Full-length article

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

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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 behaviour 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 mobilise 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.

Keywords: Amoebic gill disease; crowding stress; hydrogen peroxide; mucosal health; oxidative stress; peracetic acid

1. 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 (Subasinghe, Soto, Jia, 2009). 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 (AS, 2019).

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*) (Hannisdal, Nøstbakken, Hove, Madsen, Horsberg, Lunestad, 2020; Overton, Samsing, Oppedal, Dalvin, Stien, Dempster, 2018). 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 (Bowers, Mustafa, Speare, Conboy, Brimacombe, Sims, Burka, 2000; Mordue , Birkett, 2009; Overton, Samsing, Oppedal, Dalvin, Stien, Dempster, 2018). Another ectoparasitic infection is amoebic gill disease (AGD) caused by *Neoparamoeba perurans*, a widespread condition affecting salmonids farmed in the marine environment (Steinum, Kvellestad, Rønneberg, Nilsen, Asheim, Fjell, Nygård, Olsen, Dale, 2008). 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 (Adams, Nowak, 2003). 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 (Kiemer, Black, 1997; Pedersen, Good, Pedersen, 2012). 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 (Bechmann, Arnberg, Gomiero, Westerlund, Lyng, Berry, Agustsson, Jager, Burridge, 2019; Hjeltnes B, Bang-Jensen B, Bornø G, Haukaas A, S, 2019). 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 (Beber de Souza, Queiroz Valdez, Jeranoski, Magno de Sousa Vidal, Soares Cavallini, 2015; Kitis, 2004). 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 (Domínguez Henao, Turolla, Antonelli, 2018). 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 (Pedersen, Lazado, 2020) but its effective dose against many aquaculture pathogens is also lower than H_2O_2 (Block, 1991; Liu, Straus, Pedersen, Meinelt, 2015; Straus, Meinelt, Liu, Pedersen, 2018). The chemical behaviour of PAA in both freshwater and seawater matrices is well-described (Pedersen, Lazado, 2020; Pedersen, Meinelt, Straus, 2013) and the toxicity of PAA towards several aquaculture fish has been reported (Straus, Meinelt, Liu, Pedersen, 2018). 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 (Gesto, Liu, Pedersen, Meinelt, Straus, Jokumsen, 2018; Liu, 2017; Liu, Lazado, Pedersen, Straus, Meinelt, 2020). 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 (Soleng, Johansen, Johnsen, Johansson, Breiland, Rørmark, Pittman, Pedersen, Lazado, 2019). 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) (Espmark, Kolarevic, Aas-Hansen, Nilsson, 2015; Sveen, Karlsen, Ytteborg, 2020), crowding is an inevitable production procedure in salmon farming, such as during vaccination, transport, grading, de-licing, and chemotherapeutic bath treatments (Noble, Gismervik, Iversen, Kolarevic, Nilsson, Stien, Turnbull, 2018). 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 (Soleng, Johansen, Johnsen, Johansson, Breiland, Rørmark, Pittman, Pedersen, Lazado, 2019). 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 (Cabillon, Lazado, 2019; Lazado, 2020). 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.

2. Materials and methods

2.1. 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 (Soleng, Johansen, Johnsen, Johansson, Breiland, Rørmark, Pittman, Pedersen, Lazado, 2019). 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 (Espmark, Kolarevic, Aas-Hansen, Nilsson, 2015; Noble, Gismervik, Iversen, Kolarevic, Nilsson, Stien, Turnbull, 2018), 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 (Pedersen, Lazado, 2020). 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.

2.2. Sample collection

Sampling was performed at 4 h (for plasma and RNA) and 2 w (for plasma, RNA, histology, skin colour, 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 (Noble, Gismervik, Iversen, Kolarevic, Nilsson, Stien, Turnbull, 2018). 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).

2.3. 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 (Krasnov, Timmerhaus, Afanasyev, Jørgensen, 2011). 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.

2.4. 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 (Doneanu, Chen, Mazzeo, 2011). 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 analysed 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 analysed 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.

2.5. 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 colour (RGB; Red Green Blue) values. The overall mean and the three colour channels (red, green, blue) were measured as described earlier (Lazado, Haddeland, Timmerhaus, Berg, Merkin, Pittman, Pedersen, 2020).

2.6. 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 (Sveen, Timmerhaus, Krasnov, Takle, Stefansson, Handeland, Ytteborg, 2018). 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 (Reiser, Schroeder, Wuertz, Kloas, Hanel, 2010), with modifications (Stiller, Kolarevic, Lazado, Gerwins, Good, Summerfelt, Mota, Espmark, 2020). 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.

2.7. 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 colour, 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 equalised. 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 normalised 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.

3. 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 organisations: 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 stabilisers. 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 (Noble, Gismervik, Iversen, Kolarevic, Nilsson, Stien, Turnbull, 2018). 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.

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

The skin colour analysis revealed that PAA exposure did not affect the skin colour 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 colour 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 (Lazado, Haddeland, Timmerhaus, Berg, Merkin, Pittman, Pedersen, 2020).

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 (Smart, 1976). 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 (Kostić, Kolarević, Kračun-Kolarević, Aborgiba, Gačić, Paunović, Višnjić-Jeftić, Rašković, Poleksić, Lenhardt, Vuković-Gačić, 2017). Crowding carries a strong respiratory demand for fish (Noble, Gismervik, Iversen, Kolarevic, Nilsson, Stien, Turnbull, 2018), 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 (Esteban, 2012). 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 (Liu, Lazado, Pedersen, Straus, Meinelt, 2020; Sveen, Timmerhaus, Torgersen, Ytteborg, Jørgensen, Handeland, Stefansson, Nilsen, Calabrese, Ebbesson, Terjesen, Takle, 2016). 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.

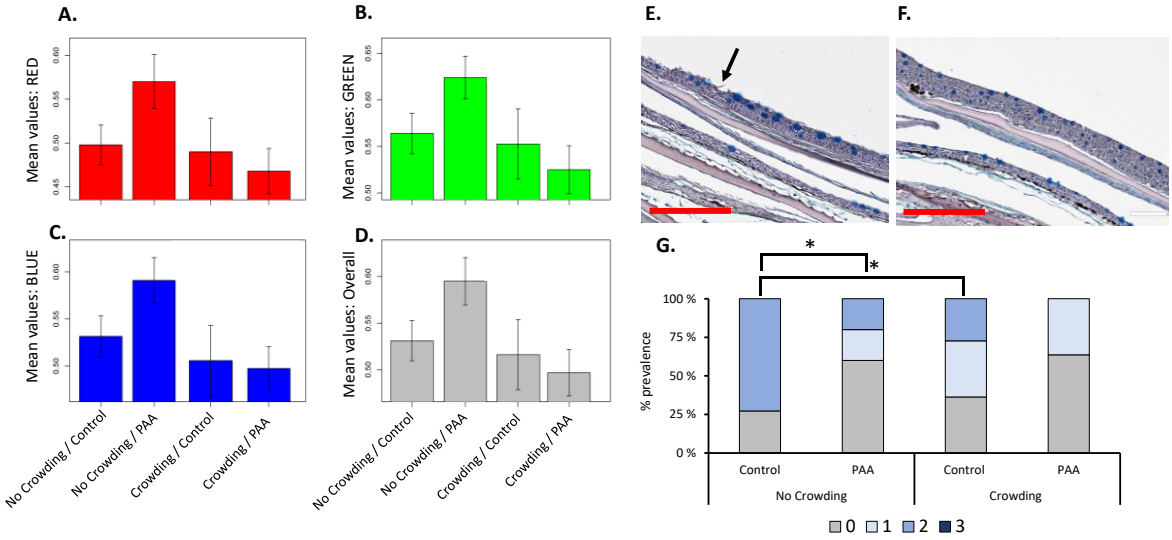

Figure 1. Macro- and micro-features of Atlantic salmon post-smolts skin 2 weeks after exposure to PAA with and without crowding history. Panels A-D: Skin colour analysis revealing the individual RGB values (**A-C**) as well as the mean values (**D**). A higher value represents lighter/brighter colours; a lower value indicates a darker colour. 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.

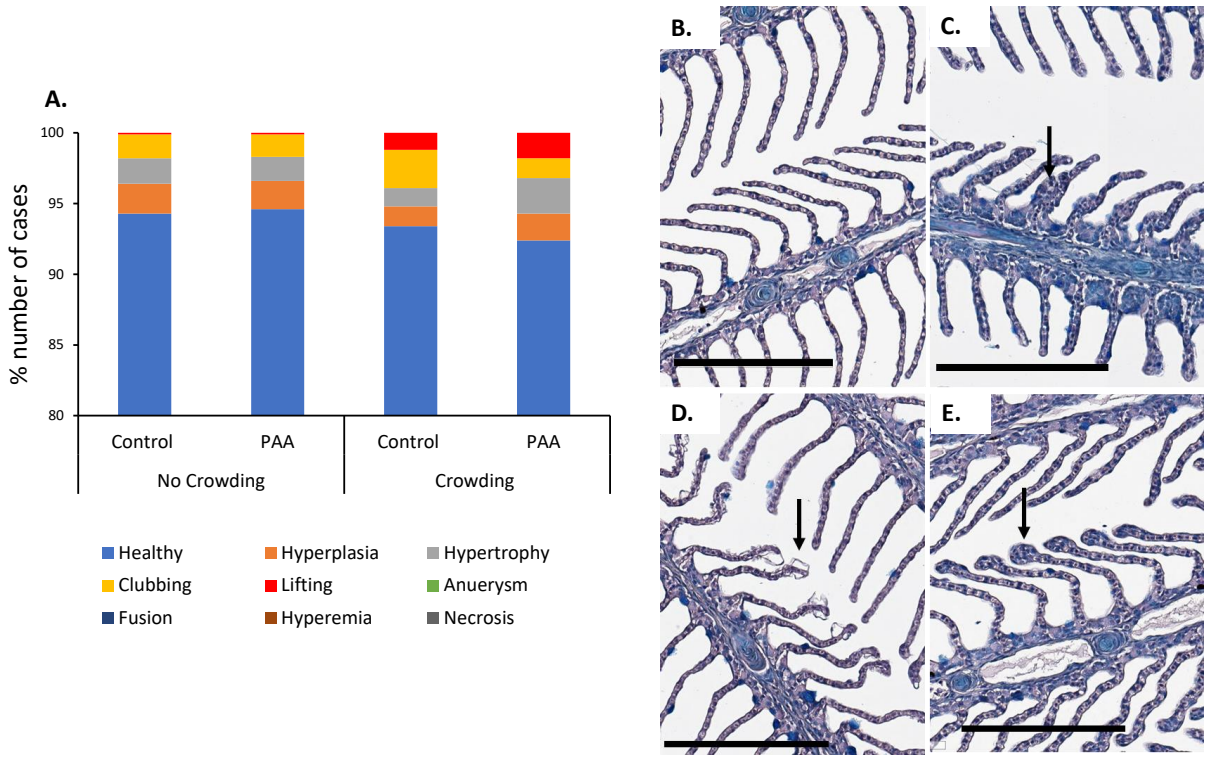

Figure 2. Histological scoring of branchial alterations in Atlantic salmon post-smolts 2 weeks after exposure to PAA with and without crowding history. Panel A: The prevalence of 9 common cases was quantified from 100 individual lamellae per fish. Only epithelial lifting was identified to exhibit inter-treatment 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.

3.2. 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 (Gesto, Liu, Pedersen, Meinelt, Straus, Jokumsen, 2018). 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 (Soleng, Johansen, Johnsen, Johansson, Breiland, Rørmark, Pittman, Pedersen, Lazado, 2019). 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* (**Supplementary File 1**), 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* (**Supplementary File 1**) 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 (Chen, Lu, Nie, Ning, Chen, 2018; Sepahi, Tacchi, Casadei, Takizawa, LaPatra, Salinas, 2017). The emblematic modulation of their transcription following crowding provides new insights into their mucosal function in fish during crowding stress.

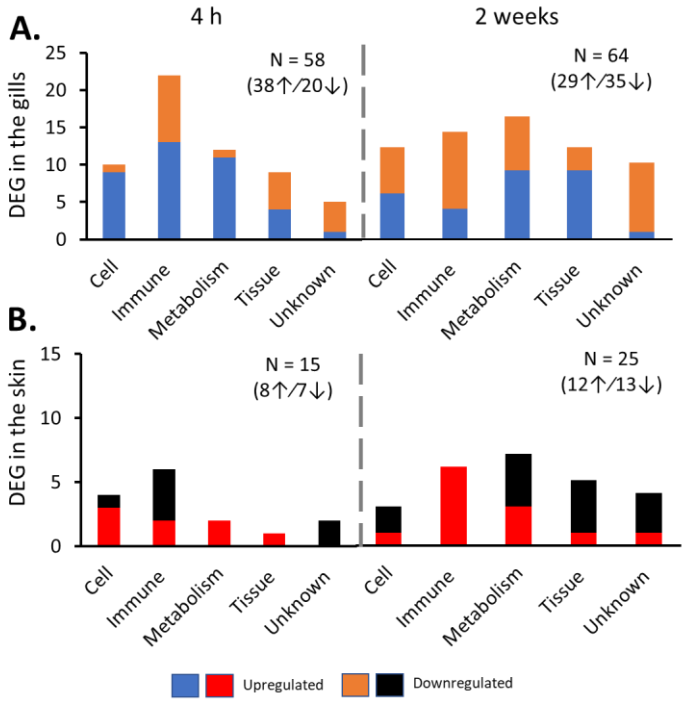

Figure 3. Differentially expressed genes (DEG) in the gills and skin of Atlantic salmon post-smolts 4 h and 2 weeks after crowding. The no-crowding control group was compared to the crowding control group to identify genes that were responsive to crowding alone. DEGs were identified with a criterion 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.

3.3. 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 (Gesto, Liu, Pedersen, Meinelt, Straus, Jokumsen, 2018; Hushangi, Hosseini Shekarabi, 2018; Liu, Straus, Pedersen, Meinelt, 2017; Liu, Lazado, Pedersen, Straus, Meinelt, 2020). 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 (Costas, Aragão, Mancera, Dinis, Conceição, 2008). 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 (**Supplementary File 1**). 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 (Livingstone, 2003; Siwik, Pagano, Colucci, 2001). 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 (Souza, Bonilla-Rodriguez, 2007). 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 (Soleng, Johansen, Johnsen, Johansson, Breiland, Rørmark, Pittman, Pedersen, Lazado, 2019). 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 (Soleng, Johansen, Johnsen, Johansson, Breiland, Rørmark, Pittman, Pedersen, Lazado, 2019), 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 (**Supplementary File 1**). 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* (**Supplementary File 1**). 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 (Ferro, Goitre, Retta, Trabalzini, 2012). 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 (Liu, Lazado, Pedersen, Straus, Meinelt, 2020; Soleng, Johansen, Johnsen, Johansson, Breiland, Rørmark, Pittman, Pedersen, Lazado, 2019), 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 (Lazado, Nagasawa, Babiak, Kumaratunga, Fernandes, 2014; Wilson, González-Billault, 2015). 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.

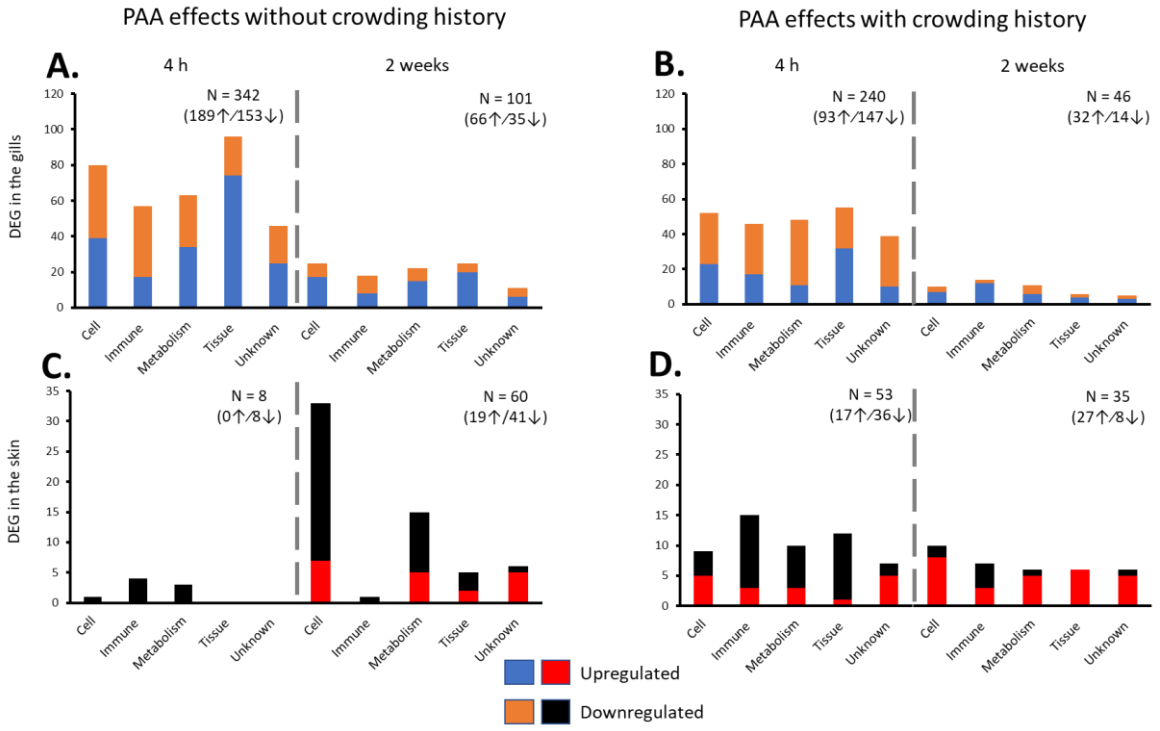

Figure 4. Differentially expressed genes in the gills and skin of Atlantic salmon post-smolts 4 h and 2 weeks after PAA exposure, with and without crowding history. PAA-exposed and control groups with no crowding history were compared to identify genes responsive to PAA treatment (Panels A, C). The same 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.

3.4. 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; Supplementary File 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 (Herrera, Mancera, Costas, 2019). The plasma free tyrosine levels have been found to increase during acute stress in fish, suggesting the importance of tyrosine during a stress episode (Costas, Conceição, Aragão, Martos, Ruiz-Jarabo, Mancera, Afonso, 2011; Vijayan, Pereira, Grau, Iwama, 1997). 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 (Singh, Kukreti, Saso, Kukreti, 2019). In addition, DNA damage associated with oxidative stress is mediated by guanine (Kawanishi, Hiraku, Oikawa, 2001). 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 (Soleng, Johansen, Johnsen, Johansson, Breiland, Rørmark, Pittman, Pedersen, Lazado, 2019). 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.

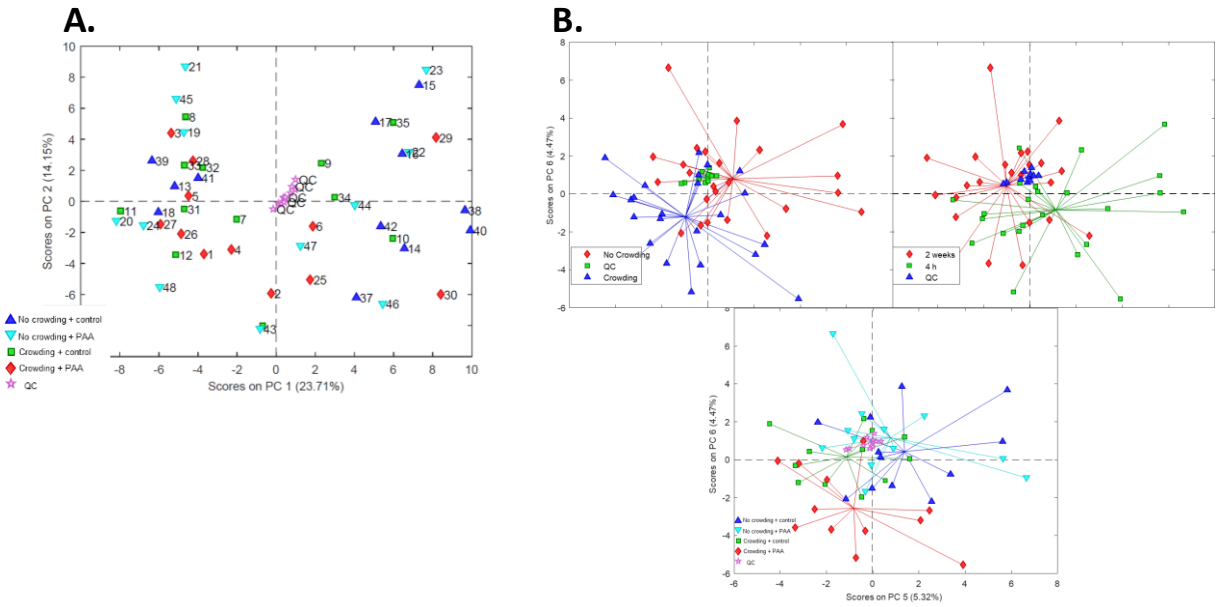

Figure 5. Plasma metabolomes of Atlantic salmon post-smolts 4 h and 2 weeks after PAA exposure with and without crowding history. Panel A: Score plot from the PCA model calculated on the relative concentrations of the variables in the reduced dataset. Data have been auto-scaled. **Panel B:** Score plots 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

3.5. 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 organisations 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 localised (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 minimised 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.

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Author contributions

C.C.L. and L.F.P. conceived the idea for the research. C.C.L. and L.F.P. designed the trial. C.C.L., L.F.P., G.T., and M.S. conducted the experiments and collected the samples. C.C.L., G.T., L.S. and M.S. performed the analyses. C.C.L., L.S. and G.T. processed and analysed the data. All authors contributed to the writing of the draft and reviewed the final version of the manuscript.

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