DEGs of which 663, 139 and 94 were identified at p value <0.001, <0.01 and <0.05, respectively. Based on PAA treatments (i.e., infected-0ppm; infected-5ppm, 30 mins; infected 10ppm, 15 mins), 545 DEGs were identified with a distribution of 201, 164, and 180 according to p value <0.001, <0.01 and <0.05, respectively. The AGD factor (i.e., uninfected-0ppm, infected-0ppm) had the lowest DEGs at 383, which were distributed as follows; 145 (p <0.001), 102 (p <0.01) and 136 (p <0.05).

Identifying the interactions of these DEGs, we found 150 DEGs common in all factors (Figure 3B). There were 398, 79 and 39 DEGs exclusively found in comparisons within timepoints, treatments and AGD status, respectively. In terms of interaction between two factors, 270 DEGs were found in both timepoint and treatment comparison, while 138 DEGs were identified for timepoint and infection. Seventy-five DEGs were different for infection and treatment.

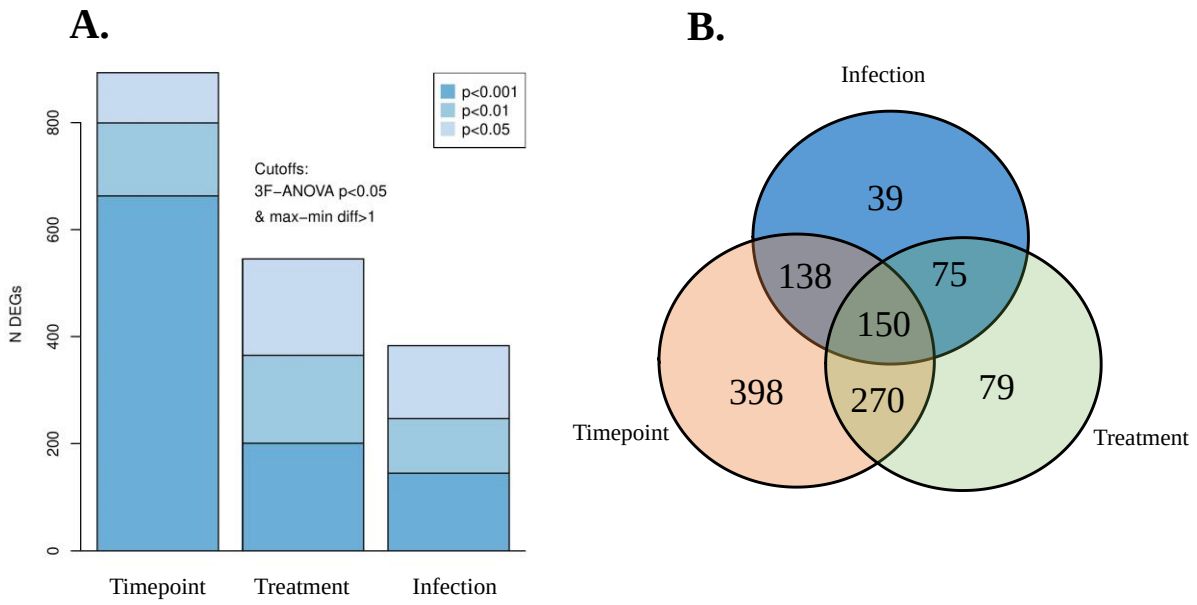

Figure 3. Differentially expressed genes (DEG) in the gills of AGD-affected Atlantic salmon treated with PAA. A) DEGs accounted based on 3 key factors – timepoint, treatment and infection status. Shades of blues indicate different p-value levels. B) Venn diagram showing the distribution of the DEGs, with significantly different expression due to the three factors.

Functional categories of DEGs in the gills

We then functionally categorised the DEGs affected by the three factors which resulted in 9 major clusters grouped according to the patterns of their regulation (Figure 4A). Cluster 1 is a large cluster consisted of 267 genes. This cluster is categorised as slightly upregulated from 2 weeks post treatment, particularly in groups affected by AGD. The general response was the induction of cell stress and ECM mucus (Figure 4B). Cluster 2 is relatively a small cluster with 16 genes, characterised by upregulation shortly after PAA treatment. Moreover, the response was stronger at higher dose, shorter exposure and involved mostly immune genes. Cluster 3 is a large cluster comprised of 250 genes, represented by down-regulated genes as early response to PAA treatment. Most of the genes in these clusters include several adaptive immune pathways with B- and T- cells. Cluster 4 is the largest cluster with 280 genes. Though large in number, expression of the genes was weakly upregulated as an early response to PAA and mostly represented by protein synthesis and metabolism related genes. Cluster 5 is also a relatively large cluster with 142 down-regulated genes, where the strongest effect was observed in AGD affected groups regardless of the treatment at 2 and 4 weeks. The group contains mainly innate and early immune response genes. Cluster 6 is a small cluster with 13 strongly down-regulated genes, which mainly consisted of Matrix metalloproteinases. At 24h post-treatment, AGD appeared to repress them, but PAA caused induction. At 4 weeks pots treatment, the strongest repression was in the PAA treated groups. Cluster 7 comprises of 29 genes with a strong up-regulation in AGD affected groups. There was a different regulation in “infected-10ppm, 15 mins” group at 24 h (higher than the other AGD groups) and 2 weeks after treatment (lower than the other AGD groups). The group includes several immune signalling genes. Cluster 8 contains 51 strongly up-regulated genes at the later time points in the AGD group, as well as in the “uninfected-untreated” group. At 2 weeks post treatment, a stronger expression was characterised in the groups exposed to PAA. The last group, Cluster 9 is combined cluster from 4 small clusters and contains 6 genes. These genes were strongly up regulated in all AGD groups.

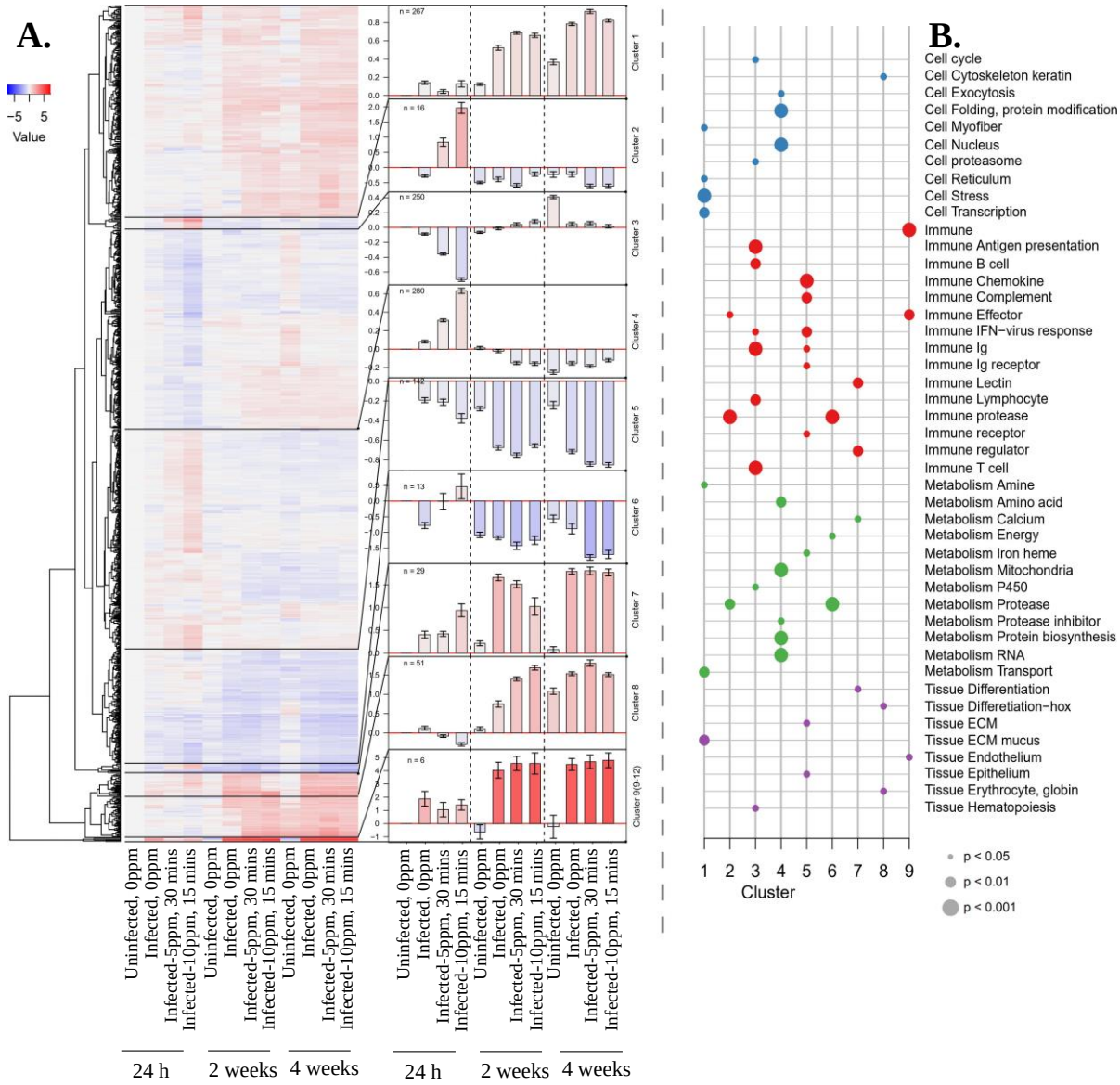

Figure 4. Functional categories of the DEGs identified in the gills of AGD-affected Atlantic salmon treated with PAA. A) The heatmap on the left shows the down- and upregulation of DEGs in a colour gradient from blue to red. The dendrogram was split into 9 sub-clusters, and the mean values for genes within these clusters are represented in bar plots (error bars show +/- standard error of the mean) in the centre. Cluster 9 includes 4 of the smallest clusters. B) Enrichment analyses of the 9 sub-clusters. The identified functional gene categories are shown along the Y-axis, and the nine clusters are arranged along the X-axis. Dots were coloured according to the higher categories (Cell, Immune, Metabolism and Tissue), and the size indicates the p-value according to Fisher's exact test.

Gill score, histological evaluation and parasite load

At 24 h after treatment, all treatment groups showed almost identical distribution of gross gill scores (GS) – at least 55-60 % of the evaluated fish exhibited a GS 1 while around 25-30 % showed GS 2 (Figure 5A). Less than 5 % exhibited GS 3. The GS distribution changed at 2- and 4-weeks post treatment, where an increased to 3 was evident in all treatment groups. At 2 weeks post treatment, all groups showed GS 2 in about 40 % of the evaluated fish while at least 25-30 % demonstrated GS 3. At 4 weeks post treatment, GS 3 accounted to about 75-80 % of the evaluated fish. There were no notable inter-treatment differences.

Parasite load from the gill swabs indicated significant temporal difference especially in 5ppm-30 mins group, where the count was highest at 2 weeks post treatment (Figure 5B). Significant inter-treatment difference was only identified at 4 weeks post-treatment where "infected-5ppm, 30mins" group significantly lower parasite load than the two other groups.

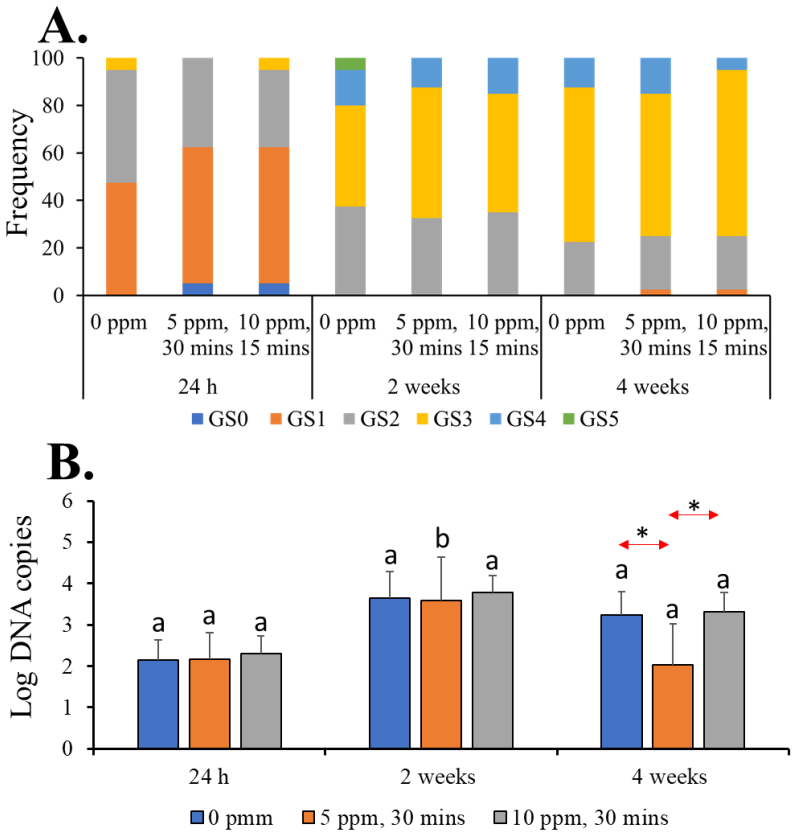

Figure 5. Gross gill scores and parasite load of AGD-affected Atlantic salmon treated with PAA. A) Frequency of occurrence of the gill scores accounted in each treatment group per time point. Each time point was represented by 10 individual fish. B) Parasite load in gills quantified by qPCR. Values represent mean ± SD of 10 individual fish per group at each sampling point. Different letters denote significant difference over time within a treatment group. Asterisk (*) indicates that the two groups at a particular time point exhibit significant difference.

In groups treated with PAA, except for the “infected 5ppm group”, some lamellar adhesions and moderate to severe lesions in lamellar vessels were found 24 hours after exposure (Table 2, Figure 6). In the uninfected fish only, mild epithelial hyperplasia not suspected to be AGD related was seen. AGD-related pathologies were likewise evaluated using a 0-to-5 point system and visual presence of amoeba was recorded as present or not present as detailed in Table 2. At 2 weeks after treatment, 80 % of the fish in “infected-0ppm” group exhibited microscopic AGD pathology scores 2 and higher, 70 % in “infected-5 ppm, 30 mins” and all evaluated fish in “infected-10 ppm, 15 mins”. At 4 weeks after treatment, 100 % of the fish in “infected-0ppm” group exhibited microscopic AGD pathology scores 2 and higher. The group “infected-5ppm,30 mins” remained having 80 % of the evaluated population with AGD pathology scores 2 and higher, whereas 90 % of the evaluated fish in “infected-10 ppm,15 mins” showed the same level of severity. The presence of amoeba corresponded to the microscopic GS, which the amount, as visually scored, increased as the disease progressed (Table 2). All evaluated fish in “infected-0ppm” demonstrated microscopic GS 2 and higher at termination. Few epitheliocysts were occasionally seen in all groups during the study.

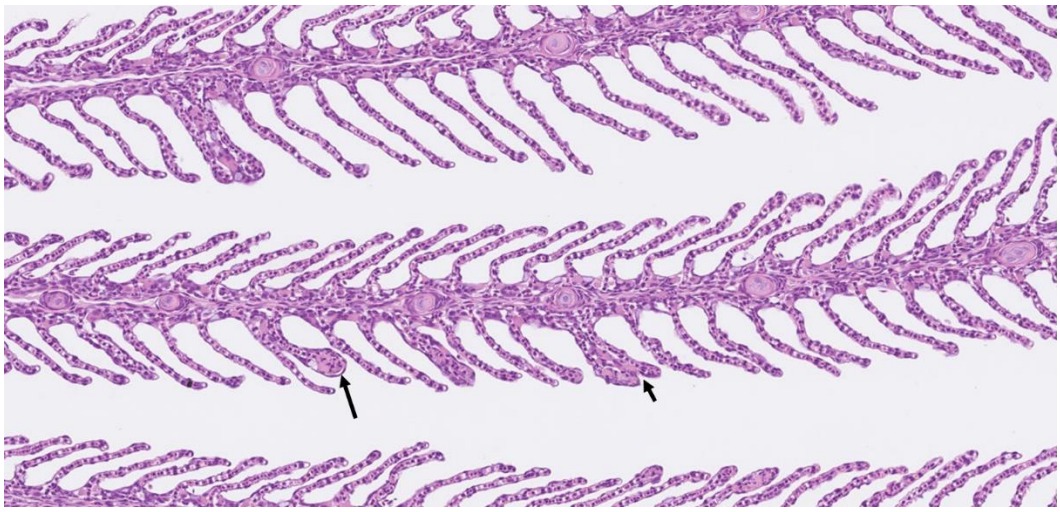

Figure 6. Lesions of lamellar vessels and lamellar adhesions (arrows). Uninfected 5 ppm sampled 24 hours after treatment.

Mucosal mapping revealed that at 24 h after treatment, mucous cell size and density did not significantly change among the treatment groups (Figure 7AB). After 4 weeks, infection with AGD led to a significant increase in mucous cell area (lme, $p<0.05$), volumetric density (lme, $p<0.05$), and defence activity (lme, $p<0.05$) relative to the uninfected group (Figure 7). The infected but untreated group (AGD, no PAA) had the mean largest cells at the mean highest density and was clearly in the vulnerable zone of Quantidoc's database (lme, all Dunnett test, $p<0.05$; Figure 8). Increasing the treatment dose to 10ppm PAA increased the mucous cell density (lme, Dunnett test, $p<0.05$) and defense activity (lme, Dunnett test, $p<0.05$) without significantly affecting the cell size. The variation in the individual response was more apparent in 10 ppm group relative to the uninfected Control.

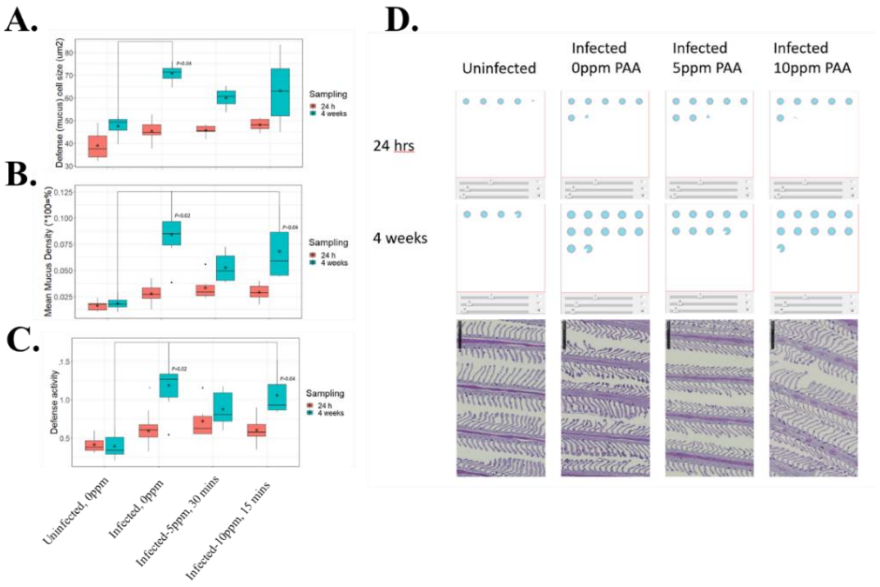

Figure 7. Features of mucous cells in the gill lamellae of AGD-affected Atlantic salmon treated PAA. A) The mean mucous cell size (µ2) and B), the mean mucous cell volumetric density (%) in the gill lamellar epithelium and C) Defence Activity calculated as (1/Area:Density)x1000. Measurements were taken at 24 h and 4 weeks after treatment. N= 10 per fish/treatment group. D) Illustration of the mucous cell size, density and defence activity in a standardised 100 x 100 µ2 epithelium (Dicer App v2) in the gill lamellae of Atlantic salmon in a Control group (uninfected) or exposed to AGD and treated with either 0ppm, 5ppm or 10 ppm PAA. The blue dots represent the mean mucous cell values per group and per time. The bottom row are sections of a representative gill from each group after 4 weeks. Note that the patchiness of the mucous cell distribution of the entire gill area is standardized through the application of the Dicer.

*Table 2. Gill histological parameters. Percentage distribution of microscopic scores of AGD pathologies in the gills of PAA treated fish. The presence of amoeba in the histological section was likewise evaluated and given as *number of samples where the parasite is present/total number of samples analysed.*

		AGD pathology			Amoeba*		
		Post-treatment			Post-treatment		
	Score	24 h	2 weeks	4 weeks	24 h	2 weeks	4 weeks
Infected-0 ppm	**0**	30	0	0			
	1	20	20	0			
	2	50	50	80	3/10	6/10	8/10
	3	0	30	20			
	4	0	0	0			
	5	0	0	0			
Infected-5 ppm, 30 mins	**0**	50	0	0			
	1	40	30	20			
	2	10	30	60	5/10	4/10	4/10
	3	0	30	20			
	4	0	10	0			
	5	0	0	0			
Infected-10 ppm, 15 mins	**0**	30	0	10			
	1	20	0	0			
	2	50	67	60	4/10	5/10	6/10
	3	0	33	30			
	4	0	0	0			
	5	0	0	0			

Comparing the changes in the mucous cell parameters to Quantidoc database which included 524 specimens within the same weight range, the uninfected-0ppm group showed mean gill mucous cell values which were near the mean of generally healthy salmon of the same size range (Figure 8A). After 4 weeks this treatment difference increased, and the infected groups regardless of the treatments were all in the “red zone” or beyond (Figure 8B).

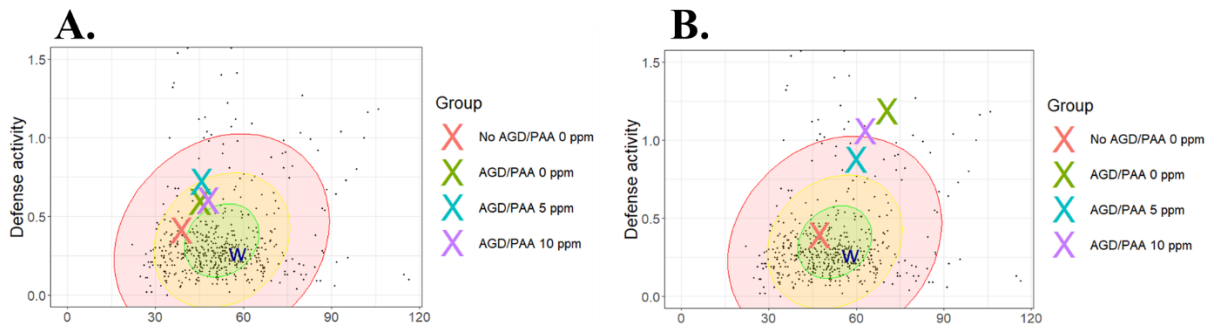

Figure 8. Comparison of group mean mucosal parameters of the gill lamellae (respiratory surface) of Atlantic salmon at 24 hours post exposure to AGD and PAA treatment relative to the Quantidoc database of Atlantic salmon between 89 and 250 g in seawater. A) 24-hrs post exposure. B) 4 weeks post exposure. Defence activity is calculated from the equation (1/(A:D)) x 1000 where A is mucous cell size and D is volumetric density in the epithelium. The W represents the mean values of gills of 7 wild adult salmon captured with permission. The individual dots are individual values from the Quantidoc database. N= 10/group, W=7.

Plasma stress indicators

Plasma cortisol level significantly increased in all groups 1 hr after stress and returned to basal level after 6 hours (Figure 9A). The “uninfected-0ppm”, “infected-0ppm” and “infected-10ppm, 15 mins” showed almost an identical patterns of cortisol response after stress, though the magnitude was higher in “uninfected-0ppm”. This was reflected in timewise inter-treatment differences especially the level of cortisol in “uninfected-0ppm” group which was significantly higher than the infected-treated groups at 1 and 3 h after stress. It was also observed that at time 0 (pre-stress), the cortisol level in “infected-0ppm” group was significantly higher than the rest of the groups. On the other hand, cortisol level 6 h post-stress in “uninfected-0ppm” group was significantly lower than the other groups.

Only the glucose level in “infected-10 ppm, 30 mins” group showed significant variations after stress, where it peaked at 3 h after stress induction and returned to pre-stress level at 6 h after stress (Figure 9B). Moreover, its level at this timepoint was significantly higher than the “uninfected-0ppm” group.

The plasma lactate level did not change after stress induction (Figure 9C).

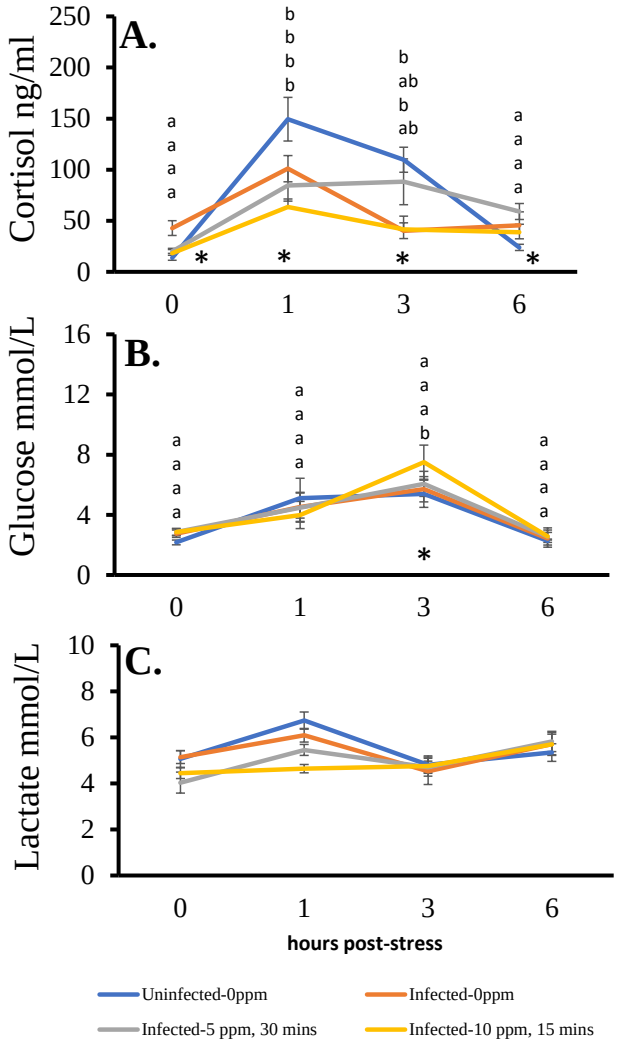

Figure 9. Changes in the plasma stress indicators after subjecting AGD-affected, PAA-treated Atlantic salmon to crowding. Stress test was performed after the last sampling. The levels of (A) cortisol, (B) glucose, and (C) lactate, were measured in 10 fish per treatment group at each timepoint. Significant difference over time in a treatment group is indicated with different letters. Groups are arranged from top to bottom: uninfected-0ppm, infected-0ppm, infected-5ppm-30mins, and infected-10ppm15mins. An asterisk (*) indicates that significant inter-treatment difference exists among the groups at that particular timepoint, which has been detailed in the text.

Discussion

Our understanding of the epidemiology and pathophysiology of amoebic gill disease (AGD) has progressed dramatically in the last years – from documentation of gross and microscopic pathologies which have already been routinely used for disease assessment to the molecular aspects of host-parasite interactions that identify biomarkers for potentially novel diagnostic assays [329, 376]. However, there is a paucity of information on the physiological consequences of a potential chemotherapeutics in AGD-affected fish. Here we showed that exposing AGD-affected fish to peracetic acid (PAA), an oxidising agent with strong biocidal activity, influenced the physiological responses to the infection and offered opportunities for improvement to develop a new candidate chemotherapeutics.

PAA may pose health and welfare risks to AGD-affected salmon

Though often regarded as one of the most eco-friendly disinfectants in aquaculture, identifying the safe dose of PAA is often presented with a challenge because the toxicity window for many fish species, including Atlantic salmon, is narrow [169, 298]. The doses used in the present study were identified in a series of studies where naïve Atlantic salmon had been exposed to PAA at different doses, duration, and frequency [170, 286]. Mortality was documented in “infected-10 ppm, 15 mins” shortly after exposure and the survival level 24 h after treatment was lower than the other exposed groups. Lesions of lamellar vessels and lamellar adhesions were identified in PAA-treated fish, which likely an additional factor that aggravated the impact on the respiratory function AGD-affected fish. This is a welfare-related issue that must be considered in using PAA as a chemotherapeutics. It was also apparent that the infected groups, regardless of the PAA treatment administered, appeared to be more susceptible to PAA toxicity. Such a response was not observed in the uninfected groups, as well as in previous PAA trials where these doses had been investigated and identified [170, 286]. This suggests that AGD might have lowered the tolerance threshold of Atlantic salmon to PAA, thereby increasing the toxicity risk. This hypothesis is further supported by the cellular responses to the treatments: while the uninfected group had the smallest and fewest cells typical of healthy gills, and infestation with AGD produced many and large mucous cells, commensurate with quite vulnerable gills (Figure 8). Unfortunately increasing the dose to 10ppm PAA induced more but not larger mucous cells in the gills. These results support a balance between mucosal protection from infection or exposure to toxins and the effective dose of PAA or other therapeutic actions, as has been found in previous studies [137, 267]. This could be related to the dysregulation in immunity of AGD-affected fish [376] that limit their adaptive capacity to the oxidant. The magnitude of behavioural changes in the infected groups were likewise greater, thus, lending support to the hypothesis that the infection might have increased the sensitivity of salmon to PAA. These results provide another dimension on PAA toxicity in Atlantic salmon, and thus, must be considered in the informed basis of its application.

Plasma indicators reveal effects of AGD on internal organ health

Respiratory disturbances associated with the proliferation and fusion of the lamellar epithelium in AGD-affected fish decreases the functional gill surface area and increases the diffusion distance in the water-blood barrier for oxygen transfer, which will result in secondary hypoxic changes in the liver [369, 377]. Here we documented that liver health was affected by AGD, as indicated by the increase in plasma level of alanine transaminase (ALT) and alkaline phosphatase (ALP). Higher levels of these analytes are often implicated to liver damage. These analytes were elevated 24 h and 4 weeks after treatment in “infected-0ppm” group indicating secondary effects of gill infection to liver function. At 4 weeks post-treatment, ALT indicated that PAA treatment might have protected the liver from AGD-associated damage, but ALP was suggesting otherwise. It would be interesting to evaluate in the future the specificity of these analytes to differentiate infection severity of AGD. Tissue leaks lactate dehydrogenase (LDH) when damaged [378]. The elevated level of plasma LDH is most likely connected to structural alterations in the gills that are associated with the pathology of AGD, which might have

released LDH into the bloodstream. Interestingly, the level did not increase following PAA treatment indicating that the oxidant did not intensify the organ damage associated with AGD. This was supported by histology. It was previously identified that at the concentrations tested, PAA caused only minor reversible gill alterations [170, 286]. Systemic indicators for AGD are not common, therefore, the changes in LDH present a potential biomarker for AGD that should be validated in future infection studies.

Infection induces systemic oxidative stress but is not exacerbated by PAA treatment

Oxidative stress, the imbalance of reactive oxygen species and the inability of the organism to scavenge and neutralise them, has been linked to the pathophysiology of many diseases [379]. It is known that PAA is a mild environmental stressor, and its ability to trigger transient oxidative stress has been thoroughly documented in Atlantic salmon [81, 287]. The results demonstrated that AGD alone or in combination with PAA treatment induced systemic oxidative stress by increasing the level of ROS (arbitrarily measured as H_2O_2) in plasma. The alteration of oxidative stress status has been implicated as a key mechanism at the late stage of AGD [380]. The significantly elevated ROS level observed at 4 weeks after treatment when gill scores were between 2-3 corroborated the earlier evidence of the involvement of oxidative stress in the pathophysiology of AGD. Increased production of ROS during infection facilitates pathogen clearance as well as contributing to signalling cascades related to inflammation, cell proliferation, and immune responses [381], and very likely these mechanisms are at play as well in AGD. Treatment with PAA did not increase the ROS level indicating that oxidative stress-inducing ability of AGD was not influenced by another strong oxidative chemical stressor.

At the mucosal level, several regulators of oxidative stress genes have been identified in the gill transcriptome and in most cases the rate of transcriptional change was higher at the early timepoints. For example, the expression of *thioredoxin reductase 3*, *glutathione peroxidase* 1 and *superoxide dismutase*, genes involved in ROS scavenging and detoxification [81], were highly affected 24 h after treatment. It was also apparent that infected groups treated with PAA had higher magnitude of change than the infected-0ppm. This demonstrates the known consequence of PAA as an inducer of transient oxidative stress [264, 286] was not interfered by the disease status of the fish. The changes in the systemic ROS, as well as in the expression of key redox genes in the gills, underscored a salient pattern that mucosal oxidative stress is triggered as an early phase response followed by a systemic redox dysregulation as a subsequent consequence during late stage.

Fish have an array of antioxidant molecules that ensure redox homeostasis and play a crucial defence role during oxidative stress [382]. At 4 weeks post treatment, plasmatic TAC significantly increased in a manner like the elevated level of ROS level in AGD-infected, PAA-treated fish. This provides evidence that AGD and PAA treatment did not impede the inherent ability of salmon to counteract elevated ROS. We have earlier shown that salmon has an efficient antioxidant system that addresses oxidative stress triggered by oxidative chemotherapeutics [264, 286]. Interestingly in the early timepoints, there was a variability in the TAC level particularly with the “infected-10pppm,15mins” group. This implies that the immediate and mid-recovery TAC responses could be influenced differentially by the high PAA dose, suggesting slight interference with the antioxidant system following treatment.

PAA modifies the gill transcriptomic response to AGD, but predominantly shortly after treatment

Several transcriptomics studies on AGD either by qPCR of large panel of genes, microarray or RNA sequencing have elucidated the molecular mechanisms associated with the onset of the disease and its progression [329, 366, 376]. However, many of the available transcriptomic data sets vary depending on the dose and virulence of the *N. perurans*, fish size, tank environment, duration of infection and use of lesion or non-lesion specific gill tissue, which often pose a challenge when performing comparisons.

Nonetheless, there are a number of mechanisms that have been implicated that likely govern host-pathogen interaction during the onset until the late stage of the disease. To our knowledge, this is the first study that explored transcriptome-wide responses of AGD-affected fish after a treatment. It is evident in the gill transcriptome data that PAA treatment of AGD-affected fish had stronger effects immediately after treatment and the infected groups (treated and untreated) had almost the same transcriptomic response thereafter. This suggests that the modulation of response to AGD by the oxidant was only transitory and did not persist during the recovery/disease progression phase. This can partly explain why gross and microscopic pathologies were almost similar among groups at 4 weeks post-treatment.

There were 4 main clusters – cluster 2, 3, 4 and 5 that conspicuously showed that oxidant treatment altered the transcriptomic responses of AGD-affected fish at 24 h after treatment. In Cluster 2, proteases involved in immunity and metabolism were substantially affected and characterised by upregulation in the PAA-treated groups where the magnitude of change was dose dependent. *Matrix metalloproteinase 13* (*mmp 13*) is expressed in wound keratinocytes and may be stimulated by the small ROS, hydrogen peroxide [383]. Given that H_2O_2 is one of constituent components of the PAA trade product, the upregulation of several *mmp13* genes may likely be a response to the oxygen radical. Moreover, this lends support to the discussion earlier that mucosal oxidative stress was triggered after treatment. In addition, the upregulation of *heat shock protein 90, alpha* (*hsp90a*) especially at the highest dose suggest stress responses from the gills. This group of genes indicate that stress might have been triggered at the gill mucosa by PAA treatment, but not significantly by AGD.

It was reported earlier that AGD-affected gills displayed an increased mRNA expression of cellular markers of immune cells, including professional antigen presenting cells (MHC-II, CD4), B cells (IgM, IgT, MHC-II) and T cells (TCR, CD4, CD8) [330]. In another study, a coordinated down-regulation of MHC I pathway-related genes during the later stages of infection was demonstrated and the down-regulation of *interferon-regulatory factor* (*irf*)-1, *independent of interferon-α*, *interferon-γ* and IRF-2 expression have been implicated to be involved [328]. In Cluster 3 we found an overrepresentation of genes responsible for T and B cell-mediated immune response, where the infected groups showed downregulation at 24 h after treatment and interestingly, the impact was magnified by oxidant treatment. The group receiving 10 ppm PAA for 15 mins showed the highest downregulation among the groups. In earlier PAA exposure studies, we did not find a strong impact of PAA to these molecules [170, 229]. This indicates that PAA may impair B- and T-cell mediated response only when there was a pre-existing infection. Further, such weakening response triggered by PAA likely contributed to an environment that allows continuous invasion and proliferation of the parasite. It was also interesting to document that B- and T-cell mediated response appeared to be marginal at 4 weeks post-infection where a number of fish already exhibited gill scores >2. This is partially contradictory to the earlier observation on the role of these molecules during the late stage of infection [330]. These results present another point for consideration in the ongoing discussion on how to devise the best approach to unravel the mechanisms behind complex disease states such as AGD [376].

Oxidative stress-inducing compounds such as H_2O_2 and PAA require a robust metabolic programming from the host to ensure efficient deployment of response for homeostasis and adaptation [229, 384]. Cluster 4 showed genes mainly involved in metabolism in mitochondria, RNA and protein, where their expression was upregulated in infected-PAA treated groups at 24 h after treatment. Moreover, the change was higher in the group that received 10 ppm PAA for 15 mins. We can speculate that this substantial change may be involved to repairing cellular damages induced by compound stressor oxidant and AGD. One of the genes which the rate of change was quite large in “infected-10, 15 mins” group was *protein arginine N-methyltransferase 3* (*prmt*), a gene responsible for methylating arginine residues in histone and non-histone proteins and has major roles in transcription and chromatin regulation, cell signalling, DNA damage response, RNA and protein metabolism, and stress [385]. This

points to its involvement in the response to the high oxidant dose. GTPases are associated with diverse cellular processes, including signal transmission, cell polarity, cell cycle progression, gene expression, material transport and construction of the cytoplasmic skeleton [386]. We have identified a significant subset of GTP-related genes that were upregulated in PAA-treated groups 24 h after treatment, including *GTPase activating protein*, *guanine nucleotide-binding protein-like 3* and *GTPase IMAP family member 7*. It has been reported previously that GTPases in mucosal organs of salmon were affected by PAA, where its regulation following exposure has been implicated to facilitate radical scavenging [170, 229]. The results concur that these molecules may be involved in the response to oxidant PAA, and this crucial function is not interfered by the disease state.

Impairment of immune response in AGD has been documented in several studies, which likely contributes to the successfully attachment, invasion and proliferation of the parasite on the gill mucosa [369]. This was exhibited in the gill transcriptome of AGD affected fish as shown by cluster 5, where most immune related genes involved in chemokine, complement and Ig mediated responses clustered, and treatment-related differences were more notable at 24 h after treatment. Cytokines in the Th1, Th2 and Th17 cell differentiation pathways are often considered to be involved in AGD immune response, where downregulation is often a hallmark response for Th1, Th17 and Tregs pathways, while pro-inflammatory cytokines (Il-1β) and Th2 cytokines exhibit upregulation [365, 387]. We have identified *interleukin 1b* (including the receptor) and several interferons to be downregulated mostly all throughout the trial in infected-0ppm as well as in two PAA-treated groups. Downregulation of these important cytokines could have facilitated the progression of the disease.

Increased mucus production is commonly associated with AGD [369], which was also identified in the current study through mucosal mapping. In particular, *mucin 5* (*muc5*) is most likely the main component of the characteristic mucus patches comprising AGD lesions [329]. We have identified *muc5* in the gill transcriptome to be heavily affected by AGD, where its expression increased as the disease developed, thus lending support to an earlier proposed involvement in AGD. Another mucus associated gene that demonstrated a higher magnitude of change in response to infection and treatment was *GMP Giant mucus protein* (*gmp*), a high molecular weight multi domain proteins specific for fish and responsive to ectoparasite salmon louse [388]. This gene was earlier shown to be affected by PAA and was implicated to be involved in mucosal protection from the chemical oxidative stressor [264].

Oxidant treatment can reduce the parasite load, but pathologies are still persistent

The spectrum of antimicrobial activity of PAA is well documented, including against relevant bacterial and parasitic pathogens in aquaculture [118, 255, 389]. Under *in vitro* conditions, PAA exhibit amoebicidal activity against *N. perurans* within the concentrations tested in this study (unpublished). PAA is in the family of oxidising agents where H_2O_2, the most well-known oxidant treatment for AGD, belongs. We evaluated for the first time the potential of PAA as a chemotherapeutics against AGD-affected salmon. The gills of AGD affected fish exhibited the classic gross pathologies such as white mucoid patches [326] and supported microscopically by evidence of multifocal hyperplasia and lamellar fusion [342]. Moreover, the parasites were likewise observed and detected in the tissue sections, indicating that infected fish used represented diseased specimens. In addition, the number of mucus cells in the gills increased in AGD affected fish which supports earlier evidence of increased mucus production during infestation [329]. From gross gill scoring, PAA treatments did not reduce the pathologies associated with the disease. The frequency of gross gill score 2 and higher increased as the recovery period progressed, and the distribution of scores showed slight differences among groups. Interestingly at termination, microscopic scoring revealed that the percentage of the population having an AGD pathology score >2 was highest in “infected-0ppm” while lowest in “infected-5ppm, 30 mins”, though difference between groups were not large. This was somehow supported by qPCR quantification where the lowest parasite load was detected in “infected-5ppm, 30 mins”. Therefore, the treatment of

AGD-affected fish with PAA resulted in equivocal disease resolution; nonetheless, 5 ppm PAA for 30 mins could reduce the parasite load and may likely require time for the pathologies to recover. It is known that fish will still have high gill gross score when they are recovering, and this likely explains the contrast between the parameters on assessing disease resolution in timeframe of this study. In an earlier treatment experiments, it was demonstrated that the efficacy of H_2O_2 against AGD was not highly dose-dependent [368]; somehow, PAA as a treatment followed such a trend. Moreover, H_2O_2 treatment does not cure the fish, but delays the development of the disease and growth of the amoeba [26, 368, 390]. A longer period of recovery after treatment is required for us to verify whether such consequences are also true for PAA.

AGD and PAA treatments alter the kinetics of stress response to as secondary stressor

We have shown earlier that periodic exposure to PAA did not affect the ability of salmon smolts to respond to a secondary stressor, but slight modifications on the kinetics of response were documented [286, 287]. For example, fish exposed to PAA had a higher cortisol response than unexposed fish. In the present study, we found that AGD and oxidant treatment did not impede the ability of salmon to respond to a secondary stressor, however, the magnitude and to some extent, the kinetics, had been altered. It appears that AGD dampened the ability to mobilise cortisol after stress because infected groups showed lower cortisol response. As an anti-inflammatory molecule, cortisol participates in the activation of immune response [391], and very likely active in resolving the AGD-related dysregulation of the host immunity, as shown in the gill transcriptome of infected fish. The lower magnitude of cortisol response in infected group suggests a potential exhaustion, in which cortisol had been extensively utilised for immune regulation as the disease progressed, thus a lesser response was demonstrated when secondary stress is encountered. Moreover, fish exposed to 10 ppm, 15 mins showed the lowest cortisol levels among the groups post stress indicating a potential compound interference caused by infection and oxidant on cortisol mobilisation. Cortisol has been found to be elevated in AGD-affected fish [348], as well as in PAA exposed salmon [286]. We documented that “infected-0ppm” had significantly higher cortisol level before stress induction, which suggest chronic stress in this group. Chronic stress from elevated cortisol may interfere with immune responses [392], and this partly explains why in the cluster of immune genes in the gill transcriptome, the responses in uninfected untreated group were marginal.

Glucose and lactate are involved in the secondary response to stress and play a key role in energy metabolism and allocation [393]. The treatment groups did not show significant changes in both parameters after stress induction except in “infected-10ppm,15 mins” where the glucose level increased at 3 h after stress. Though it is difficult to provide a solid implication of the physiological importance of such a treatment-specific response, providing the compound stressor may slightly magnify the ability of fish to mobilise glucose reserves following stress.

Conclusions

The present study revealed new insights into the physiological consequences of AGD and how they were influenced by oxidative chemotherapeutics. PAA treatment of AGD-affected fish increased the toxicity risk of PAA, hence, providing a crucial aspect that must be thoroughly considered in using this oxidant as a chemotherapeutics. AGD interfered with liver functions and induced systemic oxidative stress, nonetheless, treatment with an oxidant did not aggravate the AGD-induced alterations. These results further expanded the role of oxidative stress in the pathophysiology of AGD. The gill transcriptome elucidated the molecular changes following infection and treatments, revealing how mucosal cellular and humoral immune responses were orchestrated in response to infestation, and in some cases, modulated by PAA treatment at higher dose, but shorter exposure duration. It was apparent

that PAA treatment could interfere gill transcriptomic responses in AGD-affected fish, but only shortly after treatment. PAA treatments did not fully resolve the disease associated pathologies as gill scores further developed during recovery, although mean mucous cell measures indicate that AGD either treated or untreated, gives significantly larger and denser cells than in uninfected fish gills and may be useful in early detection of infections. Despite the equivocal treatment impacts on gross and microscopic pathologies, we documented lower parasite load in the group 5ppm, 30 mins group. AGD and PAA treatments did not impede the ability of fish to respond to secondary stress, however, infection and treatment altered the magnitude and kinetics of response indicating a potential interference of the stress axis. Lastly, the study offered new insights into host-pathogen-treatment interactions in AGD research. PAA as a treatment for AGD requires further study, particularly focusing on identifying the optimum treatment protocol. Nonetheless, the results presented here would be valuable in the evidence-driven use.

5.3.4 Effects of PAA on AGD - Influence of different PAA products

The results presented here are included in a manuscript that is currently under peer-review.

Publication 12*

The oxidative stress response repertoires in the gills and olfactory organs of Atlantic salmon display distinct patterns of regulation during infection and treatment of the parasite *Neoparameoba perurans*

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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 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 mins and 60 mins. Sampling was performed 24 h and 2 weeks after PAA treatment (equivalent to 2- and 4-weeks post parasitation). At each sampling point, gross pathology of the gills was evaluated, plasma was taken for reactive oxygen species (ROS) and total antioxidant capacity (TAC) analysis and organs (i.e., gills and olfactory organ) were dissected for histology and gene expression analysis. 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 organs; 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 organs 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. However, PAA treated groups for 30 mins 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 was 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 organs 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.

Introduction

Reactive oxygen species (ROS) refers to an array of derivatives of molecular oxygen that play a crucial role in aerobic life [394]. 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 [394, 395]. In aquatic animals, the antioxidant system is composed of enzymatic and non-enzymatic, low and high molecular mass antioxidants, that ensure that ROS are kept under a non-deleterious level [293]. The balance between the production of ROS and the systems required to mitigate ROS is called redox state. 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 [293, 396]. 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 cell 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 [397].

There are two major parasitic infestations in the sea-caged production of Atlantic salmon – 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 plight of fostering sustainable salmon farming. The causative agent, *Neoparamoeba perurans,* is a free-living and opportunistically parasitic amoeba species, that attaches to the gill lamellae [1]. AGD manifests clinically as lethargy, anorexia, congregation at the water surface and increased ventilation rate [7], which leads to respiratory distress that can result in mortality if left untreated [8, 9]. AGD is characterised by increased mucus production and the formation of white mucoid spots and plaques on the gill surface [10], 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 [11]. Recently, oxidative stress has been implicated in the late stage of AGD in farmed Atlantic salmon [380] 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 widely recognised as a sustainable disinfectant because of its rapid degradation into innocuous by-products [169]. By releasing highly reactive oxygen radicals upon its decay [117], 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 [28, 398]. 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 [397], including PAA [287]. We have shown in earlier studies that bath exposure of Atlantic salmon smolts to PAA could induce transient oxidative stress [229, 286, 287], which is a major indicator for its informed use in aquaculture. In addition, we also identified that the stress status of fish before exposure to PAA could alter the response to the chemical oxidative stressor [170]. PAA is a good candidate chemotherapeutic against a number of bacterial and parasitic infections [169] – 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 organs) of Atlantic salmon. The most widely used commercial treatment against AGD is freshwater bathing. 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 though disease resolution between laboratory trials and during actual production often differs considerably. 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.

Materials and Methods

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.

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 12 ± 1 ºC, oxygen at >85 %

saturation, salinity at 35 ppt, 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, amoebic gill disease 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 1000 parasites/L. The fish were exposed to the parasites for 1 hr. During the exposure period, the level of oxygen was routinely monitored to ensure that DO level did not go below 80 % 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.

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 in the product was empirically determined by the Technical University of Denamrk (DTUA Aqua).

After 10 days, fish were checked for their gill scores [326] 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 mins; 4) infected, AquaDes-treated group for 60 mins; 5) infected, Perfectoxid -treated group for 30 mins; 6) infected, Perfectoxid -treated group for 60 mins; 7) infected, ADDIAqua-treated group for 30 mins; 8) infected, ADDIAqua -treated group for 60 mins. 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 mins. 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 [286].

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, their 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 5 200 rpm (Heraeus Labofuge 200, Thermo Scientific, Massachusetts, USA) and plasma was separated and stored at -80 ºC 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 was placed in RNAlater (Ambion©, Connecticut, USA), kept at room temperature for 12 hours to allow proper penetration and afterwards stored at -80ºC 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 side was collected for RNA, while the right side was separated for histology, both fractions were handled similarly to the gills.

Plasma analysis

Total antioxidant capacity (TAC) in the plasma was analysed by a colorimetric assay kit (Sigma-Aldrich, Missouri, USA) previously used in salmon [81]. 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.

RNA isolation, cDNA synthesis and real-time quantitative PCR

Total RNA was isolated from the gills and olfactory organ 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 synthesised 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 ºC for 10 min, 37 ºC for 30 min and 95 ºC 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: 20 seconds of pre-incubation at 95 ºC, amplification with 40 cycles for 1 second at 95 ºC and 20 seconds at 60 ºC, and a dissociation stage for 1 second at 95 ºC, 20 seconds at 60 ºC and 1 second at 95 ºC. 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 *eleongation factor 1 alpha* (*ef1α*) for the gills, while *actb* and *hypoxanthine-guanine phosphoribosyltransferase* (*hprt1*) were used for the olfactory organ.

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 to detect 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 sec, followed by 50 cycles of denaturation at 95 °C for 3 sec and annealing at 60 °C for 30 sec. 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 in order to estimate the DNA copies per reaction.

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)

Histology treatment and 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. 2) *Quantification of non-specific pathologies*. 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” [399]. 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. In addition, measurements were carried out in 6 locations, for both epithelial and lamina propria thickness, in 3 randomly selected olfactory lamellae.

Statistical analysis

All statistical analyses were performed in SigmaPlot 14.0 (Systat Software Inc, Berkshire, UK). Before performing ANOVA, the data set were 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.

Results

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.

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 (Figure 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 mins 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 (Figure 1B). However, in the groups exposed to Perfectoxid for 30 mins and ADDIAqua for 60 mins, significant temporal difference was identified characterised by a higher level at 2 weeks post PAA treatment.

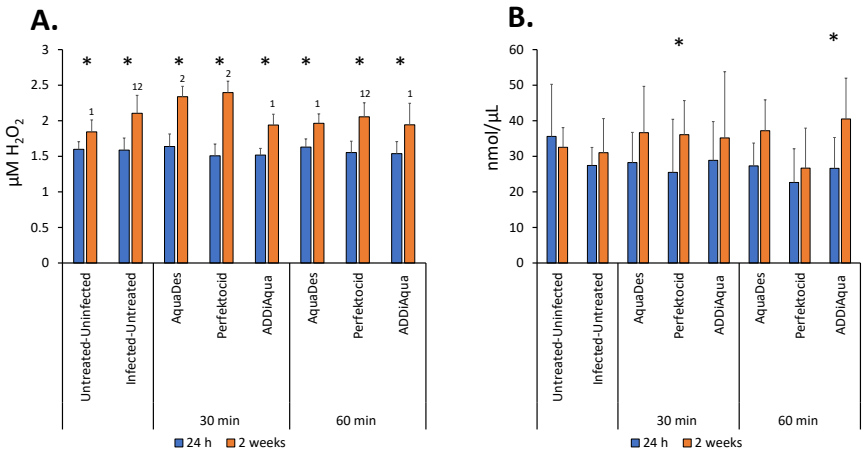


Figure 1. Plasmatic levels of reactive oxygen species (ROS, expressed as H_2O_2) and total antioxidant activity (TAC) 24 h and 2 weeks after treatment with PAA. Values are means ±SD of 10 individual fish per treatment group at a particular timepoint. Asterisk (*) indicates significant difference between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

Influence of AGD on the expression of genes related to oxidative stress in the gills and olfactory organs

We first evaluated how AGD affected the expression of genes relevant to oxidative stress in the gills (Figure 2) and olfactory organ (Figure 3) by comparing the uninfected-untreated (control) and AGD 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* (Figure 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 (Figure 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*) (Figure 3A, 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 difference, where the expression in the AGD-affected fish was significantly lower than the control at 2 weeks after infection (Figure 3B).

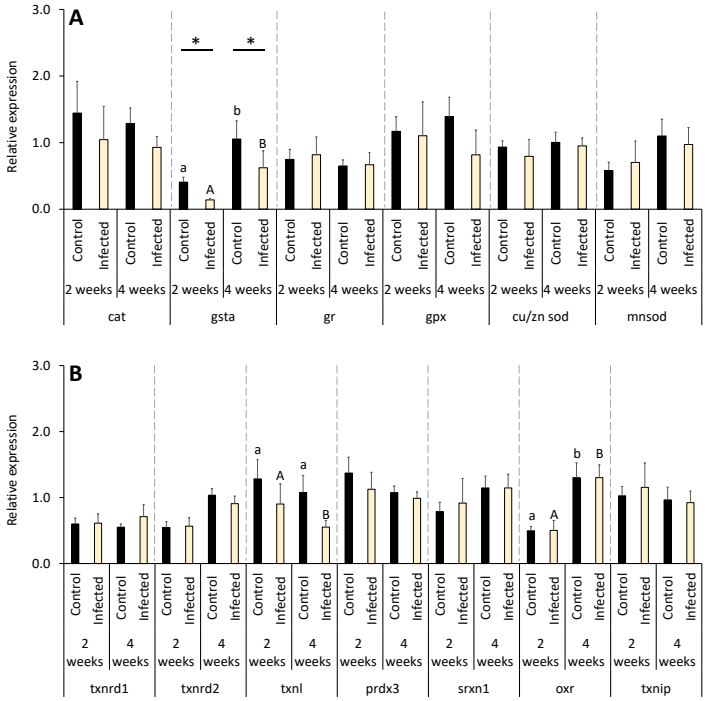


Figure 2. Changes in the expression of oxidative stress genes in the gills of AGD-affected salmon. Values are means ±SD of 10 individual fish per treatment group at a particular timepoint. Asterisk (*) indicates significant difference between control (uninfected) and infected group at a particular sampling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

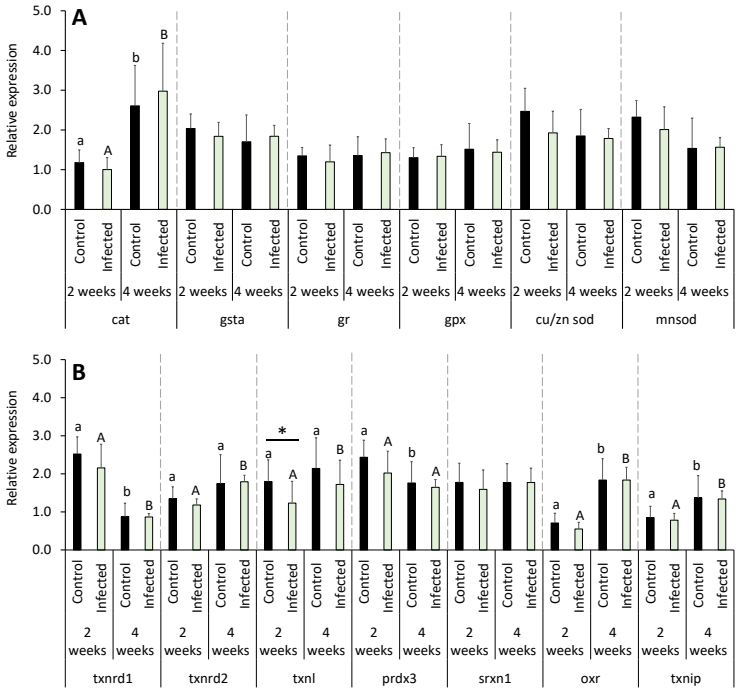


Figure 3. Changes in the expression of oxidative stress genes in the olfactory organs of AGD-affected salmon. Values are means ±SD of 10 individual fish per treatment group at a particular timepoint. Asterisk (*) indicates significant difference between control (uninfected) and infected group at a particular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

Effects of oxidant treatment on the expression of genes related to oxidative stress in the gills and olfactory organs of AGD-affected fish

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 mins or 60 mins (Figure 4). 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 demonstrated 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 cluster while of the rest of the oxidative stress genes were in a separate cluster (Figure 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. Evaluating the changes of individual genes, it was found that 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 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.

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 (Figure 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. 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.

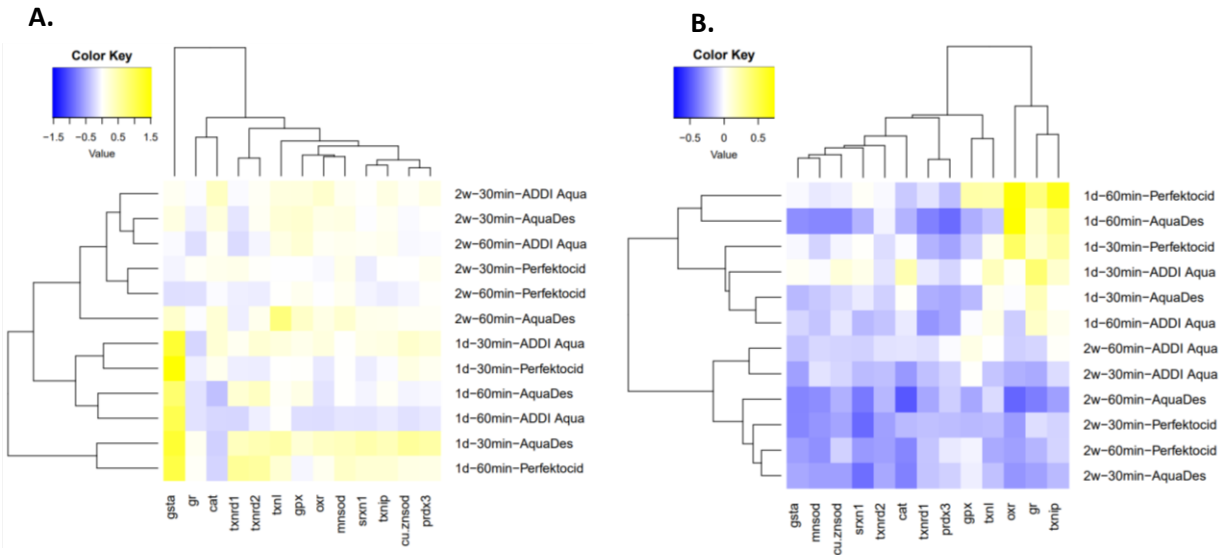


Figure 4. Changes in the oxidative stress genes in the (A) gills and (B) olfactory organs of AGD-affected Atlantic salmon.

Gross pathological gill scores and parasitic load

Gills scores were taken 24 hrs and 2 weeks after treatment (Figure 5A). At the beginning (24 hrs), all groups had a gill score between 1-2. 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 2-3. The group treated with AquaDes for 30 min had the lowest gill score among the groups. All groups treated with PAA for 30 mins demonstrated lower gill score than the infected-untreated group.

The parasitic load in the gills is shown in Figure 5B. There were no significant differences in the parasitic load across different treatments and timepoints.

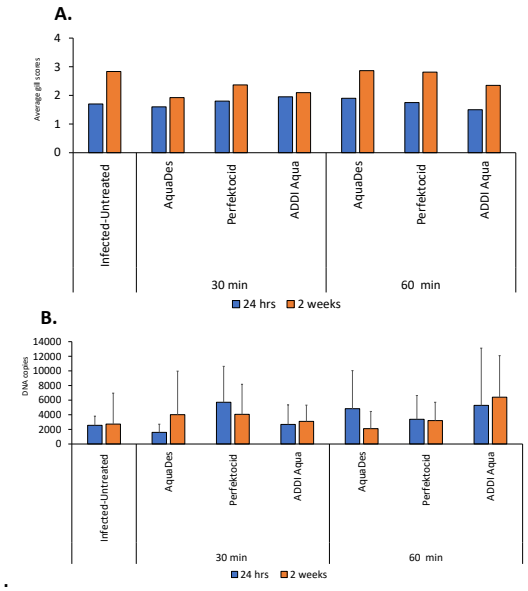


Figure 5. Level of AGD infection assessed by (A) macroscopic gill score and (B) qPCR of N. perurans from gill swabs.

Histopathological changes in relation to infection and treatment

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 (Figure 6A). Although it appears that fish treated with PAA for 60 mins demonstrated higher frequency of hyperplasia, when compared with the infected-untreated group and PAA-treated groups for 30 mins, 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 showed no significant difference to the uninfected-untreated group, including AquaDes-30, AquaDes-60 and Perfectoxid-60. 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 mins 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 (Figure 2B). At 2 weeks after treatment, mucous cells in AGD-affected groups, untreated and treated (except in ADDIAqua-30 and AquaDes60), showed significantly higher mucous cells in the gills than the uninfected-untreated group.

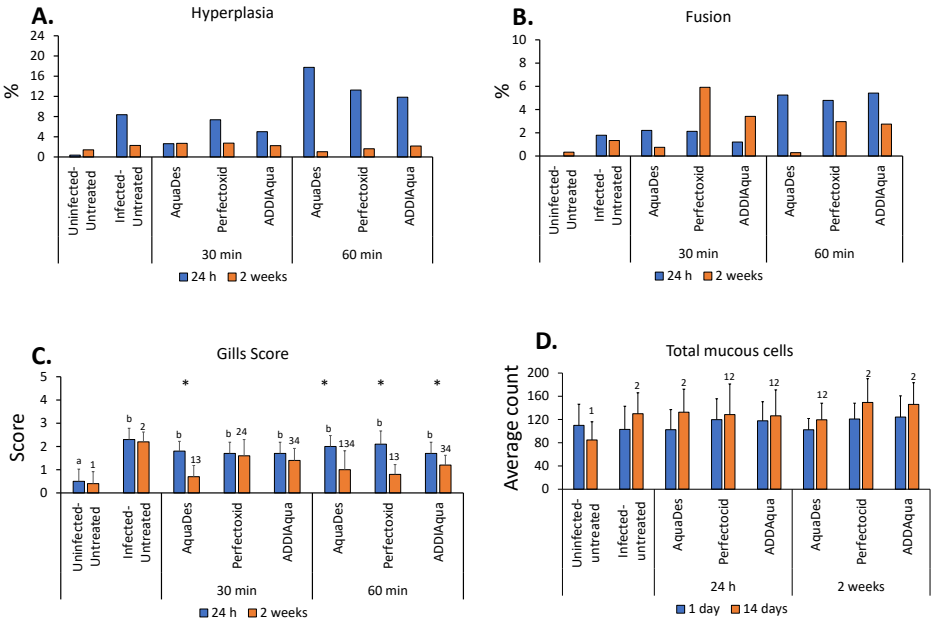


Figure 6. Microscopic evaluation of Atlantic salmon gills. Frequency of (A) hyperplasia and (B) fusion had been evaluated. (C) Microscopic gill score assessed through an injury score system is likewise indicated.

Changes in the olfactory organ

There were no significant temporal and inter-treatment changes in the thicknesses of the epithelium and lamina propria of the lamella of the olfactory organ (Figure 7).

The width of the mucosal tip of the olfactory lamella did not significantly differ 24 h after treatment (Figure 7). 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

(Figure 7). For the group treated with Perfectoxid for 60 mins, the olfactory mucosal tip was significant 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. (Figure 7).

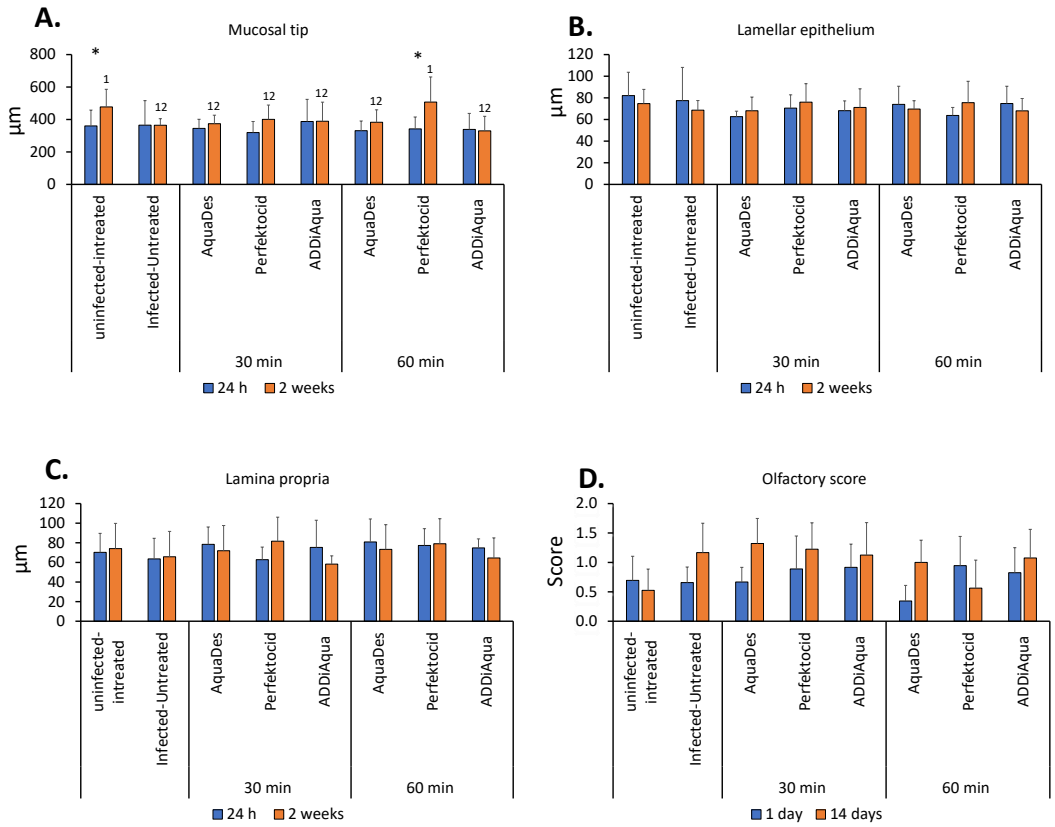


Figure 7. Microscopic evaluation of Atlantic salmon olfactory organs. Measurements were taken from (a) mucosal tip, (B) lamellar epithelium; and (C) lamina propia. The overall health status of the olfactory organ was assessed by a scoring scheme, where a higher score denotes more damage.

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.

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 [293]. 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 [400]. 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 [380]. 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 [401]. 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 [380] 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 [80, 81]. 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 [81, 170, 229, 286, 287]. Though 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.

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 [342], while gills and the olfactory organs have been shown to be sensitive to PAA [287] 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 [402], and have been shown to be vital in the protective mechanism against PAA-induced oxidative stress in the mucosal organs of salmon [264, 287]. *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 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 [403] by the neutralisation of hydrogen peroxide through decomposition[404]. 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 [405]. 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 [406, 407]. 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.

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

Unlike in the gills, where the changes in the oxidative stress genes showed a clear transient response, the olfactory organs 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 [264]. 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 [408]. We have earlier shown that PAA treatment altered the expression of *gr* in salmon, both using *in vitro* and *in vivo* models [81, 286]. 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 [409]. 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 [409], 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 [410]. 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 organs 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 [411]. 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.

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

Neoparamoeba perurans is a free-living organism in the environment, contacts and adheres mainly to the gills of Atlantic salmon [412]. Scoring of the gross pathology of the gills is the most common on-site assessment of the severity of AGD infection. Macroscopic 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 mins regardless of the product type showed relatively lower macroscopic gill scores than fish treated with PAA for 60 mins and infected-untreated group. However, the inter-treatment resolution was not very clear which was further corroborated by the PCR quantification of amoeba.

When then investigated the microstructural changes in the gills of AGD affected fish following treatment. After colonising the organ, then 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 [342, 369]. Interestingly in 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 PCR 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.

Both the macroscopic 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 pathologies in the gills.

Gills infected with *N. perurans* parasite is often characterised with an increase in mucus production, likely due to hyperplasia and hypertrophy of mucus-producing cells [329, 369]. In this study we observed that 24 h after treatment, inter-treatment differences in the number of gill mucus cells were found. However, at 2 weeks thereafter, the number of mucus cells in the gills of AGD-affected fish and those treated with PAA displayed higher mucus cell counts than the uninfected-untreated group, hence displaying the typical mucosal response to AGD. In addition, such a response to PAA of increased in mucus cell number in the gills corroborated an earlier documentation of such consequences in smolts exposed to PAA [267]. Gills exposed to an irritant stimulus increase the number of mucus cells as a protective response [413, 414], 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 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 [415].

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 [416, 417]. 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 [418]. AGD did not affect the structural integrity of the olfactory organs, neither did the treatment to PAA of AGD-affected fish. We did not visually find the amoeba in the olfactory organ; 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 organ are altered by recurrent PAA treatment [287]. We did not document such a consequence here. This apparent different is likely attributed to frequency of delivery – in the current study, we exposed salmon once to PAA while the previous study reported from 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.

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 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 mins. 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 organ. This striking response profile was not heavily influenced by either PAA product or duration of treatment. 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 pathologies related to infection. Standardisation of exposure protocol is the next step in evaluating the chemotherapeutic potential of PAA against AGD.

6 Main findings

The project explored the different aspects of PAA as a potential chemotherapeutics for Atlantic salmon. We have made significant advancements in understanding the chemistry and biology behind PAA, and ultimately identified how these innate properties influenced its efficacy as a treatment for amoebic gill disease in Atlantic salmon.

- We have established the degradation kinetics of PAA in seawater, where it follows exponential first-order decay with half-lives on the order of minutes to hours. Moreover, we have identified the effects of temperature, salinity, light and UV on the decay dynamics of PAA. Temperature, salinity and UV significantly increased PAA decay. This information is important in dosing as well as in handling discharges after treatment.

- Healthy, uninfected Atlantic salmon smolts could tolerate PAA doses from 0.6 to 10 ppm, though the risks are higher at higher doses. Moreover, the responses were dependent on exposure duration, stress status, frequency of exposure and PAA trade products. These factors are crucial in defining the frameworks of optimal treatment protocol using PAA.

- Using an array of tools – from molecular to whole organismal response - the project provided the most comprehensive evaluation of the physiological consequences of PAA application in an aquaculture species to date. We have identified the mechanisms of how PAA affects the fish and the countermeasures the fish mount to address the physiological pressures from this potent oxidant. Evidence have been established that though PAA is considered a stressor, Atlantic salmon could effectively respond to the demands of PAA, at least within the concentrations tested in the study.

- Atlantic salmon mucosal organs responded to PAA – with the gills and olfactory organs as the most responsive. Skin was not heavily affected by PAA, even at higher concentrations. The effects of PAA on these mucosal organs could be observed shortly after treatments.

- PAA, as an oxidant, was demonstrated to induce both mucosal and systemic oxidative stress. Nonetheless, this striking effect was transient and carried moderate long-term effects. Intermittent administration appeared to increase the oxidative stress-inducing potential of PAA.

- We have provided new insights into the pathophysiology of AGD. We have corroborated earlier evidence that oxidative stress is involved in the disease development, though the trials showed a variable profile. Moreover, we have demonstrated that induction of mucosal oxidative stress preceded the systemic dysregulation of ROS balance, particularly during the early onset of infection. Metabolomics revealed two new markers in plasma that have the potential to differentiate between gill scores.

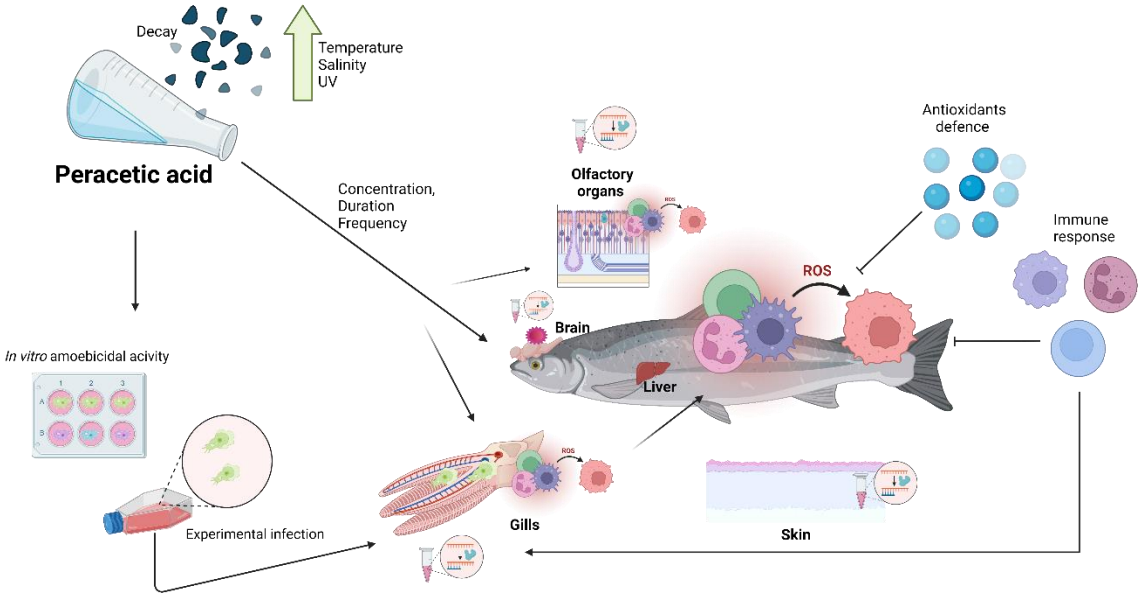
- AGD increases the toxicity of PAA in Atlantic salmon. This was demonstrated both by prominent behavioural changes during treatment and increased post-treatment mortality in PAA-treated AGD-infected fish. This response was dose dependent.

- Under *in vitro* conditions, PAA exhibited amoebicidal activity. PAA treatment of AGD-affected fish did not provide an unequivocal treatment resolution. The gross gill scores continued to increase following infection. Histological lesions associated with infection likewise persisted, though in some cases, a longer recovery period maybe necessary. Nonetheless, PAA treatment of AGD-affected fish appeared to reduce the parasitic load which was influenced by the treatment protocols.

- The type of PAA product elicited different responses from AGD-affected fish. This provides evidence that different trade products can be a strong factor for consideration in selecting PAA as a treatment option. PAA likewise affected the responses of the gills to AGD, and most of this interference was observed at the early stage of recovery.

- Additional studies are needed for PAA to be used as a treatment for AGD. Further treatment optimisations are required, especially by taking into considerations the risk factors identified in PERAGILL.

- **Nonetheless, we have advanced in the holistic understanding of one of the most sustainable disinfectants in aquaculture - PAA. We believe that this knowledge is not only valuable in the discussion of treatment options for a gill health issue, but also in other application of PAA in aquaculture, in particular as a routine disinfectant in systems operating in recirculation technology.**



Advancements in the understanding of the chemistry and physiology of peracetic acid (PAA) in Atlantic salmon smolts. *Peracetic acid is a strong oxidant that rapidly degrades in water. Its decay kinetics are influenced by temperature, salinity and UV. Exposure of naïve healthy Atlantic salmon smolts to PAA induces changes in organs important for defence (i.e., gills, olfactory organ and skin), metabolism (i.e., liver) and behaviour and stress (i.e., brain). The gills and olfactory organs are the most sensitive organs to PAA. These changes have been documented using a wide array of platforms – molecular, biochemical and histological, employing both traditional and modern tools, including -omics and AI-based strategies. PAA is a strong trigger of mucosal and systemic oxidative stress, which is counteracted by an array of molecules, including antioxidants and cellular and humoral mediators of immune response. Effects on behaviour and stress, though minimal in naïve fish, are likely associated with the dysregulation in the brain. Under in vitro conditions, PAA exhibits amoebicidal activity. We have developed an infection model based on an isolate from a field outbreak. AGD induces mucosal (i.e., gills) and systemic oxidative stress. Moreover, AGD triggers a local immune response, where most of the genes have been identified to be downregulated. This downregulation is perhaps involved in the invasion, and importantly, to disease progression. PAA treatment alters the gill immune responses to AGD but only during the early phase after treatment. PAA treatment appeared to lower the parasitic load, but gross and microscopic pathologies associated with AGD persisted even after treatment. (Illustration was created in BioRender).*

7 Deliverables

7.1 Oral Presentations at conferences/meetings

1. Lazado, C.C. A slimy insight into 3Rs: What can we learn from fish mucosal surfaces. UK Royal Society for the Prevention of Cruelty to Animals (web). February 23, 2022.

2. Lazado, C.C., Cabillon, N.A., Furtado, F., Osorio, J., Aas, L.H., Breiland, M.W., Johansen, L.H., Mota, V., Ytteborg, E. The nasal mucosa of Atlantic salmon – where chemoreception meets immunity. Frisk Fisk 2022, Bergen, Norway, May 30-31, 2022.

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7.2 Poster presentation at conferences/meetings

1. Lazado, C.C., Alipio, H.R., Timmerhaus, G., Breiland, MW., Johansen, L.H., Burgerhout, E., Pedersen, L.F., Strand, D. Metabolomics elucidates the adaptive responses of Atlantic salmon to stress and infection. 7th International Symposium on Genomics in Aquaculture (GIA2022), Granada, Spain, May 4-6, 2022.

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3. Voldvik, V., Lazado, C.C. Circadian rhythms of oxidative stress responses in the gill mucosa of Atlantic salmon. 1st International Symposium on Mucosal Health in Aquaculture MHA2019, Oslo, Norway, September 11-13, 2019.

4. Soleng, M., Johansen. L.H., Johansson, G.S., Breiland, M., Johnsen, H., Pedersen, L.F., Lazado, C.C. Coordination of systemic and mucosal stress responses in Atlantic salmon treated with an organic peroxide. 1st International Symposium on Mucosal Health in Aquaculture MHA2019, Oslo, Norway, September 11-13, 2019.

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7.3 Peer-reviewed articles (published)

1. Lazado, C.C., Breiland, MW., Furtado, F., Burgerhout, E., Strand, D. 2022. The circulating metabolome of *Neoparamoeba perurans*-infected Atlantic salmon *(Salmo salar)*. *Microbial Pathogenesis*. 166, 105553.

2. *Osório, J., Stiller, K.T., Reiten, B.K., Kolarevic, J., Johansen, L.H., Afonso, F., Lazado, C.C. 2022. Intermittent administration of peracetic acid is a mild environmental stressor that elicits mucosal and systemic adaptive responses from Atlantic salmon post-smolts. *BMC Zoology*. 7, 1.

3. Lazado, C.C., Timmerhaus, G., Breiland, M.W., Pittman, K., Hytterød, S. 2021. Multiomics provide insights into the key molecules and pathways involved in the physiological adaptation

of Atlantic salmon (*Salmo salar*) to chemotherapeutic-induced oxidative stress. *Antioxidants*. 10, 1931.

4. Haddeland, S., Lazado, C.C., Merkin, G., Myre, O. J., Okubamichael, M., Pedersen, L.F., Pittman, K. 2021. Dynamic morphometrics of mucous cells reveal the minimal impact of therapeutic doses of peracetic acid on Atlantic salmon gill health. *Aquaculture*. 534, 736315.

5. Lazado, C.C., Sveen, L., Soleng, M., Pedersen, L.F., Timmerhaus, G. 2021. Crowding reshapes the mucosal but not the systemic response repertoires of Atlantic salmon post-smolts to peracetic acid. *Aquaculture*. 531, 735830.

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12. Soleng, M., Johansen, L.H., Johnsen, H., Johansson, G.S., Breiland, M.W., K., Rørmark, L., Pittman, Pedersen, L.F., Lazado, C.C. 2019. Atlantic salmon (*Salmo salar*) mounts systemic and mucosal stress responses to peracetic acid*. Fish & Shellfish Immunology*. 93, 895-903.

7.4 Peer-reviewed articles (under revision/under review)

1. Carletto, D., Breiland, M.W., Hytterød, S., Timmerhaus, G., Lazado, C.C. Recurrent oxidant treatment induces dysregulation in the brain of transcriptome of Atlantic salmon (*Salmo salar*) smolts. *Under revision in Toxicology Reports*.

2. Furtado, F., Carletto, D., Strand, D., Timmerhaus, G., Breiland, M.W., Afonso, F., Lazado, C.C. The molecular repertoire of oxidative stress display distinct patterns of regulation in the gills and olfactory organ of Atlantic salmon (*Salmo salar*) during infection and treatment of the parasite *Neoparameoba perurans*. *Under review in Fish & Shellfish Immunology*.

7.5 Peer-reviewed articles (in preparation)

1. Lazado, C.C., Strand, D., Breiland, M.W., Timmerhaus, G., Gjessing, M., Hytterød, S., Merkin, G., Pedersen, L.F., Pittmann, K., Krasnov, A Mucosal immune and stress responses of *Neoparamoeba perurans*-infected Atlantic salmon (*Salmo salar*) treated with peracetic acid shed light on the host-parasite-oxidant interactions.

2. Lazado, C.C., Strand, D., Breiland, M.W., Timmerhaus, G., Gjessing, M., Hytterød, S., Pedersen, L.F. Different peracetic acid products provide varying treatment impacts against amoebic gill disease in Atlantic salmon.

3. Lazado, C.C., Sundaram, A., Breiland, M.W. Comparative high-through put sequencing of Atlantic salmon gills and olfactory organs.

4. *Ytteborg, E., Lazado, C.C., Hansen, R.I., Johansen, L.H. The skin mucosal barriers of lumpfish (*Cyclopterus lumpus*) are altered by production-related stressors in salmon farms.

*Trials described in this paper are not directly under PERAGILL, but the project participated by delivering competence and providing the PAA products.

7.6 Thesis

1. Sindre Haddeland. Thesis: *Benchmarking healthy gills in Atlantic salmon (Salmo salar) in seawater recirculating aquaculture system after repeOralated peracetic acid exposure*. University of Bergen, Norway. (Completed)

2. Malene Soleng. Thesis*: Systemic and mucosal stress responses of Atlantic salmon exposed to peracetic acid*. UiT – The Arctic University of Norway, Norway. (Completed)

3. Francisco Furtado. Thesis*: Regulation of molecules involved in the oxidative stress response of Atlantic salmon infected with amoebic gill disease and treated with peracetic acid*. University of Lisbon, Portugal. (Completed)

4. Karoline Koppen Vågnes. Provisional title: Behavioural and physiological responses of Atlantic salmon to a commercial disinfectant in aquaculture. DTU/NTNU. (On going)

7.7 In the press

1. Researchers develop new treatment for AGD
https://thefishsite.com/articles/researchers-develop-new-treatment-for-agd

7.8 Award

The results from this study have been instrumental for the project leader (Lazado) in receiving the 2021 No Recopa's 3R Prize.

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