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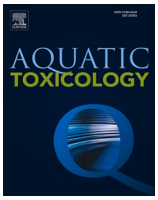

Consequences of continuous ozonation on the health and welfare of Atlantic salmon post-smolts in a brackish water recirculating aquaculture system

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ABSTRACT

This study investigated the biological consequences of 45-day continuous ozonation on Atlantic salmon (*Salmo salar*) post-smolts in a brackish water recirculating aquaculture system (RAS). There was no significant difference in survival, operational welfare indicators, and average weight at termination between the ozone-treated and control groups. Plasma biochemical analyses revealed that the creatinine level was significantly higher in the ozone-treated group than in the control at termination. Histological evaluation of skin health showed no significant difference between the two groups. On the other hand, quantitative histopathology disclosed that the ozone group exhibited a better gill health status than did the control group, particularly at the end of the trial. Mucosal transcriptomics revealed a distinct response profile between the gills and skin. At day 45, there were no differentially expressed genes (DEG) identified in the skin, in contrast to 242 ozone-induced DEGs in the gills. Assessment of the transcriptomic profiles over time revealed that temporal effects were of greater impact in skin compared to gills, regardless of the treatment. The treatment did not result in metabolomic dysregulation and the overall profile lent support to the transcriptomics data that temporal effects had a greater influence on the changes observed. Exposure to handling-confinement stress revealed that ozone treatment did not alter the ability of post-smolts to respond to a secondary stressor. In summary, the suite of health and welfare indicators collectively indicated that continuous ozonation resulted in minimal physiological perturbations in salmon post-smolts. The results are expected to contribute to optimising the rearing conditions for post-smolts in RAS.

1. Introduction

Recirculation technology is becoming more and more common in fish farming. A case in point is Atlantic salmon (*Salmo salar*) aquaculture, where technological innovations in closed systems highlight the advances of recent years (Lazado and Good, 2021). Closed-containment systems (CCS) are aquaculture systems that are separated from the surrounding ecosystem by an impermeable or semi-permeable wall. Recirculation aquaculture system (RAS) is essentially a CCS with fish tanks, filtration system and water treatment incorporated into one production loop. The system is also quite distinct with its limited water exchange. RAS is projected to contribute to improving farming sustainability, as these systems ensure site flexibility, reduced water usage, lower effluent volumes, and better environmental control (Gonçalves and Gagnon, 2011). Since water is one of the fundamental resource inputs, the system must therefore be equipped with an efficient strategy for its treatment and re-use.

Ozone (O_3) is widely regarded in aquaculture because of its disinfection property and the ability to improve water quality, thereby creating an optimal environment for growth, survival, and performance

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of cultured species (Davidson et al., 2011; Gonçalves and Gagnon, 2011; Good et al., 2011; Reiser et al., 2010; Stiller et al., 2020). Ozone is a very unstable molecule, and decomposes very quickly in the water (Leynen et al., 1998). Because of its high oxidative potential, some of the pollutants and metabolic by-products accumulating within low and near-zero exchange systems are oxidised; hence, rendering them less harmful for the cultured fish and making the rearing environment more favourable (Spiliotopoulou et al., 2018; Summerfelt et al., 2004, 2009).

Despite these beneficial features of ozone, toxicity issues remain a serious concern (Davidson et al., 2011; Gonçalves and Gagnon, 2011; Leynen et al., 1998; Li et al., 2014; Reiser et al., 2010, 2011; Stiller et al., 2020). If not adequately controlled, excess residual ozone remaining in the culture water could cause significant harm or even mass mortality to cultured fish (Leynen et al., 1998; Summerfelt et al., 2004). Ozone toxicity is of more significant concern in systems with higher salinities because of the persistence and higher occurrence of by-products, such as free bromine and bromoamines, which at higher concentrations can be toxic to fish (Schröder, 2011; Stiller et al., 2020; Summerfelt et al., 2004). We have found earlier that 350 millivolts (mV) were the safe upper limit for ozone for brackish water-adapted salmon. Beyond this threshold, ozone became toxic, causing substantial mass mortality and severe consequences for health and welfare (Stiller et al., 2020). This threshold was in agreement with the toxicity of ozone with other marine organisms such as in European seabass (*Dicentrarchus labrax*) (Li et al., 2014) and Atlantic halibut *(Hippoglossus hippoglosus*) (Tango and Gagnon, 2003).

Even though there exists substantial documentation of the chemical behaviour, water quality improvement, and toxicity of ozone in aquaculture systems (Leynen et al., 1998; Li et al., 2014; Spiliotopoulou et al., 2018; Summerfelt et al., 2004; 2009), knowledge about the physiological consequences of ozone in fish is fragmentary. Chronic ozone exposure could result in altered redox homoeostasis, eventually causing an oxidative stress state (Rivas-Arancibia et al., 2009). In both salmon and juvenile turbot (*Psetta maxima*, L.), exposure to ozone affected the expression of several antioxidant defence genes, which implied the mustering of countermeasures against ozone-induced oxidative stress (Reiser et al., 2011; Stiller et al., 2020). In addition, ozone targets the mucosal surfaces, as shown by the alterations in the histostructural features of mucosal organs, particularly the gills (Cabillon and Lazado, 2019; Good et al., 2011; Reiser et al., 2010; Stiller et al., 2020). There are several benefits and risks in the use of ozone in aquaculture and exploring the responses in a multi-platform approach will provide important information regarding its biological effects and as such enable optimisation and development for ensuring water quality that safeguards fish health and welfare.

An earlier study demonstrated that salinity at about 12‰ had favourable effects on the growth, survival, and welfare of salmon in RAS; it was proposed as a cost-efficient production strategy before post-smolts are transferred to open sea cages (Ytrestøyl et al., 2020). This study aimed to determine the health and welfare impacts on salmon post-smolts of continuous ozone application in brackish water RAS using the safe upper threshold earlier identified in flow-through (FT) systems (Stiller et al., 2020). The multi-platform approach to study the effects of ozone allowed a thorough documentation of the consequences of its application, an attempt that will advance post-smolt production in brackish water RAS.

2. Materials and methods

2.1. Ethical use of animals in research

The study adhered to the guidelines and protocols of European Union Directive 2010/63/EU and was approved by the Norwegian Food Safety Authority under FOTS ID 21630.

2.2. Experimental set-up

The experiment was conducted at the Nofima Centre for Recirculation Aquaculture (NCRA) in Sunndalsøra, Norway. The fish (Bolaks strain) used in the trial were produced at the research station and were reared in a freshwater FT system before their use in the experiment. Smoltification was artificially induced (Handeland and Stefansson, 2001), and a seawater test (i.e. 34 ppt for 72 hrs), including measurement of plasma chloride level, was conducted to verify the smoltification status of the fish for the trial. Seawater tolerance was achieved when the fish were able to regulate plasma chloride levels to less than 150 mmol L^{-1} during the test.

The full technical specifications of the recirculation aquaculture facility at NCRA are described in an earlier publication (Terjesen et al., 2013). Briefly, the water treatment of the semi-commercial RAS was composed of a microscreen belt filter, a moving bed bioreactor, a degasser column, and two holding sump units. Prior to the return to fish tanks, water was oxygenated and oxygen saturation in tanks was kept above 85% during the experiment. The total RAS water volume was 38 m^3, the water exchange rate was approximately 20% of the water system (volume/day), and system hydraulic retention time was about five days. Two identical RAS units with a total volume of 38 m^3 were employed in the trial. Before the transfer of fish to the recirculation units, the water from the two systems was mixed by crossing over water from one system to another. This was done to ensure identical water quality conditions at the start of the experiment. RAS 1 was dedicated to the ozone group while RAS 2 was the control. Each RAS unit included three octagonal tanks with a volume of 3.3 m^3, a diameter of 2 m, and a water depth of approximately 0.85 m. Each tank was stocked with 250 smolts with an average starting weight of 98.1 ± 0.4 g (mean ± standard deviation, SD). The smolts were allowed to acclimatise for three weeks before the ozonation commenced. During this acclimation period and in the whole duration of the ozonation trial, fish were fed in excess by 3 mm Skretting Nutra Olympic pellets (Skretting, Stavanger, Norway) and the following water parameters were maintained: dissolved oxygen = >90% saturation, pH = 7.4–7.5, temperature = 12.5 ± 0.2 °C, salinity = 12 ppt, photoperiod = continuous lighting.

After the acclimation period, ozone, generated from an OZAT Ozone Generator, CFS-14 2 G (Ozonia Degrémont Technologies Ltd., Zurich, Switzerland) with a set point of 530–535 mV, was injected into the water before it entered the Salsnes filter of RAS 1. Expressing the ozone exposure level as oxidation–reduction potential (ORP, in millivolts, mV) allowed us to corroborate the recommended upper safe threshold of ozone for brackish water-adapted salmon that was identified earlier by our group (Stiller et al., 2020). The system was continuously ozonated, achieving an average tank ORP value of 334±22 mV throughout the trial. An ORP probe (OxyGuard International A/S ORP probes with Redox Manta controllers, Farum, Denmark) was placed after the microscreen of the belt filter of each experimental RAS unit and continuously logged the ORP value every 5 mins. To ensure that fish were exposed to the target concentration, a handheld ORP metre (Multi 3620 IDS & SenTix ORP-T 900, WTW, Weilheim, Germany) was used to check the ORP level in the experimental tanks several times each day. For the control group, the mean ORP value in the tank was 282 ± 13 mV. The experiment ran for 45 days. In addition to the water quality parameters mentioned above, the levels of ammonia (NH_4—H; average during the trial: control = 0.06±0.03 mg/L; ozone = 0.24±0.14 mg/L) and nitrate (NO_3—N: control = 0.02±0.01 mg/L; ozone = 0.06±0.04 mg/L) were measured 3 times a week.

We acknowledge the technical limitations of the present study with the use of 1 RAS-3 tanks set-up. Since our focus was on fish, the fact that we had only one system with ozone allowed us to have similar conditions within all 3 tanks, something that might be difficult to achieve with 1 tank 1 RAS solution. Therefore, the present study discussed the consequences from a fish perspective and not on the system effects of continuous ozonation.

2.3. Sampling

There were three extensive tissue samplings: prior to ozone application (day 0; time point 0; T0), mid-point (day 20, T20), and termination (day 45; T45). Feeding was restricted 24 h prior to sample collection. Ten fish were netted out from each tank and were euthanised with an overdose of Finquel (0.5 mg/L, MSD Animal Health, Netherlands). The length and weight of each fish were measured, and the evaluation of 14 operational welfare indicators was executed by blind evaluation using the FISHWELL scoring handbook (Noble et al., 2018) (Supplementary File 1). This scoring strategy followed a 0-to-3-point system where 0 means in excellent condition while 3 indicates severely compromised state. Blood was collected from the caudal artery using a heparinised vacutainer (BD Vacutainer™, NJ, USA), centrifuged for 10 min at 5200 rpm, and plasma was collected and stored at −80 °C until analysis. Skin samples (ca 2 cm x 1 cm) below the dorsal fin and second gill arch were collected and divided into two portions. The first portion, which was intended for gene expression analysis, was suspended in RNAlater™ (Ambion, USA), kept at room temperature overnight to allow penetration, and subsequently stored at −80 °C until RNA extraction. The remaining portion was stored in 10% neutral buffered formalin (BiopSafe®, Denmark) for histological processing.

2.4. Stress test

Twenty-four hours after the last sampling of the trial, a subset of the remaining fish was subjected to a stress test composed of netting, air exposure, and confinement. Before the stress test was performed, 10 fish from each tank were netted out and euthanised with an anaesthetic overdose, and then plasma was collected as described above (section 2.3). This fish group served as the pre-stress fish (T0). The stress test protocol was as follows: 20 fish per tank ($n = 60$, per treatment group) were netted, exposed to air for 15 s then confined in a bucket for 15 mins, achieving a density of around 250 kg m^3. During the confinement, oxygen was continuously logged, and aeration was provided to ensure that DO level was above 85% saturation. Thereafter, the fish were transferred to a recovery tank where they were allowed to recover for 6 hr. Plasma samples were collected from five fish at 1, 2, and 6 h after stress, following the protocol described above (section 2.3).

2.5. RNA isolation and microarray analysis

Total RNA was isolated from the skin and gill tissues of fish collected at T0 and T45 and their quality was assessed as described earlier (Lazado et al., 2020). Microarray analysis (Supplementary File 2) was performed using a custom-designed Atlantic salmon DNA oligonucleotide microarray SIQ-6 (Agilent Array, ICSASG_v2) and the pro-processing of the data was carried out in Nofima's bioinformatics package STARS (Salmon and Trout Annotated Reference Sequences) (Krasnov et al., 2011; Lazado et al., 2020).

2.6. Histology

Tissue samples for histology were processed, stained with Periodic Acid Schiff-Alcian Blue (AB-PAS), and digitised using a slide scanner (Aperiod CS2, USA). Histological scoring of the gills followed a previously published protocol (Lazado et al., 2020; Stiller et al., 2020). Two hundred forty lamellae taken from six different locations (40 lamellae per location) of the gill section were investigated per fish. Three randomly selected regions of the skin, which were ca 500 μm wide per area, were used to score the skin health status (i.e. general appearance and surface quality) of fish using a semi-quantitative 4-point (i.e. 0 is the best while 3 is the worst) scale system (Stiller et al., 2020) (details are provided in Supplementary File 2).

2.7. Plasma biochemistry

Plasma samples collected during the three sampling events were analysed for cortisol (Demeditic Diagnostics GmbH, Kiel, Germany), lactate (Pentra C400, HORIBA ABX SAS, Montpellier, France) glucose (Pentra C400), alkaline phosphatase (Pentra C400), lactate dehydrogenase (Pentra C400), creatinine (Pentra C400), and total antioxidant capacity (Sigma-Aldrich, Missouri, USA). On the other hand, plasma samples collected during the stress test were analysed for cortisol, lactate, and glucose. All determinations were done in duplicates.

2.8. Plasma metabolomics

The detailed protocol for the preparation and processing of samples for metabolomics is provided in Supplementary File 2. Briefly, plasma filtrate was subjected to UPLC using a slightly modified version of an earlier protocol (Doneanu et al., 2011). Peak areas were extracted using Compound Discoverer 2.0 (Thermo Scientific) and the compounds were identified at four levels of accuracy.

2.9. Data handling

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 (Systat Software Inc., London, UK) .The external welfare scores, plasma parameters, and histological scores were subjected to a two-way ANOVA followed by multiple pairwise comparisons by Holm-Sidak test. A t-test was used to identify the difference in average weight between groups at termination. All tests for statistical significance were set at $P < 0.05$. Normalised expression data were compared between groups using linear modelling in the Bioconductor package limna. Differential gene expression (ozone versus control) significance cut-off was adjusted p-value < 0.01. Analyses for Gene Ontology and Reactome pathway enrichment were also performed ($P < 0.05$). For the metabolomics data, the Benjamini-Hochberg correction was employed, with an acceptable false-positive rate set at 0.1. A compound was considered significantly affected by the treatment when P-value from t-test was less than the Benjamini-Hochberg critical value. A detailed description of the statistical tests and pipeline for the analyses of microarray and metabolomics data are given in Supplementary File 2.

3. Results

3.1. Mortality, external welfare, and production performance

The control and ozone-treated groups showed comparable survival rates (i.e. ozone group = 99.4%; control group = 100%) at termination. Daily visual inspection during the trial disclosed no apparent differences on social and feeding behaviour between the two treatment groups. Likewise, there was no significant difference (i.e. ozone: 272±7.2 g vs control: 280±4.5 g; $P = 0.820$) in the final average weight between the two treatment groups.

The study focused on five of the most frequently occurring damages (i.e. skin damages and damages to the dorsal, caudal, pectoral, and pelvic fins) (Fig. 1, Supplementary File 1). The majority of the skin damages on both sides of the fish, regardless of the treatment, were accounted for by scale loss (> 60 % of the cases per timepoint) (Fig. 1A). There was a significant temporal difference observed in the skin damages on the right sides of the fish ($P = 0.013$), especially in the ozone group ($P = 0.020$) (Fig. 1a); such trend was not observed on the left side of the fish (treatments, $P = 0.927$; time, $P = 0.185$; treatmentXtime $P = 0.626$). The average scores were between 1.0 and 2.0. More than 60% of the accounted damages in the four fin groups were active damages with average scores of 1.5 to 2.0 (Supplementary File 1). Except in the dorsal fin, active damages were relatively higher in the ozone than the control group especially at day 45, though at least 60 % of these damages were

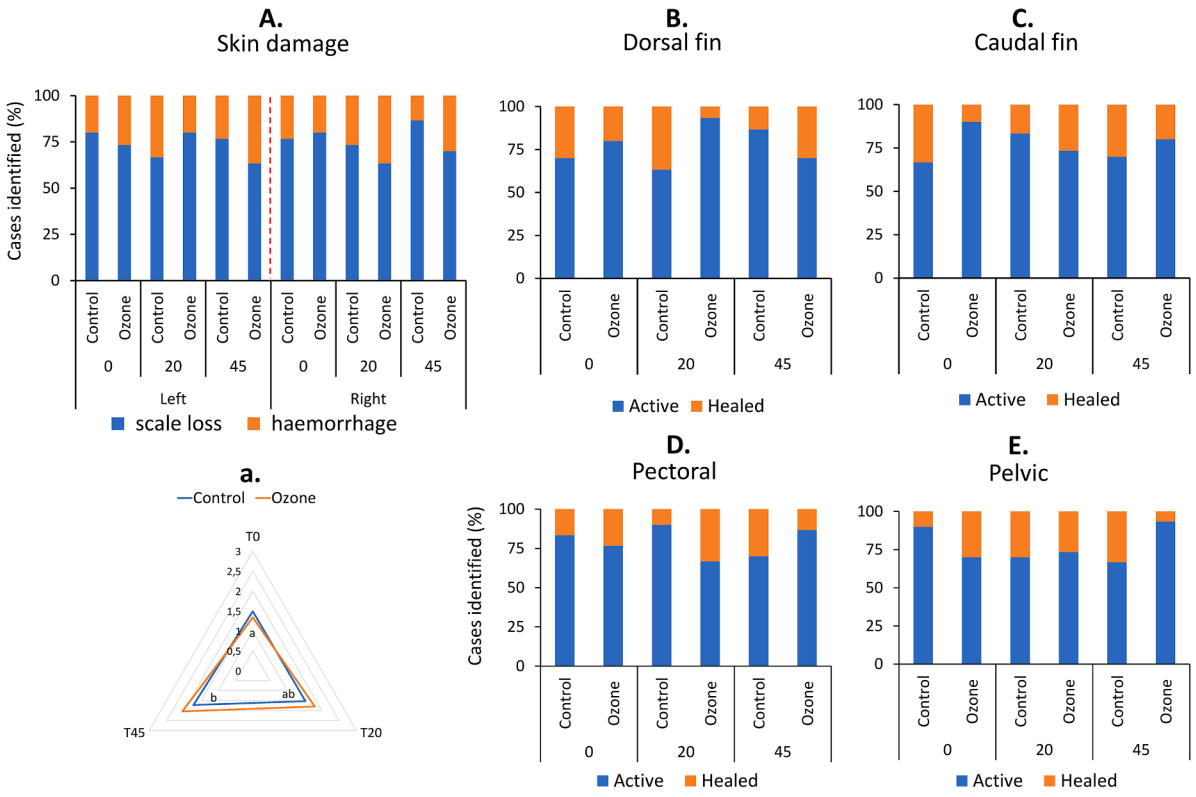


Fig. 1. Representative external welfare indicators of Atlantic salmon. A-E) Damages to skin and fins were frequently occurring damages in all sampled fish. a) Radial chart showing the mean scores of the skin damage on the right side of the fish. Different letters indicate significant temporal difference in the ozone group. No significant difference was identified in the control group. $N = 30$ fish per sampling point, per treatment group.

scored <2. Damages in all fin types were not significantly different between treatments (dorsal, $P = 0.992$; caudal, $P = 0.586$; pectoral, $P = 0.497$; pelvic, $P = 0.623$) and over time (dorsal, $P = 0.733$; caudal, $P = 0.979$; pectoral, $P = 0.071$; pelvic $P = 0.996$) (Fig. 1B-E).

3.2. Plasma biochemical indicators

There were no significant inter-treatment differences identified in plasma cortisol (treatments, $P = 0.846$; time, $P = 0.976$; treatmentXtime $P = 0.832$), lactate (treatments, $P = 0.612$; time, $P = 0.085$; treatmentXtime $P = 0.668$), and glucose (treatments, $P = 0.677$; time, $P = 0.058$; treatmentXtime $P = 0.694$) (Table 1). Alkaline phosphatase (marker for hepatobiliary injury) appeared to increase over time for both treatment groups ($P = 0.027$), nonetheless, no significant inter-treatment differences were identified in all timepoints ($P = 0.358$). Lactate dehydrogenase (for tissue damage) did not significantly change (treatments, $P = 0.138$; time, $P = 0.063$; treatmentXtime $P = 0.128$). Creatinine (for renal function) significantly increased through time in both groups ($P = <0.001$), and the highest level for both groups was identified at termination. Moreover, creatinine was significantly higher in ozone group by two-fold compared with the control group at this time point ($P = <0.001$; Table 1). Total antioxidant capacity in plasma significantly changed over the course of the trial for both treatment groups ($P=<0.001$), but no inter-treatment differences were observed (treatments, $P = 0.845$; treatmentXtime $P = 0.771$; Table 1).

Table 1
Plasma biochemical parameters of Atlantic salmon. Different letters indicate significant difference over time in a treatment group, while different numbers denote a significant difference between control and ozone-treated group in a timepoint. The values presented here are mean±SD of 12 individual fish per treatment group.

Parameter	Timepoint (day)					
	0		20		45	
	Control	Ozone	Control	Ozone	Control	Ozone
Cortisol (ng/ml)	17.7 ± 3.0	15.7 ± 5.3	20.3 ± 7.2	14.5 ± 4.4	21.3 ± 4.3	19.1 ± 5.1
Lactate (mml/L)	4.57±0.51	4.40±0.28	5.38±0.32	5.78±0.45	5.82±0.40	4.95±0.33
Glucose (mmol/L)	4.13±0.15	4.23±0.09	5.05±0.38	5.17±0.27	4.62±0.18	4.78±0.27
ALP (U/L)	163.7 ± 16.3^{a}	175.8 ± 3.5^{a}	207.0 ± 18.5^{b}	236.4 ± 26.8^{b}	230.0 ± 9.6^{b}	244.1 ± 26.8^{b}
LDH (U/L)	770.9 ± 141.6	1000.3 ± 214.9	619.4 ± 84.5	960.1 ± 233.3	632.0 ± 205.7	407.6 ± 92.0
Creatinine (μmol/L)	4.5 ± 0.4^{a1}	3.9 ± 0.2^{a1}	18.6 ± 9.39^{ab1}	7.9 ± 1.0^{a1}	36.6 ± 10.9^{b1}	75.9 ± 10.4^{b2}
TAC (nmol/μL)	13.6 ± 0.2^{a}	12.8 ± 0.4^{a}	16.2 ± 0.9^{a}	16.2 ± 0.7 ^{a}	17.7 ± 1.0^{b}	19.1 ± 0.8^{b}

ALP: Alkaline phosphatase; LDH: Lactate dehydrogenase; TAC: Total antioxidant capacity..

3.3. Skin and gill health status

Overall, the skin health status was not significantly affected by the treatments (general appearance, $P = 0.094$; surface quality, $P = 0.263$, Fig. 2A). The average scores in both groups in all timepoints did not exceed 2 in a scoring scale of 0 to 3. There was an apparent tendency that the scores for both evaluated parameters were increasing through time (which also meant worsening in the status; general appearance, $P = 0.017$; surface quality, $P<0.001$), though there were no significant treatment x time interactions (general appearance, $P = 0.076$; surface quality, $P = 0.225$, Fig. 2B, C).

Around 90% of the evaluated lamellae in both groups were characterised as healthy at T0 ($P = 0.911$, Fig. 2D,E). At T20, the number of evaluated healthy lamellae in both groups decreased compared with T0. The number of healthy lamellae in the ozone group decreased by approximately 20% while the control group registered approximately 10% reduction relative to T0 level, though not statistically significant (control, $P = 0.112$; ozone, $P = 0.296$). Cases of clubbing ($P = 0.012$) were significantly higher in the ozone compared to the control group. There was also a tendency of a slight increase in the number of lifting and hyperplasia cases, however, these changes were not statistically significant (Fig. 2F-H). At termination, the number of healthy lamellae in the control group decreased by even more: it was 24% and 16% lower compared with T0 ($P = 0.043$) and T20 ($P = 0.112$), respectively. The number of healthy lamellae in the ozone group was almost identical to the cases recorded at T0 (91.3% vs 88.8 %). In addition, all recorded pathologies at this time point, such as lifting ($P = 0.042$), clubbing ($P = 0.045$), and hyperplasia ($P = 0.075$), revealed that the number of cases was higher in the control compared to the ozone group.

3.4. Transcriptomic changes in the skin and gills

Prior to treatment, no significant differences were observed between the gene expression profiles (i.e. number of differentially expressed genes (DEGs) of ozone and control samples of each tissue type (FDR-adjusted $P < 0.01$). At T45, no DEGs were identified in the skin between control and ozone groups. In contrast, 242 ozone-induced DEGs were identified in the gills compared to the control group (Fig. 3B; Supplementary File 4). Of these DEGs, 191 displayed upregulation while 51 showed downregulation. The top 10 significantly upregulated and downregulated genes are given in Fig. 3B.

We also compared "ozone vs control" groups regardless of tissue types and timepoints. The expression of 70 genes was significantly altered (relative to the control group), and about 78 % (55) of these DEGs were upregulated while the remaining displayed downregulation. Of these 70 DEGs, 10 genes were also significantly altered in the ozone vs control (Gills T45) groups. This overlap was considered significant (hypergeometric test $P < 0.001$).

Time-related effects were of greater impact in skin compared to gills. This was true in both the control and ozone groups. Moreover, the number of DEGs identified was greater in the ozone-treated group compared to the control of the same tissue. In the skin, there were 329 DEGs in the control group, while 789 were identified in the ozone group. On the other hand, 124 DEGs were identified in the gills of the control group and 437 in the ozone group.

3.5. GO terms and reactome pathways affected by ozone

A functional enrichment analysis was performed on the DEGs identified in the association tests (section 3.4). Some of the enriched GO terms in the gills of the ozone-exposed group at T45 include

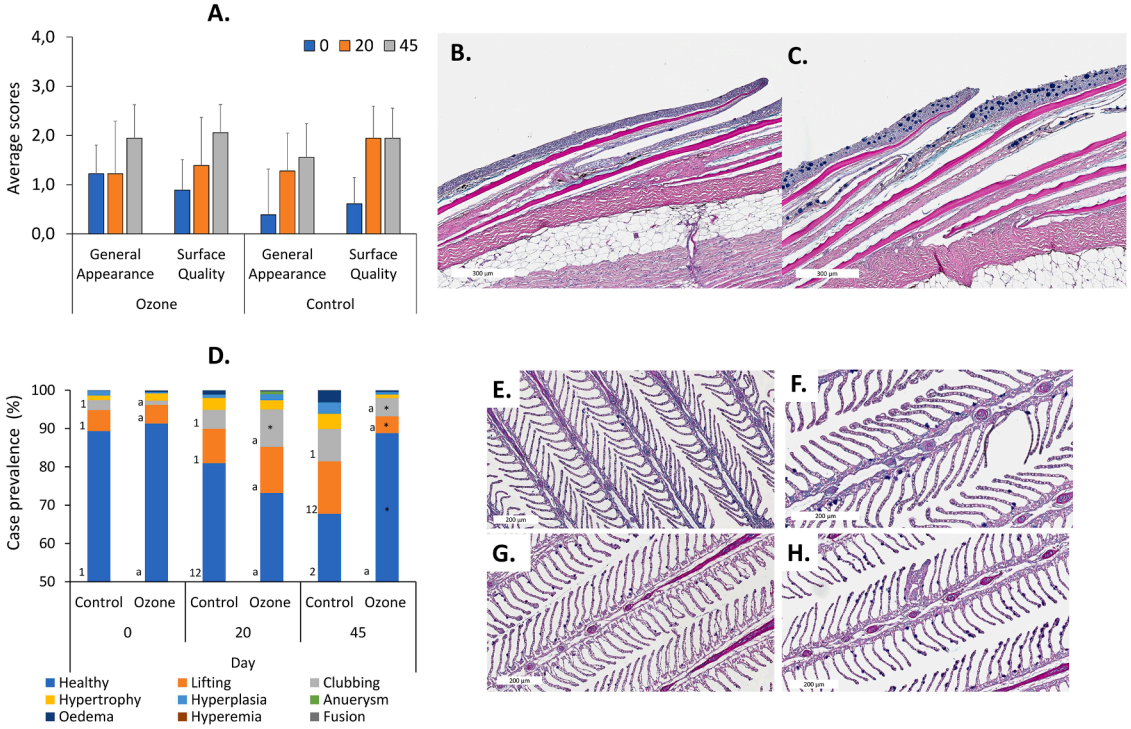


Fig. 2. Histological assessment of skin and gill health. A) Skin health was assessed using two key parameters, 1) General appearance and 2) Surface quality, using a rating scale of 0 (best) to 3 (worst) . Both evaluated parameters did not show significant differences between treatments and across timepoints. Representative photographs of B) healthy skin; defined histostructures, with a smooth surface and intact epithelium, and C) a slightly compromised skin; rough surface, the epithelium is slightly missing. Note the mucous cells (blue) in this slide. D) Gill health was assessed by counting the prevalence of key pathologies, including the healthy lamella. Significant difference through time was indicated either by different numbers (for control group) or letters (for ozone group). Significant difference between groups for a particular pathology in each timepoint was denoted by asterisk (*). Representative photographs of E) healthy gills, and some of the pathologies, F) clubbing, G) lifting and H) hyperplasia. Measurements and evaluations were carried out using tissue samples from 12 fish per treatment group.

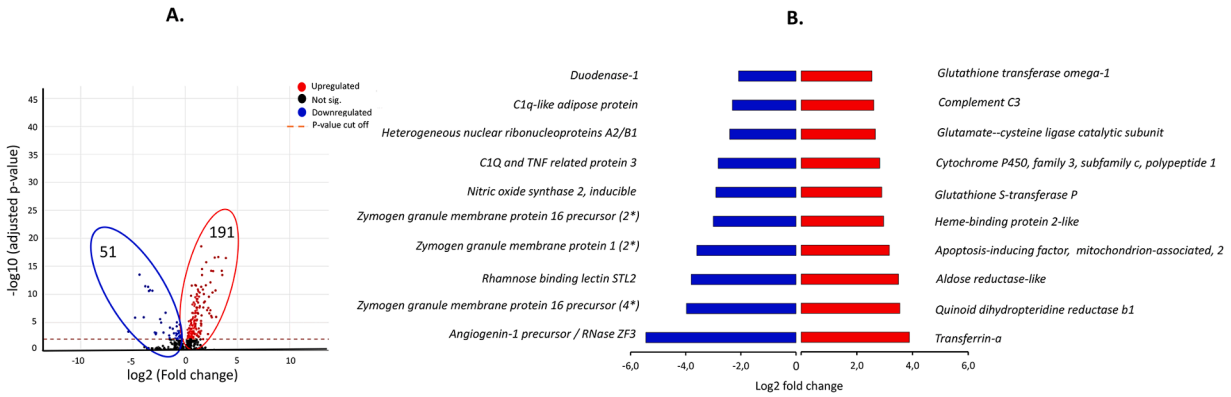


Fig. 3. Differentially expressed genes (DEGs) in the gills on day 45. A) A volcano plot showing significance (as log10 transformed p-values) against magnitude (log2 (fold change)). Significant features are represented as either red (upregulated) or blue (downregulated) dots; non-significant features (black). Numbers inside the demarcation indicate the number of DEGs. The horizontal orange line represents the applied p-value threshold for DEG. B) List of 10 representative transcripts that were either significantly upregulated or downregulated. Complete list of DEG is provided in Supplementary File 4.

oxidation–reduction process, oxidoreductase activity, glutathione biosynthetic process, monooxygenase activity, and haem binding, where the first two mentioned GO terms represent the terms with substantial upregulated features (Fig. 4A). The Reactome pathway analysis further revealed that cholesterol biosynthesis, steroid metabolism, cellular responses to external stimuli, and cellular responses to stress were some of the major pathways affected by ozone in the gills (Fig. 4B).

We then identified the GO terms and Reactome pathways affected over time in both groups. Comparing T0 and T45 in the ozone group, the DEGs in the skin and gills were both enriched in GO terms such as transferase activity and steroid metabolic process (Fig. 4A) and pathways such as cholesterol biosynthesis and metabolism of steroids

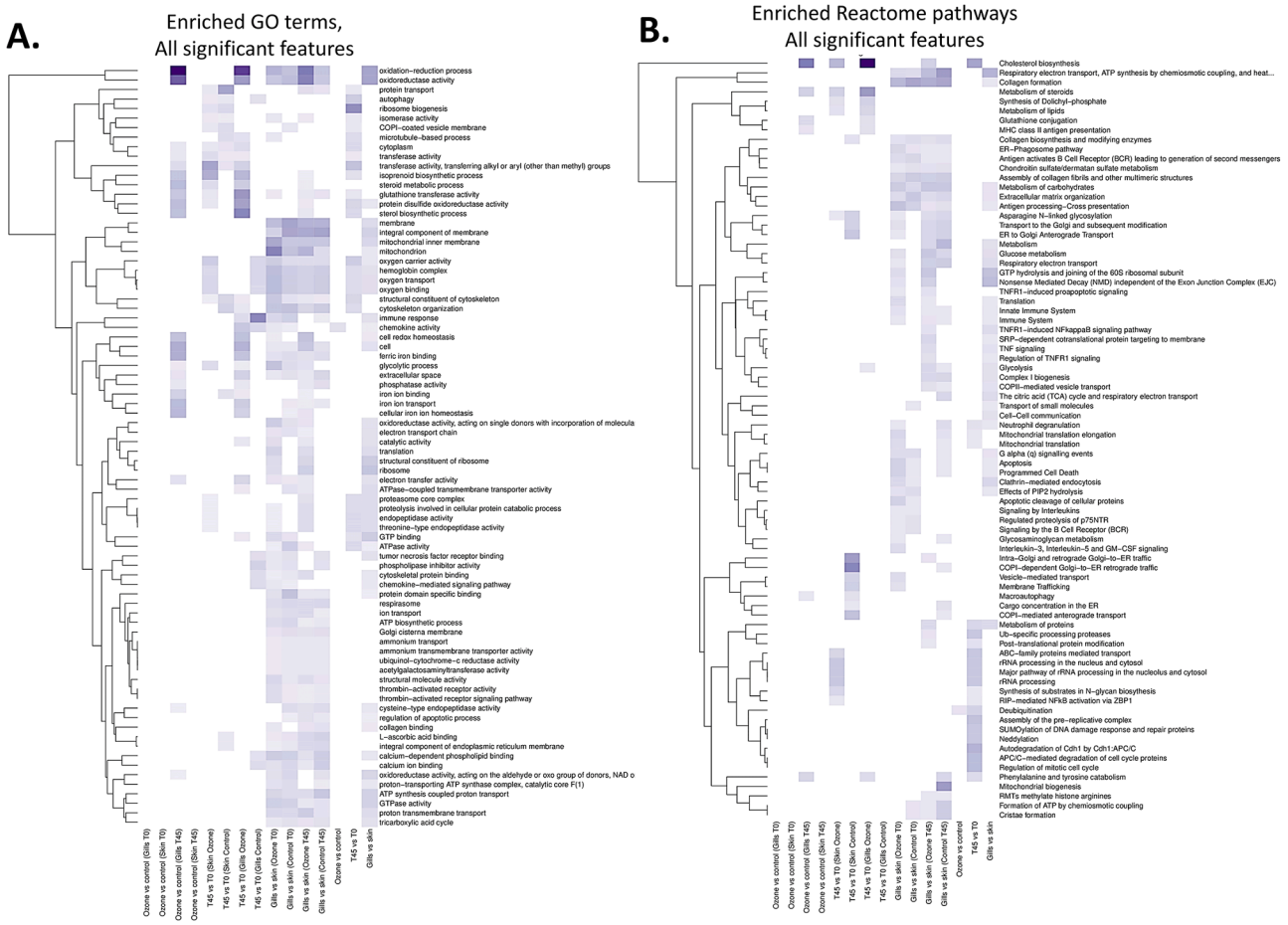


Fig. 4. Heatmaps of significantly enriched A) GO terms and B) Reactome pathways for different contrasts. When performing tests for enrichment, terms were restricted to include only those with two or more genes. Only the top 80 terms/pathways are displayed. Results are shown for the union of significant up- and downregulated features. Note that different genes of a given GO term/Reactome pathway may be both up- and downregulated within a single comparison. The union of up- and downregulated genes is assigned based on the -log10 (enrichment p-value), with lighter colours implying less significant enrichment. Hierarchical clustering was applied to terms (rows). The most significant terms were clustered according to Euclidean distance using the complete linkage method.

(Fig. 4B). For the control group, temporal effects on the DEGs in the gills identified the enrichment in GO terms such as immune response and Reactome pathways such as activation of NF-κB in B cells. For the equivalent contrast in the skin, enriched GO terms included protein transport and RNA binding, whereas Reactome pathways related to Golgi and ER transport were enriched.

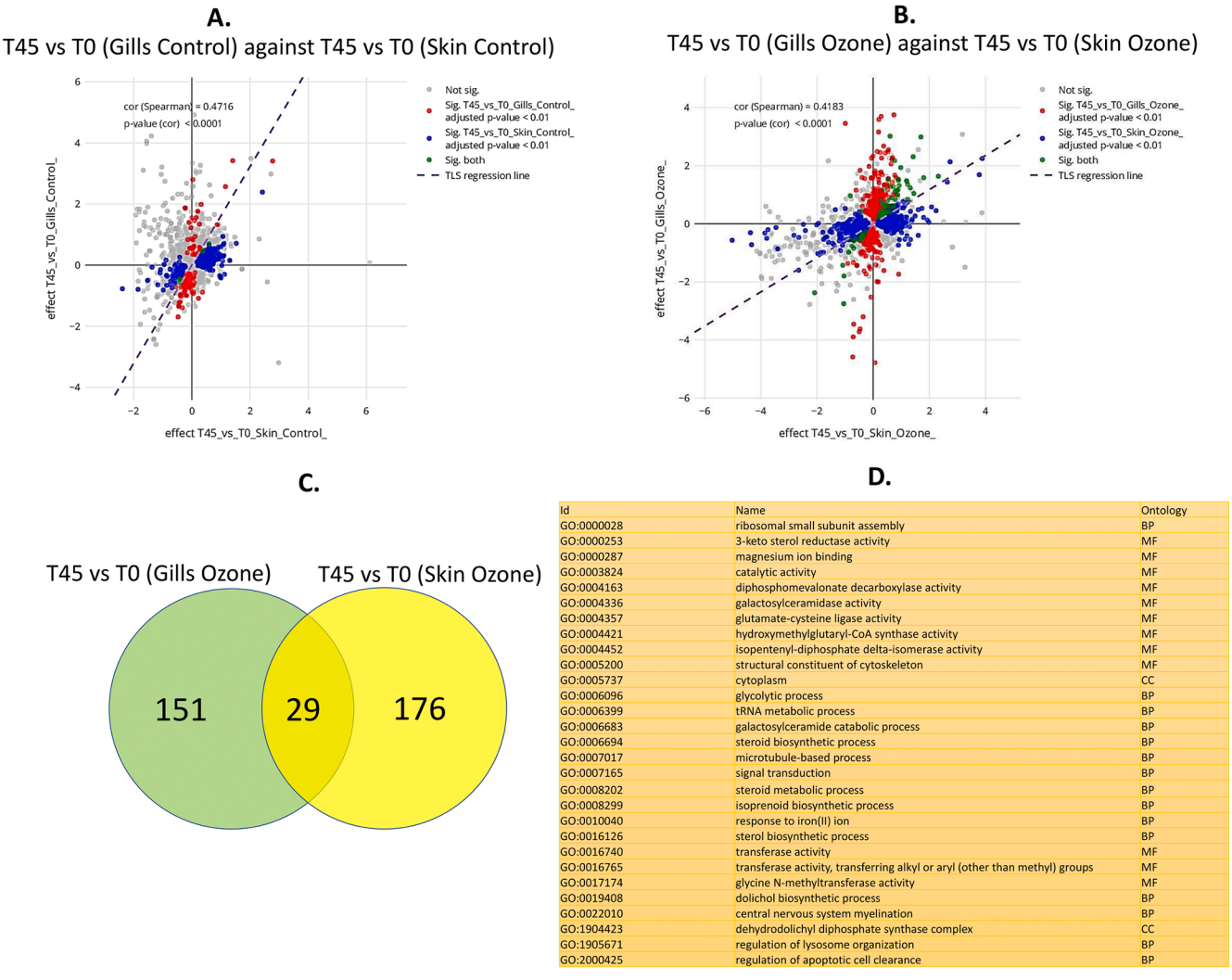
3.6. Congruence analysis – gills versus skin

A congruence analysis was performed to compare the two tissues to identify the genes and pathways commonly impacted in both salmon gills and skin. To study the impact of time, the contrasts T45 vs T0 control and T45 vs T0 ozone for skin and gills were compared. In both comparisons, there was a significant correlation between the fold change in gene expression of skin and gills when considering all genes available for the analysis (Fig. 5A,B).

When considering only significantly DEGs, ozonation for 45 days altered the expression of 74 genes in both tissues. This overlap was considered statistically significant (hypergeometric test $P<0.001$). The overlap was further assessed for GO terms between T45 vs T0 (gills ozone) (180 GO terms) and T45 vs T0 (skin ozone) (205 GO terms). About 29 GO terms overlapped between the two sets when not considering direction of change and was significant ($P<0.001$) (Fig. 5C). These GO terms include catalytic activity, signal transduction, and transferase activity commonly enriched the DEGs in both tissues upon 45-day ozonation (Fig. 5D).

3.7. Plasma metabolomes

A total of 563 compounds were detected in the samples, of which 80 were annotated on level 3, 35 on level 2b, 45 on level 2a, and 24 on level 1. The PCA plot revealed an apparent distinction between the plasma metabolomes of control and ozone groups at T0 and at T45 (Fig. 6A). There were only two metabolites significantly different between ozone and the control groups at T45 – serine and arginine, where the levels were significantly higher in the ozone group (Fig. 6B). Comparing between T0 and T45, the metabolomic consequences were more pronounced in the control than in the ozone group. There were 72 and 92 significantly affected metabolites in the ozone and control groups, respectively. From these, 21 metabolites were exclusively identified in the ozone group while 41 were detected in the control. Some of the level 1 metabolites that were significantly and exclusively affected in the



Id	Name	Ontology
GO:0000028	ribosomal small subunit assembly	BP
GO:0000253	3-keto sterol reductase activity	MF
GO:0000287	magnesium ion binding	MF
GO:0003824	catalytic activity	MF
GO:0004163	diphosphomevalonate decarboxylase activity	MF
GO:0004336	galactosylceramidase activity	MF
GO:0004357	glutamate-cysteine ligase activity	MF
GO:0004421	hydroxymethylglutaryl-CoA synthase activity	MF
GO:0004452	isopentenyl-diphosphate delta-isomerase activity	MF
GO:0005200	structural constituent of cytoskeleton	MF
GO:0005737	cytoplasm	CC
GO:0006096	glycolytic process	BP
GO:0006399	tRNA metabolic process	BP
GO:0006683	galactosylceramide catabolic process	BP
GO:0006694	steroid biosynthetic process	BP
GO:0007017	microtubule-based process	BP
GO:0007165	signal transduction	BP
GO:0008202	steroid metabolic process	BP
GO:0008299	isoprenoid biosynthetic process	BP
GO:0010040	response to iron(II) ion	BP
GO:0016126	sterol biosynthetic process	BP
GO:0016740	transferase activity	MF
GO:0016765	transferase activity, transferring alkyl or aryl (other than methyl) groups	MF
GO:0017174	glycine N-methyltransferase activity	MF
GO:0019408	dolichol biosynthetic process	BP
GO:0022010	central nervous system myelination	BP
GO:1904423	dehydrodolichyl diphosphate synthase complex	CC
GO:1905671	regulation of lysosome organization	BP
GO:2000425	regulation of apoptotic cell clearance	BP

Fig. 5. Set interactions of significant features in the skin and gills of treatment groups. Scatter plot comparing significant features in A) T45 vs T0 (Gills Control) against T45 vs T0 (Skin Control) and B) T45 vs T0 (Gills Ozone) against T45 vs T0 (Skin Ozone). Features are represented by points. The colour of the point indicates which set the feature is assigned to. For each feature, the log2 (fold change) in the T45 vs T0 (Gills Control)/ T45 vs T0 (Gills Ozone) contrast (y-axis) and the log2 (fold change) in the T45 vs T0 (Skin Control)/ T45 vs T0 (Skin Ozone) contrast (x-axis) are shown. C) Venn diagrams of significant GO terms in T45 vs T0 (Gills Ozone, green) against T45 vs T0 (Skin Ozone, yellow). D) List of the overlapping 29 GO terms.

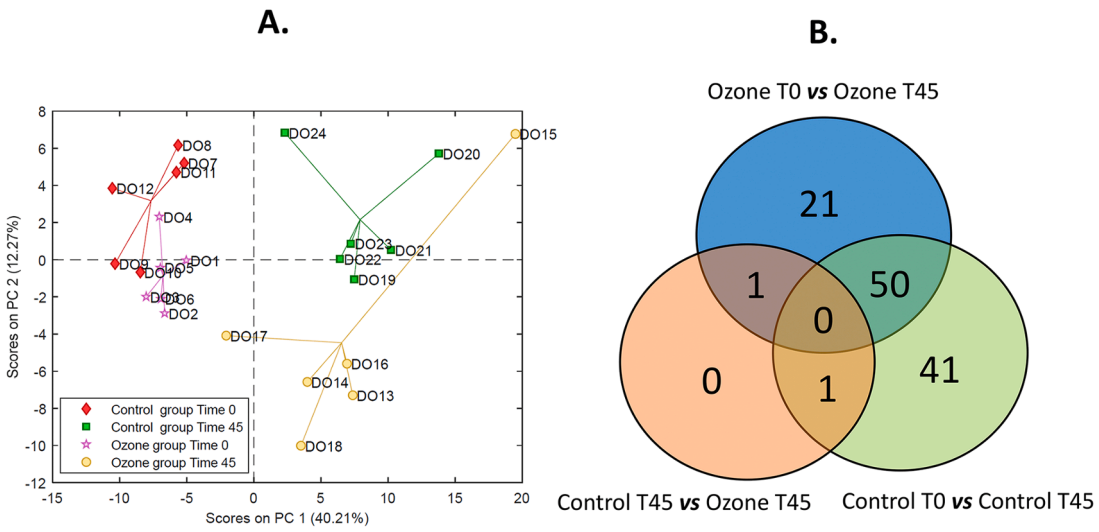


Fig. 6. Plasma metabolome of Atlantic salmon. A) Score plot from PCA model calculated on the relative concentrations of the annotated variables in the reduced dataset. Data have been auto scaled. The plot demonstrates three clear groupings: T0 samples on the left of the plot, T45 samples on the right, and a distinction between ozone and control groups. B) Multiple univariate analyses (i.e. for 184 metabolites) were performed to identify how different factors (i.e. time, treatment) induced metabolite-specific response (Supplementary File 5). Venn diagram for differentially abundant metabolites in plasma from different comparisons.

ozone group include palmitoylcarnintine (C16), histidine, and glutamic acid. Fifty metabolites were common between the two time-treatment contrasts, and from these, five were level 1 metabolites, namely, inosine, docosahexaenoic acid, methionine sulfoxide, citrulline, and pentose.

3.8. Responses to handling-confinement stress

Fish from both groups responded to the stressor as indicated by the changes in the plasma cortisol and lactate levels post-stress (Fig. 7). Plasma cortisol increased by at least fourfold 1 hr after stress in both groups, and the level was still significantly elevated even after 6 h relative to the pre-stress level (Fig. 7A). The cortisol level was significantly higher by 30% in the ozone group compared to the control 1 hr post-stress ($P = 0.026$). Such a significant inter-treatment difference was not identified thereafter. Though not statistically significant, a similar trend was identified in the lactate level post-stress (Fig. 7C). Plasma glucose level remained unchanged in both groups following exposure to a stressor (Fig. 7B).

4. Discussion

One of the fundamental principles in rearing fish under the captive environment, such as RAS, is the requirement of optimal conditions for growth, health, and welfare. Using molecular, histological, biochemical, and visual gross indicators, the study revealed the ability of salmon to

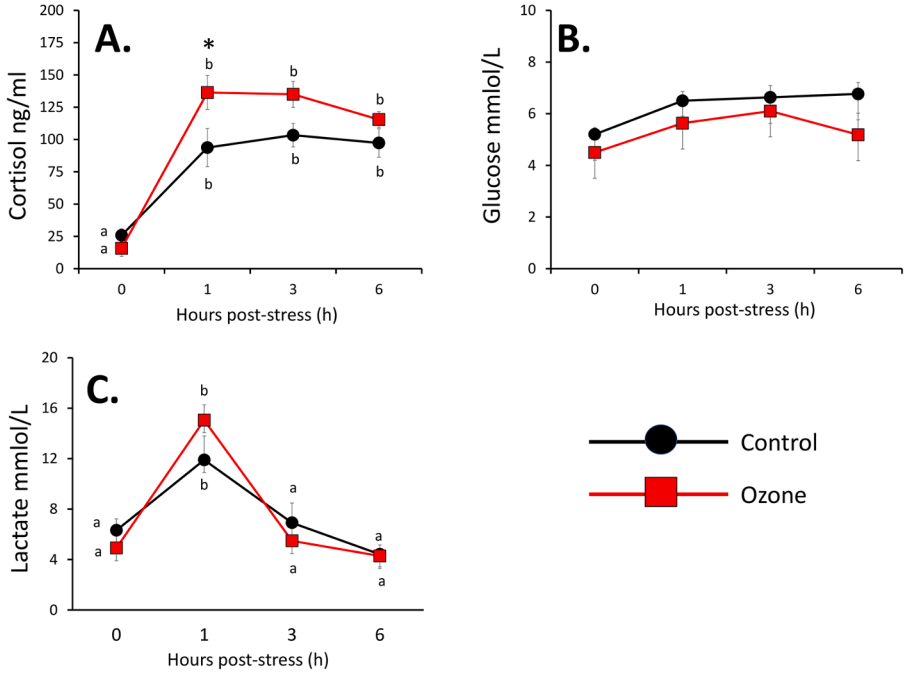


Fig. 7. Plasma stress parameters (A: cortisol; B: glucose; C: lactate) of Atlantic salmon. Values are presented as mean ± SD of 12 individual fish per treatment group. Different letters indicate significant difference within a treatment group. Asterisk (*) denotes a significant difference between treatment groups at that timepoint.

adapt to an environment with continuous ozonation, and these adaptative responses provide strong evidence that the previously proposed ozone threshold (<350 mV) (Stiller et al., 2020) carried minimal health and welfare concerns and did not impair production performance.

Overall, the documented gross pathologies were subclinical (i.e. score mostly below 2 in a scale of 0-to-3) for the 14 welfare indicators, where damages to the skin and fins were the most prevalent. Loose scale is common in salmon during smoltification (Handeland et al., 2013) and transfer to RAS (Lazado et al., 2020; Timmerhaus et al., 2021). The lack of pattern and differences between treatments and over time indicate that the production stage (i.e. early phase of post-smoltification and transfer to RAS) was likely the major contributory factor for the observed prevalent scale loss. Fin condition is often employed as an indicator of fish welfare based on rearing conditions (Ellis et al., 2008). The type of fin damage for both groups was predominantly active, meaning that the alteration was new or ongoing. Increased stocking densities and high levels of suspended solids have been found to be predisposing factors for fin damages since they can trigger behavioural changes such as aggression and increased foraging (Ellis et al., 2008; Timmerhaus et al., 2021; Wedemeyer, 1996). The apparent difference in water turbidity (Fig. 8) could lead to higher damage scores in the control group, though a higher sample size is required to make a robust conclusion.

Plasma biochemical indicators remained unchanged, implying that continuous ozonation did not trigger internal organ damage (i.e. indicated by LDH, ALP) and metabolic imbalance (i.e. indicated by cortisol, lactate, and glucose); the latter is substantiated by the plasma metabolomics data (Fig. 6). For a healthy adult salmon, the plasma creatinine level is 26–46 μmol (Sandnes et al., 2006). The increase in creatinine level in the ozone group which was far beyond the basal threshold suggests that the treatment interfered with renal function. To our knowledge, this is the first paper to report the effects of ozone on fish renal function. Unfortunately, we did not collect kidney samples for histology. Therefore, it is challenging to provide a conclusive remark on the potential damaging impact of ozone to the kidneys of salmon; though based on the data and the increasing trend with time this cannot be excluded and requires further studies to confirm the extent of the impact and on whether salmon can eventually adapt and recover.

Earlier studies have reported that ozonation could impact the skin and gills at varying degrees, though often the severity was dependant on concentration (Good et al., 2011; Reiser et al., 2010; Stiller et al., 2020). This study supported the earlier observations that salmon gills are more sensitive to ozone than the skin (Figs. 3-5, (Stiller et al., 2020)). At T45, the ozone group exhibited a higher number of healthy filaments and lower cases of key pathologies such as lifting, clubbing, hypertrophy, and hyperplasia. Though these pathologies are generally considered unspecific tissue responses to numerous infectious and non-infectious stimuli (Good et al., 2011; Sutherland and Meyer, 2007), their increased prevalence in the control group collectively indicates that continuous ozonation may likely provide an environment that promotes relatively superior gill health. Suspended solids affect water turbidity, which influences the gill health status (Au et al., 2004). Low-dose ozonation of the RAS water lowers suspended solids (Davidson et al., 2011) and turbidity (Gonçalves and Gagnon, 2011) levels, where particle coagulation is an important mechanism for this effect (Rebhun and Lurie, 1993). The difference in the turbidity between the treatment groups likely contributed to the significant observations of the branchial histological status. Poor gill health is often reported in salmon reared in RAS (Figenschou and Hillestad, 2019). The use of ozone may offer a potential strategy to improve the gill health of salmon in this kind of production system, which should be explored further in the future.

To our knowledge, this is the first study in salmon to demonstrate a systemwide molecular response to continuous ozonation; in particular, it offers insights into the molecular processes involved in the physiological adaptations at the mucosa. The gene expression profile of the two mucosal tissues was very distinct, indicating the tissue specificity of ozone-induced changes. The mucosal transcriptomes at termination lent support to histology that the gills were significantly more responsive to ozone than was the skin. It was documented in juvenile turbot that transcriptional changes related to ozone were greater in the gills compared with other tissues studied (Reiser et al., 2011). This presents strong evidence that ozone primarily targets the gills, and they are, thus, a good organ to study how fish respond to ozone. The profound susceptibility of the gills to ozone and suspended solids can be likely attributed to the amount of surface area exposed to water (Koppang et al., 2015), less complex structures than the skin (Lazado et al., 2020), and its plasticity to environmental changes (Sollid and Nilsson, 2006). It was identified that time-related effects were of greater impact in the skin compared with the gills, regardless of the treatment. It would be interesting to explore in the future whether growth plays a role in this striking profile. This temporal feature further signifies the difference between the skin and gills in their adaptation to the RAS environment.

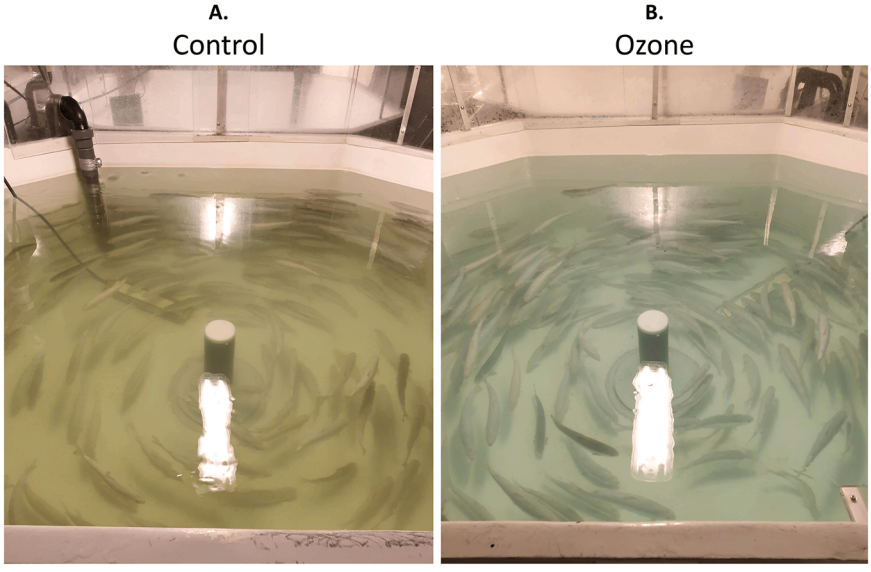


Fig. 8. Apparent impact on water turbidity following continuous ozonation. Photos were taken at termination. Photo A) shows a representative tank from the control group, while B) reveals the colour difference in the group that received ozone.

Although the two tissue types had distinct gene expression profiles as highlighted by enrichment analysis by both the GO terms and Reactome pathways, the expression of 74 features was altered significantly in both tissues over the course of 45 days in the ozone group. This suggests that the mechanisms involved in mucosal responses to ozone shared a common regulatory pattern, which could be explored further to gain more insights into oxidant-mucosa interplay in fish. Transcriptomics revealed that oxidative stress may have been triggered at the mucosa, especially in the gills. Ozone, as an oxidant, is a potential trigger of oxidative stress. Several studies have shown that the expression of antioxidant genes was affected by ozone treatment, which was pinpointed to the countermeasures the mucosa employed in response to ozone challenge (Reiser et al., 2011; Stiller et al., 2020). The enrichment of genes involved in the process (i.e. the gluthathiones) demonstrates that oxidative stress was locally triggered and that the gills mobilised countermeasures to protect the mucosa.

The metabolic cost of ozone through the analysis of circulating metabolomes have been reported in higher animal models (Shore, 2019), though it remains unexplored in fish. Continuous ozonation did not result in a large-scale metabolomic dysregulation. In fact, the impact was minimal since only two metabolites, i.e. serine and arginine, were found to be significantly altered in the ozone-exposed group at T45. Serine and arginine are amino acids, and their changes indicate that ozone likely interfered with amino acid metabolism. This interaction has been linked earlier in non-fish models (Sharma and Graham, 2010), though the extent of interference in the present study could not be strongly established. Ozone may result in DNA breaks; hence, the increase in these amino acids, especially arginine, may likely participate in DNA repair since such a protective mechanism has been reported earlier (Cui et al., 2011). Metabolomic profiling demonstrated that there was a strong time-related effect, and such a tendency was more pronounced in control than in the ozone-exposed group. This temporal feature can be attributed to the normal ageing process of an organism that the circulating metabolome profile has been implicated to be dependant upon (Yu et al., 2012).

Key plasma stress parameters did not significantly change, indicating that the concentration applied did not trigger chronic stress to salmon post-smolts, thus creating minimal welfare risk. Steroid metabolism, an important process in mounting a response during a stressful episode was significantly affected by ozone in both skin and gills as shown by the interference in the expression of genes within GO term steroid metabolic process and Reactome pathway metabolism of steroids. This difference indicates that ozone treatment might have triggered mucosal stress responses but induced minimal changes at the systemic level, though the whole mechanism of such interaction remains an open question.

Ozonation did not alter the ability of post-smolts to respond to secondary stressors, which were husbandry manipulations common in normal production practice. The cortisol and lactate levels appeared to be higher in the ozone-exposed group compared with the control 1 hr after stress. The implication of this tendency requires further corroboration. Nonetheless, the ability to return to a comparable level, as with the control, indicates that such effect on the kinetics of stress response was merely transient and bears little pervasive impact. This further substantiates the propositions that the ozone level tested here does not cause a welfare concern.

In summary, we have identified that ozonation 1) did not impact production performance of salmon post-smolts negatively, 2) did not significantly alter the external morphology of fish, 3) did not induce changes in key plasma biochemical markers of internal organ health, 4) had a far greater impact to the gills than the skin as shown by histology and transcriptomics, 5) did not result in metabolomic disarray, and 6) did not affect the ability of fish to respond to a secondary stressor. There was strong temporal response profile documented in the present study and this may be explained by: 1) some of the parameters (i.e. gene expression, metabolome) that are highly influenced by growth; and 2) fish came from a flow-through system before they were transferred to RAS. We have unpublished evidence that this transition and the time fish spent in RAS influenced some physiological parameters, including those reported in this study, which was indicative of adaptation to a new environment. Future research must be directed at understanding the system effects of continuous ozonation by employing a 1 RAS – 1 tank system, and the quantification of the different disinfection by-products of ozone and their individual and compound effects on salmon health and welfare.

CRediT authorship contribution statement

Carlo C. Lazado: Conceptualization, Supervision, Formal analysis, Data curation, Writing – original draft, Writing – review & editing. **Kevin T. Stiller:** Supervision, Writing – review & editing. **Britt-Kristin Megård Reiten:** Supervision, Writing – review & editing. **João Osório:** Formal analysis, Writing – review & editing. **Jelena Kolarevic:** Conceptualization, Supervision, Writing – review & editing. **Lill-Heidi Johansen:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare no competing interests.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2021.105935.

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