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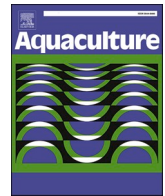
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# Differential sensitivity of mucosal organs to transient exposure to hydrogen sulphide in post-smolt Atlantic salmon (*Salmo salar*)

Hanna Ross D. Alipio<sup>a,b</sup>, Julie Hansen Bergstedt<sup>c</sup>, Carlo C. Lazado<sup>b,\*</sup>

<sup>a</sup> Aquaculture and Fisheries Group, Department of Animal Sciences, Wageningen University & Research, PO Box 338, 6700 AH Wageningen, The Netherlands

<sup>b</sup> Nofima, The Norwegian Institute of Food, Fisheries and Aquaculture Research, 1433 Ås, Norway

<sup>c</sup> Technical University of Denmark, DTU Aqua, Section for Aquaculture, The North Sea Research Centre, PO Box 101, 9850 Hirtshals, Denmark

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## ABSTRACT

Mortality related to hydrogen sulphide (H<sub>2</sub>S) has recently become a serious concern in Atlantic salmon (*Salmo salar*) farming, particularly in saline recirculating aquaculture systems (RASs), where the risk of H<sub>2</sub>S formation is high. H<sub>2</sub>S has a distinct odour of rotten eggs, and its production is associated with the anaerobic bacterial decomposition of protein and other sulphur-containing organic matter. Significant advances have been made in elucidating its formation in RAS, but the biological consequences of this toxicant in salmon remain elusive. We report the physiological consequences of transient exposure of post-smolt Atlantic salmon to H<sub>2</sub>S. The fish were exposed to one of three levels of H<sub>2</sub>S for 1 h: 0 µM (unexposed), 0.6 µM (low exposure), and 1.2 µM (high exposure). Fish were allowed to recover for 24 h and then sampled for gene expression, histology, and metabolomics analyses. Molecular profiling was performed on a subset of genes with known functions in sulphide detoxification, mucins, immunity, and stress responses, which focused on the gills, olfactory organ, skin, and distal gut. With the exception of *interleukin 10*, all genes studied were significantly affected in the skin, where high H<sub>2</sub>S triggered significant upregulation. Stress-related genes were mostly affected in the gills, where the high H<sub>2</sub>S level also induced significant upregulation. Downregulation of the marker genes was identified in the olfactory organ especially in the low-dose group. The distal gut was less sensitive to H<sub>2</sub>S, regardless of the dose. Histological health scoring of the four mucosal organs revealed no substantial structural alterations and only sporadic cases of mild-moderate unspecific tissue damage. High-throughput metabolomics revealed that transient H<sub>2</sub>S exposure had a substantial mucosal impact rather than a systemic impact, as shown by changes in skin mucus metabolome. Functional annotation indicated that 10 metabolomic pathways were significantly affected in the skin mucus, including tRNA charging, the superpathway of branched-chain amino acid biosynthesis, and glucosylate biosynthesis from phenylalanine. The physiological alterations following transient exposure to H<sub>2</sub>S showed that the mucosal organs exhibited distinct response profiles, where transcriptional impacts were more pronounced in the skin and gills. The results contribute to a better understanding of the biological functions of exogenous H<sub>2</sub>S in teleost fish, as well as the development of mitigation strategies for salmon-production facilities and the risk of H<sub>2</sub>S exposure.

## 1. Introduction

Technological innovations to increase production and ensure optimal health and welfare in farming conditions have contributed significantly to the rapid growth of the aquaculture industry (FAO [Fao, 2020](#)). This is reflected in the development of Atlantic salmon (*Salmo salar*) aquaculture in the last 10 years. Historically, land-based farming of salmon has depended on flow-through systems (FTSs) or partial reuse systems. Recently, there has been a dramatic shift to recirculating

aquaculture systems (RAS), which offer several benefits, including stringent biosecurity measures, substantial control of physicochemical parameters, protection from unpredictable environmental conditions, less restriction on location, and reduced water usage, among others ([Bergheim et al., 2009](#); [Lazado and Good, 2021](#)).

Despite the ability of these systems to control the environment and maintain a high biomass production, the technology is presented with several challenges. In particular, mass mortality events have occurred in recent years in RAS-based farming of salmon, many of which were

\* Corresponding author.

E-mail address: [carlo.lazado@nofima.no](mailto:carlo.lazado@nofima.no) (C.C. Lazado).

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attributed to hydrogen sulphide ( $\text{H}_2\text{S}$ ) exposure (Dalsgaard, 2019). Norwegian and Danish RAS facilities have reported incidents of acute mortalities in salmon smolt production associated with  $\text{H}_2\text{S}$  formation (Hjeltne et al., 2017; Sommerset et al., 2020).  $\text{H}_2\text{S}$  is a highly toxic gas characterised by an odour of rotten eggs (Harbison et al., 2015). It is mainly produced by sulphate-reducing bacteria (SRB) that uses sulphate ( $\text{SO}_4^{2-}$ ) as a terminal electron acceptor when nitrate ( $\text{NO}_3^-$ ) is not available, and this process produces  $\text{H}_2\text{S}$  as a metabolic by-product.

In traditional smolt farms using mainly freshwater, the average  $\text{SO}_4^{2-}$  concentration is 2.2 mg/L (Kristensen et al., 2009), while in seawater, the  $\text{SO}_4^{2-}$  concentration is >1000 times higher. Therefore, increased use of seawater in RAS systems in Norway may partly explain the high number of  $\text{H}_2\text{S}$  mortality events in the last years. The formation of  $\text{H}_2\text{S}$  is also possible in freshwater RAS, but the risk is relatively low compared with saline systems (Rojas-Tirado et al., 2021). RAS can be considered as an ecosystem within an ecosystem because the microbial communities residing in these systems play a crucial role in keeping the balance of metabolic by-products of fish and the microbial community. Dissolved inorganic and organic matter are crucial energy sources for microbial communities, and the degradation of organic matter under anaerobic conditions results in  $\text{H}_2\text{S}$  production (Dalsgaard, 2019; Kiemer et al., 1995; Muyzer and Stams, 2008). In RAS, fish risk occasional exposure to low chronic levels of  $\text{H}_2\text{S}$  or acutely elevated levels.  $\text{H}_2\text{S}$  peaks often carry the highest risk as they are detrimentally associated with  $\text{H}_2\text{S}$ -related mass mortalities. Although information on the background levels of  $\text{H}_2\text{S}$  in marine RAS is limited, a recent study on  $\text{H}_2\text{S}$  sensor development found that the background level was around 0.01  $\mu\text{M}$  in salmon farms (Lien et al., 2022). The reported toxic concentrations of  $\text{H}_2\text{S}$  for different fish species range from 1  $\mu\text{M}$  to 41.08  $\mu\text{M}$  (0.034 to 1.4 mg/L) (Bagarinao and Vetter, 1989; Bagarinao and Vetter, 1990), hence it was previously recommended to keep the concentration not exceeding 0.15  $\mu\text{M}$  to avoid negative effects such as high mortalities (Boyd, 2014). This limit for marine aquaculture systems is approximately 15 folds higher than the background levels previously found in salmon RAS farms.

Compared to other chemical parameters routinely monitored in RAS, such as oxygen, nitrogenous compounds, and carbon dioxide, there is a significant knowledge gap about the formation, toxicity, and monitoring of  $\text{H}_2\text{S}$ . Although advances have been made in identifying potential hotspots of  $\text{H}_2\text{S}$  production in RAS and chemical-mediated mitigation strategies (Bergstedt et al., 2022; Rojas-Tirado et al., 2021), the understanding of how  $\text{H}_2\text{S}$  affects the physiology, health, and welfare of salmon is still inadequate. Understanding these consequences is crucial in devising risk-assessment protocols and mitigation plans for production systems where the risk of  $\text{H}_2\text{S}$  formation is high.

Given that  $\text{H}_2\text{S}$  is released into the water column, the mucosal surfaces of fish (the gills, skin, olfactory organ, and gut) interact with it more than any other organs. Mucosal surfaces are considered the interface between the internal and external environments, providing an indispensable first line of defence in fish (Cabillon and Lazado, 2019). In mammalian systems,  $\text{H}_2\text{S}$  has been shown to be a strong mucosal toxicant, and exposure can result in mucus disruption and inflammation (Buret et al., 2022). In one of the earlier studies on salmon, exposure to chronic sublethal levels of  $\text{H}_2\text{S}$  (maximally 7.8  $\mu\text{M}$ ) over a period of 18 weeks resulted in histological changes in the gills, including clubbing and thickening of the secondary lamellae. In addition, progressive liver damage was revealed by diffuse hepatic necrosis and diffuse vacuolar degeneration in 80% of the samples at termination (Kiemer et al., 1995). In channel catfish (*Ictalurus punctatus*), exposure to 0.5 mg/L (14.67  $\mu\text{M}$ )  $\text{H}_2\text{S}$  at 20°C resulted in hypernea and eventually to a respiratory arrest after 30 min of exposure (Torrans and Clemens, 1982). In the same study,  $\text{H}_2\text{S}$  level at 0.1 mg/L (2.93  $\mu\text{M}$ ) also inhibited 74% of cytochrome oxidase activity of the gills, which is an important component of respiratory process. Both responses were considered as protective adaptation responses against acute sulphide poisoning.  $\text{H}_2\text{S}$  research on other salmonids (e.g., rainbow trout and Chinook salmon) is relatively advanced compared with that on Atlantic salmon, especially when it comes to

function-based understanding (Olson, 2015; Olson et al., 2006). For example, endogenous  $\text{H}_2\text{S}$  in rainbow trout gill chemoreceptors has been identified to be involved in oxygen sensing (Olson et al., 2008). In addition, it was also found that endogenous  $\text{H}_2\text{S}$  participates in adrenergic stress response in rainbow trout by directly eliciting catecholamine secretion via membrane depolarization (Perry et al., 2009).

The present study investigated how transient exposure to  $\text{H}_2\text{S}$  affects the physiology of Atlantic salmon, particularly the mucosal and metabolic responses. We focused on profiling  $\text{H}_2\text{S}$ -triggered responses on the four mucosal organs: the gills, olfactory organ, skin, and distal gut. Using molecular and histological approaches, we found that transient exposure to  $\text{H}_2\text{S}$  differentially affected the mucosal defences. Further, mucosal and systemic metabolomics provided new insights into the physiological changes associated with  $\text{H}_2\text{S}$  exposure.

## 2. Materials and methods

### 2.1. Ethical use of animals in research

All fish handling procedures complied with the Guidelines of the European Union (Directive 2010/63/EU) and approved by Danish Veterinary and Food Administration (permit:2022-15-0201-01138).

### 2.2. Experimental fish

Prior to the exposure study, Atlantic salmon smolts (*Salmo salar*) were sourced from BioMar A/S, Denmark, and were held in 2m x 2m flow-through tanks at DTU-Aqua (Technical University of Denmark-National Institute of Aquatic Resources) in Hirtshals, Denmark. The smoltification status of the experimental fish was verified by quantifying the plasma chloride level ( $\text{Cl}^-$  around 140 mmol/L). The following conditions were maintained: salinity of 33 ppt, temperature at  $15 \pm 1^\circ\text{C}$ , pH 7.8–7.9, dissolved oxygen >90% saturation, photoperiod cycle of 15 L:9D, and water depth of 70 cm. The fish were fed a commercial diet (BioMar A/S) with a daily ration of 1.2% bodyweight per day and remained in the holding tank until reaching an approximate weight of 100 g. Fish were kept in the rearing tanks to acclimatize for 4 weeks before starting the experiments. The food was restricted for 24 h before transfer to  $\text{H}_2\text{S}$  exposure tanks.

### 2.3. $\text{H}_2\text{S}$ exposure setup

The exposure tank was set up in a temperature regulating room with an ambient temperature of around 15°C. The tank was a flow-through tank with dimensions of 76 cm x 60 cm x 40 cm and contained ~180 L of water with a flow rate of 25 L/h. Probes for monitoring the pH, dissolved oxygen, temperature, and  $\text{H}_2\text{S}$  levels were installed in each tank setup (Firesting Pro, Pyroscience, Aachen, Germany). These probes were connected to a computer that recorded the concentration of each parameter in 5-min intervals. A small mixing container was placed next to the tank with a dose controller, which allowed for the mixing and gradual passage of both water and  $\text{H}_2\text{S}$  before flowing into the exposure tank (Fig. 1). Detailed description of experimental design is reported in Bergstedt and Skov (2023).

### 2.4. Acclimation and $\text{H}_2\text{S}$ transient exposure

A total of 24 post-smolt Atlantic salmon (average weight:  $102 \pm 10$  g) were used and divided into three groups of treatments comprising eight fish each. For each exposure event, only four fish were used due to the limitation of the experimental unit. Therefore, there were two exposure events for each treatment group. The exposure trials were carried out over a six-day period. Fish from the holding tank were randomly selected and transferred to individual PVC tubes, which were securely covered with a net on both ends (Fig. 1) to prevent escape from the exposure tank, lessen fish interactions, and avoid disturbance. They

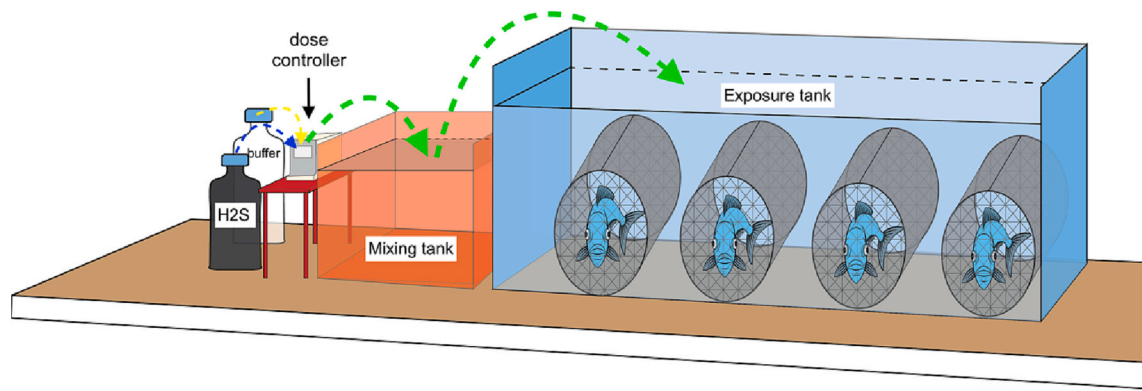


Fig. 1. Illustration of the H<sub>2</sub>S exposure setup.

were kept in the H<sub>2</sub>S exposure tank for 24 h for acclimation under the following conditions: salinity of 33 ppt, temperature at  $15 \pm 1^\circ\text{C}$ , pH 7.5–7.7, and dissolved oxygen  $>85\%$  saturation. To minimize any disturbance, the setup was shielded with non-transparent plastic.

Following the 24 h acclimation, fish were exposed to either 0.6  $\mu\text{M}$  (low) or 1.2  $\mu\text{M}$  (high) of H<sub>2</sub>S concentration. The exposure doses were chosen based on preliminary experiments of Atlantic salmon exposed to gradually increasing concentrations of H<sub>2</sub>S. Here 0.6  $\mu\text{M}$  was recognised as the lowest concentration (0.6  $\mu\text{M}$ ) that induced an effect on fish oxygen consumption, and at 1.2  $\mu\text{M}$  fish started to show signs of distress. As the fish were kept for an hour during the chosen concentrations it was imperative that the concentration caused a response, but were not as high as to cause respiratory impairment and risk mortalities (Bergstedt and Skov, 2023). The control group (0  $\mu\text{M}$ ) was handled in a similar manner to the H<sub>2</sub>S-exposed groups, but the system was not dosed with H<sub>2</sub>S. For the purpose of this paper, we denoted the H<sub>2</sub>S-exposed groups as low and high to facilitate an easy cross referencing. The H<sub>2</sub>S exposure was performed by stopping the flow of water in the tank and gradually increasing the flow of 0.1 M Na<sub>2</sub>S·9H<sub>2</sub>O (Sigma-Aldrich, MA, USA) stock solution to the mixing tank. Once the desired concentration in the exposure tank was achieved, the H<sub>2</sub>S dosing time commenced and then terminated after 1 h. To prevent an increase in the pH, 0.1 M HCl was dosed to the tank simultaneously with the sulphide stock solution. Immediately following the termination of transient exposure, the water flow controller was turned on, and the exposure tank system was replaced with new water until residual H<sub>2</sub>S was undetectable. The fish remained in the tank for 24 h of recovery before tissue sampling was performed.

## 2.5. Tissue sampling

Fish were humanely euthanised by an overdose of 0.1 g/L amino-benzoate for 5 min, and the weight of individual fish was recorded. Skin mucus was collected below the lateral line using a highly absorbent swab FLOQSwab® (COPAN Diagnostics, Murrieta, CA, USA) and snap-frozen in dry ice. Blood samples were withdrawn from the caudal artery with a heparinised vacutainer (BD Vacutainer™, Loughborough, UK). Skin samples with a size of  $\sim 2\text{ cm} \times 1\text{ cm}$  were collected below the dorsal fin. A small portion without the muscle was dissected for qPCR analysis, and the rest of the skin portion with muscle was separated for histology.

The entire second gill arch was collected. Both qPCR and histology samples were collected. Then, the olfactory rosettes were dissected and divided in two. The left side was used for qPCR, while the right side was used for histology. Subsequently, the fish was opened ventrally to collect 1 cm of the distal intestine for both qPCR analyses and histology. All tissues for gene expression analyses were suspended in RNAlater® (Sigma-Aldrich, MA, USA), kept at  $4^\circ\text{C}$  overnight to allow penetration, and stored at  $-80^\circ\text{C}$  until RNA isolation. The tissues designated for

histological evaluation were preserved in 10% formalin, kept at room temperature overnight for fixation, transferred to 70% ethanol, and kept at  $4^\circ\text{C}$  until tissue processing.

## 2.6. RNA isolation, quantification, and complementary DNA synthesis

The total RNA collected from tissues was isolated using an Agencourt RNAdvance™ Tissue Total RNA Purification Kit (Beckman Coulter Inc., CA, USA) with the aid of a Biomek 4000 automated workbench station (Beckman Coulter, Inc., CA, USA). The isolated RNA was quantified using a NanoDrop 8000 Spectrophotometer (ThermoFisher Scientific, USA). The complementary DNA (cDNA) was synthesised from 500 ng of template RNA using a High-Capacity cDNA Reverse Transcription Kit (Beckman Coulter, Inc., CA, USA) in a 20- $\mu\text{L}$  reaction, and thermocycling was carried out in a Veriti™ 96-Well Thermal Cycler 7 (Applied Biosystems, California, USA) with the following conditions: 10 min at  $25^\circ\text{C}$ , 30 min at  $37^\circ\text{C}$ , and 5 min at  $95^\circ\text{C}$ .

## 2.7. Real-time quantitative PCR

The quantification of gene expression was performed in a QuantStudio™ 5 Real-Time PCR system (Applied Biosystems) using Power SYBR Green PCR Master Mix (Applied Biosystems). Briefly, a 10- $\mu\text{L}$  reaction mixture contained 4  $\mu\text{L}$  of  $10\times$  diluted cDNA, 5  $\mu\text{L}$  of SYBR green, and 0.5  $\mu\text{L}$  of each 10  $\mu\text{M}$  forward and reverse primer of the target genes listed in Table 1. No RT control was included. All samples were run in duplicate with the following thermocycling conditions: 2 min of pre-incubation at  $95^\circ\text{C}$ ; amplification with 40 cycles for 15 s at  $95^\circ\text{C}$  and 1 min at  $60^\circ\text{C}$ ; and a melt curve stage of 15 s at  $95^\circ\text{C}$ , 1 min at  $60^\circ\text{C}$ , and 15 s at  $95^\circ\text{C}$ . The relative gene expression was calculated by the  $2^{-\Delta\Delta\text{Ct}}$  method using the geometric mean of three housekeeping genes: *elongation factor 1- $\alpha$*  (*elf1a*),  *$\beta$ -actin* (*actb*), and *acidic ribosomal protein* (*arp*).

## 2.8. Histological processing

Prior to processing, the gills, skin, and olfactory rosettes were decalcified in 10% Titriplex 3 $\times$  solution (Sigma Aldrich) for 48 h. An automated tissue processor (TP1020, Leica Biosystems, Nussloch, Germany) was used for the dehydration, clearing, and paraffin infiltration process. Processed tissues were fixed and embedded in paraffin using a modular tissue-embedding system (Leica EG1150H, Leica Biosystems, Nussloch, Germany) and were then cut into 5- $\mu\text{m}$  sections using a rotatory microtome (Leica RM2165, Leica Biosystems, Nussloch, Germany). The sections were carefully transferred onto microscope slides (Surgipath, Leica Biosystems, Illinois, USA) and heat-fixed at  $60^\circ\text{C}$  overnight (TS 9026, Termacks, Sweden). The sections were stained with Periodic Acid Schiff-Alcian Blue (AB-PAS) using an automated stainer (ST5010, Leica Biosystems), and the whole slide was scanned using a slide scanner (Aperio CS2 slide scanner, Leica Biosystem).



**Table 1**

Target and reference genes used for qPCR analysis.

Gene name	Abbreviation	Sequence (5'-3')	Reference
Acidic ribosomal protein	arp	F: TCATCCAATTGCTGGATGACTATC R: CTTCCCACGCAAGGACAGA	(Sanden and Olsvik, 2009a)
$\beta$ -actin	actb	F: CCAAAGCCAACAGGGAGAA R: AGGGACAACACTGCCTGGAT	(Sanden and Olsvik, 2009a)
Elongation factor 1- $\alpha$	elf1a	F: GAATCGGCTATGCCTGGTGAC R: GGATGATGACCTGAGCGGTG	(Garcia de la serrana, Johnston de Garcia and Johnston, 2013)
Mucin 5 ac-like	muc5ac	F: GACCTGCTCTGTGGAAGGAG R: AGCACGGTGAATTCAGTTCC	(Sveen et al., 2017)
Mucin 5b-like	muc5b	F: ATTAAGAGCGATGTCTTCACAGC R: AAGCACATGAGTCTCTCACACAA	(Sveen et al., 2017)
Mucin 2-like	muc2	F: GAGTGGGCTCTCAGATCCAG R: GATGATGCGGACGGTAGTTT	(Sveen et al., 2017)
Interleukin-1 $\beta$	il1b	F: AGGACAAGGACCTGCTCAACT R: CCGACTCCAACCTCAACACTA	(Ingerslev et al., 2009)
Interleukin-8	il8	F: GAAAGCAGACGAATTGGTAGAC R: GCTGTTGCTCAGAGTTGCAAT	(Soto-Dávila et al., 2019)
Interleukin-10	il10	F: GGGTGTACGCTATGGACAG R: TGTTTCCGATGGAGTCGATG	(Ingerslev et al., 2009)
Sulphide:quinone oxidoreductase 1	sqor1	F: GGATAGGAAGTATGATGGCTACAC R: GGTCATAGGGAATGTCTCCA	This study
Sulphide:quinone oxidoreductase 2	sqor2	F: CCAACATCATGTACAACACGTC R: GCATCTCATACTCAACACTTCAG	This study
Sulfite oxidase	suox	F: TGCTCTGAGTATAAGGTGGGTGAG R: GGTGATGTAGTTGTGCGAGAG	This study
Catalase	cat	F: GGGCAACTGGGACCTTACTG R: GCATGGCGTCCCTGATAAA	(Olsvik et al., 2013)
Heat shock protein 70	hsp70	F: CCCCTGTCCCTGGGTATTG R: CACCAGGCTGGTTGTCTGAGT	(Solberg et al., 2012)
Heat shock protein 90	hsp90	F: CCACCATGGGCTACATGATG R: CCTTCACCGCTTGTCAITC	(Olsvik et al., 2013)
Glutathione peroxidase	gpx	F: GATTCGTTCCAACTTCTCTGCTA R: GCTCCAGAACAGCCTGTTG	(Solberg et al., 2012)
Peroxiredoxin 3	prdx3	F: TTTAAAGCTACAGCTGTCCAC R: GACAAACAAACGTGAAATCGAG	(Furtado et al., 2022)
Glutathione S-transferase	gsta	F: AGGGCACAAGTCTAAAGAAGTC R: GTCTCCGTGTTGAAAGCAG	(Lazado and Voldvik, 2020)
Caspase-3	casp3	F: ACAGCAAAGAGCTAGAGGTCCAACAC R: AAAGCCAGGAGACTTTGACGCAG	(Espe et al., 2015)

## 2.9. Histomorphological evaluation

Tissue morphology evaluation was done using a semi-quantitative scoring system based on a modified scoring system developed specifically for each mucosal tissue (Supplementary Table 1). Briefly, slide images of each mucosal tissue were evaluated based on the following scoring scheme: 1 (normal morphology), 2 (mild pathological signs), 3 (moderate pathological signs), and 4 (severe pathological signs). To address bias, scoring was performed twice at two different time points, and the final score was expressed as the average of the two. Important pathological changes in each tissue were observed and noted. Subsequently, mucus cells surrounding the peripheral mucosal epithelium were quantified and expressed as an average count from three random sites of each tissue based on the described criteria (Supplementary Table 2).

## 2.10. Skin mucus and blood metabolomics

Analysis was carried out using a Thermo Scientific Vanquish LC coupled to a Thermo Q Exactive HF MS. An electrospray ionisation interface was used as an ionisation source. The analysis was performed in negative and positive ionisation modes. UPLC was performed using a slightly modified version of the protocol (Doneanu et al., 2011). Peak areas were extracted using Compound Discoverer 3.1 (Thermo Scientific). Identification of compounds was performed using four levels: level 1: identification by retention times (compared against authentic in-house standards), accurate mass (with an acceptable deviation of 3 ppm), and MS/MS spectra; level 2a: identification by retention times (compared against in-house authentic standards), and accurate mass

(with an acceptable deviation of 3 ppm); level 2b: identification by accurate mass (with an acceptable deviation of 3 ppm), and MS/MS spectra; and level 3: identification by accurate mass alone (with an acceptable deviation of 3 ppm). Sample analysis was carried out by MS-Omics.

## 2.11. Data analysis

Significant differences in gene expression, tissue score, and mucus cell count were analysed using SigmaStat Statistical Package (Systat software, London, UK). Assumptions for analysis of equal variances (Brown-Forsythe) and normality (Shapiro-Wilk) of the datasets were tested, and if they failed, they were log-transformed before analysis (Supplementary Table 3). One-way ANOVA was used, and multiple pairwise comparisons were performed using the Holm-Sidak method. Kruskal-Wallis ANOVA was used for non-parametric data, followed by a similar pairwise comparison procedure.

All values are expressed as means with their corresponding standard deviation (SD), and the significance level was set at  $p < 0.05$ . Additionally, principal component analyses (PCAs) were done using R Studio Desktop version 1.4 (RStudio, PBC, Boston, MA). A heatmap was generated using GraphPad Prism version 9.0.0 (GraphPad Software, La Jolla, USA).

Normalised data provide input for testing the statistical hypothesis, in which metabolites significantly differ between sample groups identified. Statistical comparisons were performed using linear modelling in the Bioconductor package limma. Significance values ( $p$ -values) were adjusted for multiple testing by controlling the false discovery rate. For each comparison (e.g. group A vs. group B), a positive log<sub>2</sub>(fold change)

indicates upregulation in group A relative to group B, and vice versa.

Gene set enrichment analysis (GSEA) was done using the fgsea R package. The pathway set for the GSEA analysis were MetaCyc pathways (<https://metacyc.org/>; downloaded 30 April Guo et al., 2021). The universe for the analysis consisted of all compounds with a BioCyc ID and an annotation level between 1 and 3, as provided by MSOmics ApS. Each analysis was performed by ranking according to a statistic. GSEA evaluates whether involvement in each interest tends to be over-represented at the extremes of the ranked statistic. The statistical significance (nominal  $p$ -value) of the over-representation was assessed using a method based on an adaptive multi-level split Monte Carlo scheme.

### 3. Results

#### 3.1. Gene expression profile in mucosal organs of post-smolt Atlantic salmon following transient exposure to H<sub>2</sub>S

Fig. 2 shows the expression profile of four groups of genes: mucins (*muc5ac*, *muc5b*, and *muc2*), interleukins (*il1b*, *il8*, and *il10*), sulphide-detoxification (*sqor1*, *sqor2*, and *suox*), and stress-related genes (*gpx*, *cat*, *prdx3*, *hsp70*, *hsp90*, *casp3*, and *gsta*) in four mucosal organs (the gills, distal gut, olfactory organ, and skin). In the gills (Fig. 2A), *sqor1* was significantly downregulated by 3.3-fold relative to the control when exposed to the low level of H<sub>2</sub>S. The expressions of the two other sulphide detoxification genes (*sqor1* and *suox*) were not significantly affected by the transient exposure to H<sub>2</sub>S. Mucins and interleukins were also not affected considerably by H<sub>2</sub>S exposure in the gills. All stress-related genes except *gsta* showed significantly higher expression than the control when exposed to both low and high levels of H<sub>2</sub>S. This transcriptional response followed a dose-dependent profile.

Transient exposure to H<sub>2</sub>S did not significantly alter the expression of the marker genes in the distal gut (Fig. 2B) except *prdx3*, which was downregulated by 1.8-fold when exposed to the low level of H<sub>2</sub>S compared with the control. The expression levels of interleukins and stress-related genes in the olfactory organ were significantly downregulated by the low H<sub>2</sub>S level compared to the control by at least 3.1-fold (Fig. 2C). At the high level of H<sub>2</sub>S, the transcript levels of *suox* and *gpx* were upregulated by 3.7 and 1.7-fold compared to the control,

respectively. In the skin (Fig. 2D), 14 of the 16 investigated genes were significantly upregulated by least 1.1-fold relative to the control following exposure to low levels of H<sub>2</sub>S. In addition, the transcript levels of sulphide-detoxification genes, stress-related genes, and mucin genes (except *muc2*) were significantly upregulated by the high level of H<sub>2</sub>S by up to 4.4-fold compared to the control (Fig. 2D).

Principal components 1 and 2 explained 66.8% of the variation in the entire gene expression dataset (Fig. 3). Component 1 (Dim1) explained 57.5% of the variability in the expression levels of all the genes behaving similarly in cluster 1 (Fig. 3A). Component 2 (Dim2) explained an additional 9.3% of the variability in the dataset, which is mainly explained by the genes responding similarly in cluster 2 (Fig. 3A). Combining all sample component scores in a 2-dimensional PCA plot shows a well-defined clustering between treatment groups and controls (Fig. 3A). The molecular responses in the high level of H<sub>2</sub>S exposure showed large variation from the control group (Fig. 3A), as also revealed by the pairwise analysis shown in Fig. 2.

Based on the overall response, there are two clusters of genes that showed similar expression tendencies: cluster 1, including the genes *muc5ac*, *muc5b*, *muc2*, *sqor1*, *sqor2*, and *il10*, and cluster 2, which comprises the genes *suox*, *il1b*, *il8*, *gpx*, *cat*, *prdx3*, *casp3*, *hsp70*, and *hsp90* (Fig. 3A). Another PCA plot of all component scores shows clustering of the four mucosal organs in response to H<sub>2</sub>S (Fig. 3B). The gills and skin displayed the highest variability of molecular responses, while the olfactory organs and gut demonstrated had the least variations among samples and treatment groups.

#### 3.2. Histology and morphometric measurements

Evaluation of the organs based on damage scores showed significant histo-pathological changes in the gills, distal gut, and olfactory organ following exposure to the high level of H<sub>2</sub>S in comparison with the control, although the magnitude was minimal (Fig. 4, Fig. 5). The tissue damage score in the gills was significantly higher in the group exposed to high levels of H<sub>2</sub>S compared to the control and low-exposure groups (Fig. 4A). The gills of the control group displayed normal morphology (Fig. 5A), but exposure to either low or high levels of H<sub>2</sub>S resulted in severe cases of epithelial lifting (Fig. 5B and C).

The distal gut in the control group exhibited normal and healthy

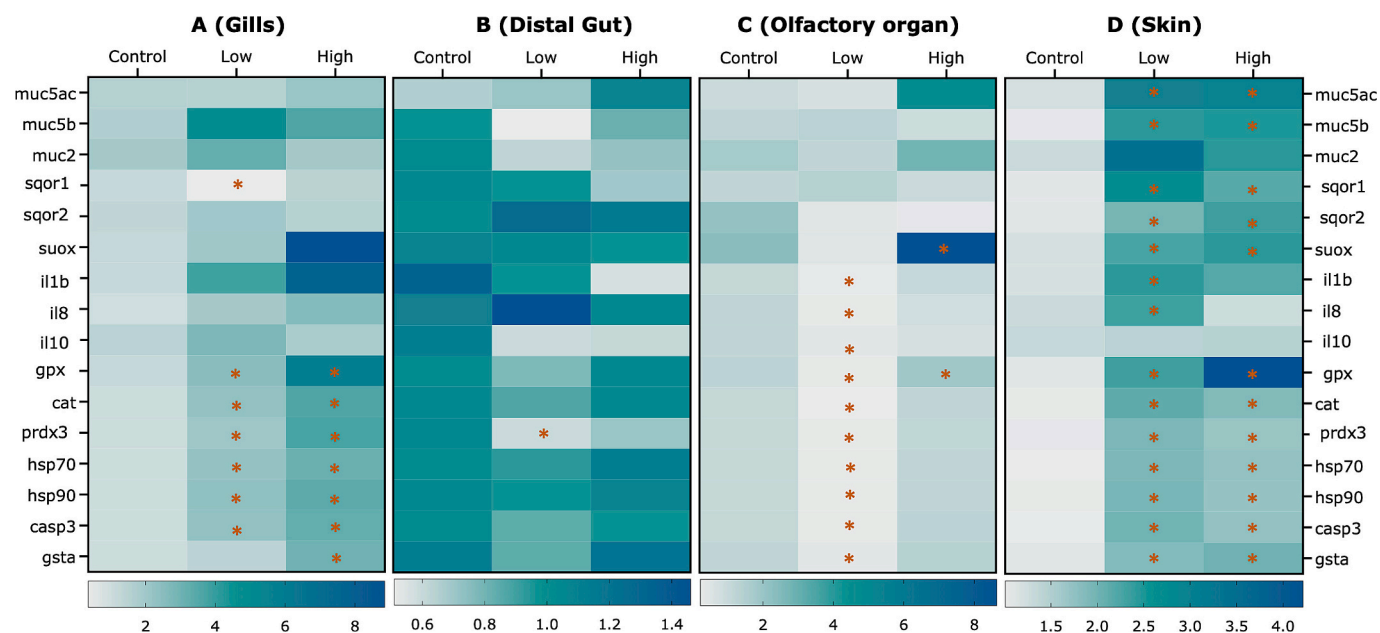


Fig. 2. Gene expression profiles in (A) gills, (B) distal gut, (C) olfactory organ, and (D) skin of Atlantic salmon transiently exposed to different H<sub>2</sub>S levels for 1 h: control (0 μM), low (0.6 μM), and high (1.2 μM). The gradients indicate a mean of  $n = 8$ ; stars denote a statistically significant difference from the control ( $p$ -value < 0.05) inferred by pairwise comparison.

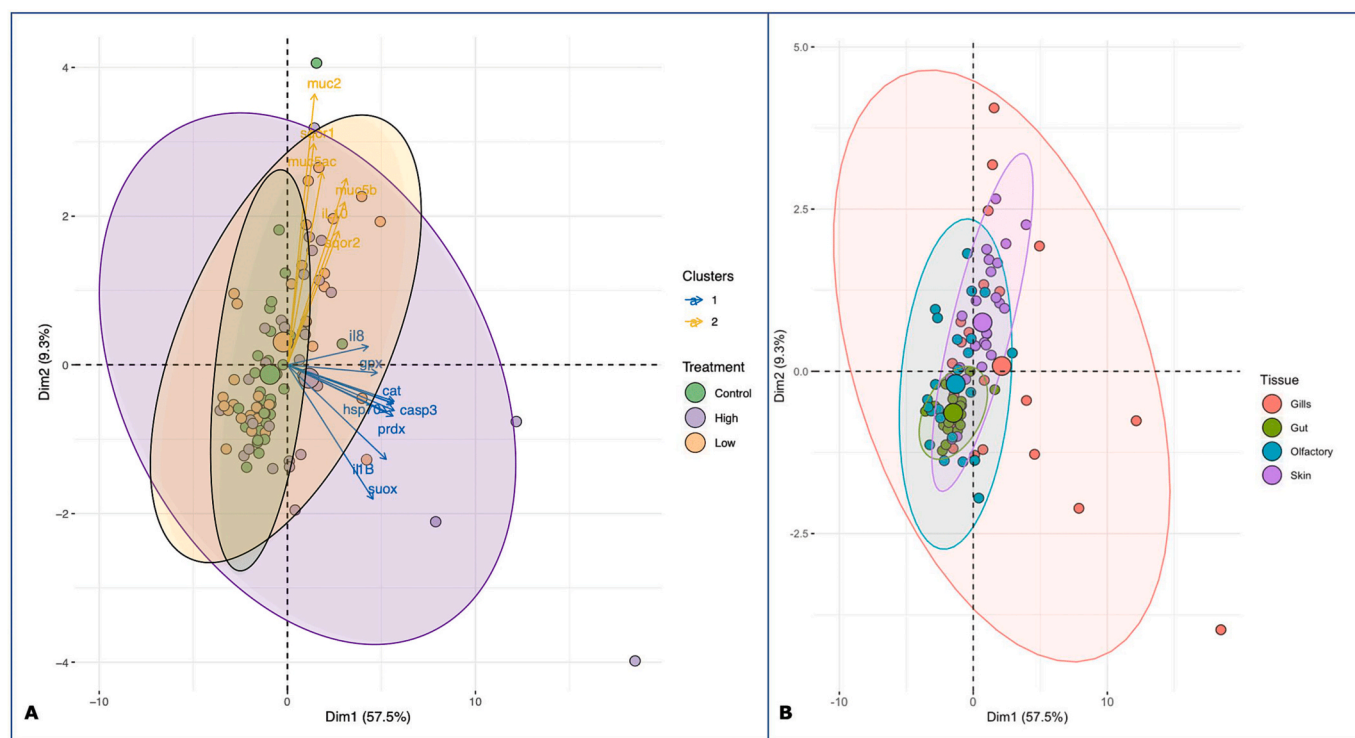


Fig. 3. Principal Component Analysis (PCA) plot showing treatment and tissue clustering (using k-means at 95% confidence interval) across 16 investigated genes of Atlantic salmon. (A) Cluster of molecular response expressed according to treatment levels of H<sub>2</sub>S. Ellipsoids used to show separations between treatments of control (0  $\mu$ M), low (0.6  $\mu$ M), and high (1.2  $\mu$ M) H<sub>2</sub>S exposure; arrows and labels show the direction of the component loading associated with each gene. (B) Cluster of molecular responses expressed according to type of mucosal organs. Ellipsoids used to show separations between tissues of gills, distal gut, olfactory organ and skin.

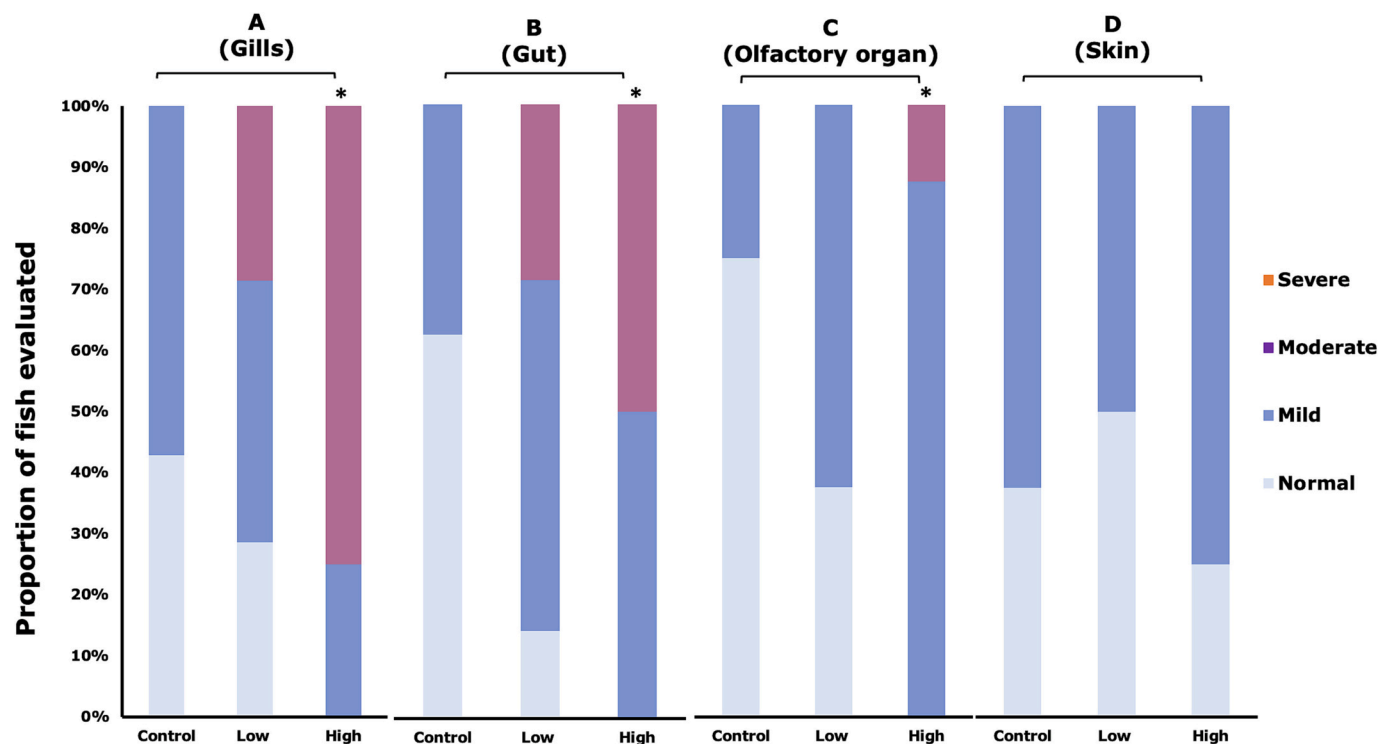
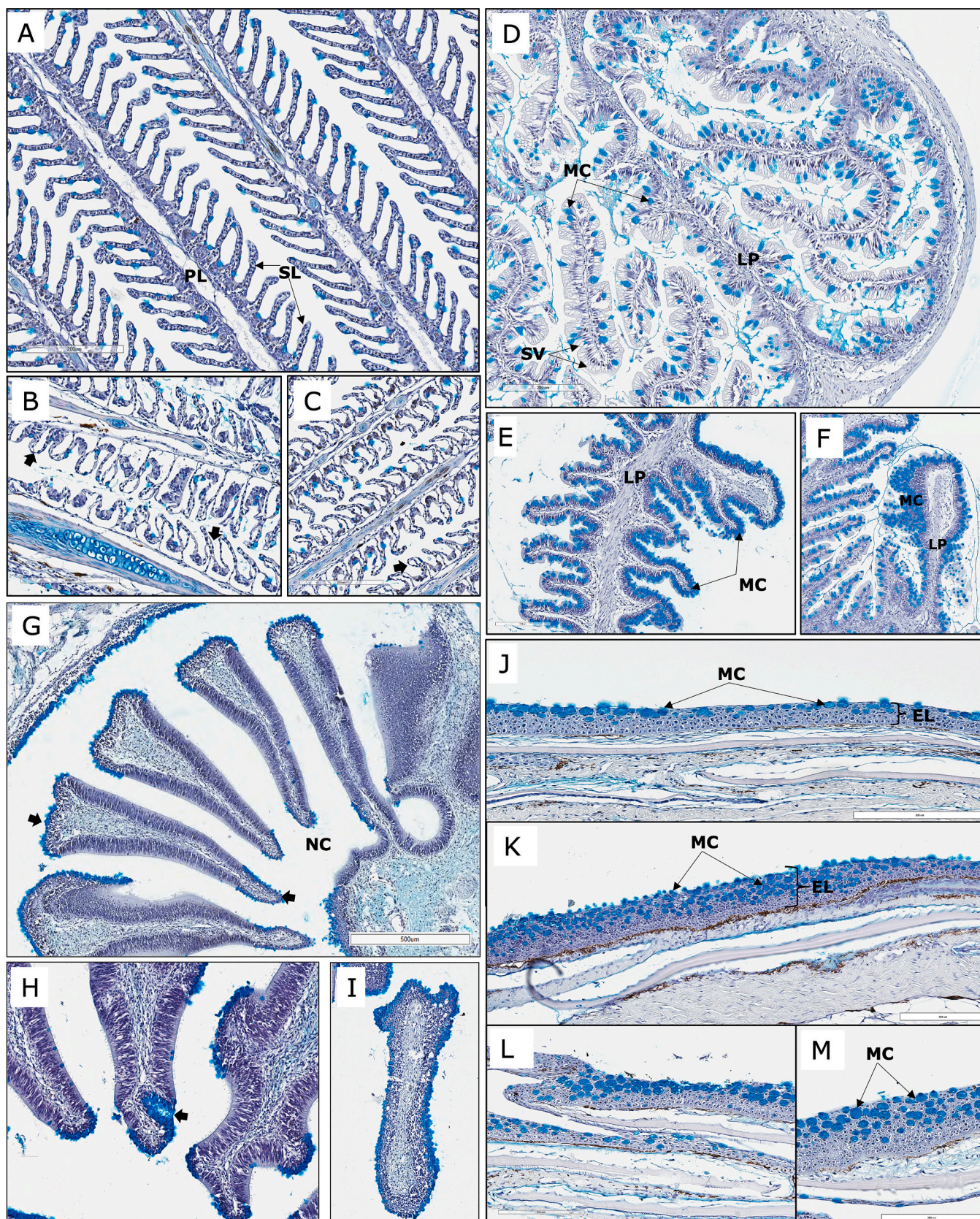


Fig. 4. Morphological evaluation of the four mucosal organs: (A) gills, (B) distal gut, (C) olfactory organ, and (D) skin of Atlantic salmon transiently exposed to different levels of H<sub>2</sub>S: control (0  $\mu$ M), low (0.6  $\mu$ M), and high (1.2  $\mu$ M). Histological damage scores are either normal, mild, moderate, or severe and presented as the proportion of fish evaluated showing these different scores ( $n = 8$ ). Stars denote statistically significant differences from control ( $p$ -value < 0.05) inferred by pairwise comparison.





**Fig. 5.** Representative photos of gills from control (A) and H<sub>2</sub>S-exposed (B, C) groups, gut from control (D) and H<sub>2</sub>S-exposed (E, F) groups, olfactory organ from control (G) and H<sub>2</sub>S-exposed (H, I) groups, and skin from control (J) and H<sub>2</sub>S-exposed (K, L, M) groups. Lifting (thick black arrow) in secondary lamellae (SL) in gills (B, C). Widened lamina propria (LP) in the distal gut (E, F). Moderately rough surface of the epithelial layer (EL) of the skin (L, M). Mucus cells (MC) = blue coloured cells. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



morphology characterised by large supranuclear vacuoles occupying almost the entire apical part of the enterocytes, mucus cells distributed on the mucosal folds, and well-defined structure of the lamina propria (Fig. 5D). The fish exposed to H<sub>2</sub>S showed variable histopathological changes, but those exhibiting severe cases were characterised by a reduction in the length of the simple and complex folds, and the surface was almost completely covered with mucus cells in comparison with the control. In addition, the width of the lamina propria was slightly increased in comparison with the control (Fig. 5E and F). These morphological changes in the distal gut also demonstrated significantly higher tissue damage scores in the high-exposure group compared to the control and low exposure groups (Fig. 4B).

The olfactory organ in the control group showed normal structure, in which mucus cells were densely distributed on the mucosal tip of the olfactory lamella (Fig. 5G). Although minor morphological changes were observed, such as rough epithelial surface and local lesions, sporadic cases showed a high number of mucus cells distributed not only on the tip but throughout the olfactory lamellar epithelium as a response to H<sub>2</sub>S (Fig. 5H and I). The group exposed to high levels of H<sub>2</sub>S showed significantly higher tissue damage scores than the control and low exposure groups (Fig. 4C).

The skin did not show any significant differences, as shown in tissue damage score following H<sub>2</sub>S exposure (Fig. 4D). In some cases, in H<sub>2</sub>S-exposed groups, the epithelial layer of the skin became noticeably rough with a dense population of mucus cells (Fig. 5K, L, and M). Statistical analysis of the mucus cell count in all four mucosal tissues did not show any significant differences among treatments (Fig. 6).

### 3.3. Skin mucus and blood metabolomes of H<sub>2</sub>S-exposed Atlantic salmon

A total of 867 compounds were detected in the blood, of which 126 were level 3, 58 were level 2b, 70 were level 2a, and 61 were level 1 (Fig. 7A). A total of 1965 compounds were detected in the skin mucus samples, of which 285 were level 3, 136 were level 2b, 63 were level 2a, and 55 were level 1. Compounds were further annotated by linking the information provided by the service provider to the HMDB database, and both levels 1 and 2a showed 100% annotation.

PCA plots revealed the separation of H<sub>2</sub>S-exposed groups from the control for both skin mucus and blood (Fig. 7B and C). We then analysed the number of differentially affected metabolites ( $p < 0.05$ ) in the H<sub>2</sub>S-exposed fish and found two different profiles between the low and high H<sub>2</sub>S groups in the two biological matrices (Fig. 7D). In skin mucus, there were 105 significantly affected metabolites in the low group, while 65 metabolites were affected in the high group. In blood, 56 significantly affected metabolites were in the low group, while 144 were affected in the high group. Level 1 metabolites that significantly changed in the “low vs. control” comparison in the skin mucus included hypoxanthine (Fig. 7E) and taurine (Fig. 7F), with the level in the H<sub>2</sub>S-exposed fish increasing in the latter while decreasing in the former. There were 11 level 1 metabolites that were significantly affected in the “low vs.

control” comparison in the blood, such as 1-methyl adenosine (Fig. 7G), where high H<sub>2</sub>S increased its abundance, while 4-guanibutyric acid (Fig. 7H) decreased following transient exposure to a high level of H<sub>2</sub>S.

GSEA of MetaCyc pathways was performed for all comparisons in both skin mucus and blood to detect global functional effects of H<sub>2</sub>S exposure (Fig. 7I). GSEA is suitable for functional analysis even in the absence of significantly differentially abundant features. Compounds are ranked by  $p$ -value and direction of effect (abundance fold change), and GSEA evaluates whether compounds involved in a functional pathway of interest tend to be overrepresented at extremes of the ranked list. At the FDR-adjusted  $p < 0.25$  threshold recommended for GSEA, there were 10 significantly perturbed MetaCyc pathways detected in the “high exposure vs. control” comparison in the skin mucus, including tRNA charging, superpathway of branched-chain amino acid biosynthesis, and glucosinolate biosynthesis from phenylalanine. In blood, L-citrulline degradation was the only significantly perturbed pathway found in the “high exposure vs. control” comparison.

## 4. Discussion

Despite the known toxicity of H<sub>2</sub>S in fish (Torrans and Clemens, 1982) and the recent mass mortality events in the industry, little is known about the consequences of exposure to H<sub>2</sub>S in the physiology of Atlantic salmon. We report that H<sub>2</sub>S is a potent modulator of the defences in Atlantic salmon mucosa. Further, we have revealed that H<sub>2</sub>S-triggered transcriptional alterations were more pronounced in the skin and gills compared with the olfactory organ and distal gut. Nonetheless, histological changes associated with the transient exposure were marginal. Physiological impacts of transient H<sub>2</sub>S exposure were further explored through metabolomics, revealing that perturbations in the skin mucus metabolome were more substantial than in the blood metabolome.

### 4.1. Transient H<sub>2</sub>S exposure induces changes in the expression of genes involved in sulphide detoxification and immunity in the skin

Among the four mucosal organs investigated, the skin appears to be the most responsive to transient exposure to H<sub>2</sub>S, as revealed by the pronounced changes in the expression of marker genes for sulphide detoxification, mucin, immunity, and stress response. The responsiveness of the skin is most likely related to the large surface area in contact with H<sub>2</sub>S in the water. H<sub>2</sub>S toxicity is often associated with the inhibition of cytochrome C oxidase, which eventually impairs cellular aerobic respiration (Bagarinao and Vetter, 1990). Mitochondrial detoxification of sulphide is an important ability of marine fishes to tolerate and survive in sulphide-rich environments (Bagarinao and Vetter, 1990).

The genes *sqor1*, *sqor2*, and *suox* encode for enzymes involved in the mitochondrial catabolism of toxic H<sub>2</sub>S into non-toxic sulphide compounds (Olson, 2012; Quinzii et al., 2017). The upregulation of these genes, particularly in the skin, suggests that the mucosa mounts an H<sub>2</sub>S detoxification response and may facilitate a protective mechanism against H<sub>2</sub>S toxicity. On the other hand, the transcript level of *sqor1* in the gills decreased following transient exposure to H<sub>2</sub>S, suggesting that the function of *sqor1* likely differs among mucosal organs. This may also indicate that at the concentrations tested, transient exposure may inhibit the inherent ability of the mucosa to detoxify H<sub>2</sub>S, at least through *sqor1*.

Mucins are the major macromolecular components of mucus, an emblematic component of every mucosal surface. A plethora of stimuli, such as toxicants, are strong regulators of MUC gene expression, which can occur at both the transcriptional and posttranscriptional levels (Voynow and Fischer, 2006). *Muc5ac* and *muc5b* expression in the skin were upregulated by transient exposure to H<sub>2</sub>S, regardless of the level. Previous studies have also observed upregulation of *muc5*-type genes in the mucosa when Atlantic salmon and common carp (*Cyprinus carpio*) were exposed to various external stressors such as oxidants (Osório et al., 2022) and copper and polyvinyl chloride microparticles (Hoseini

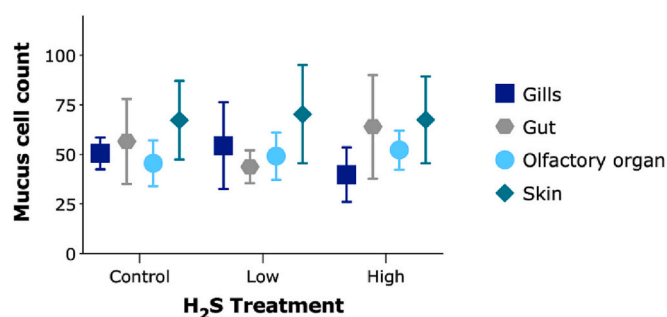
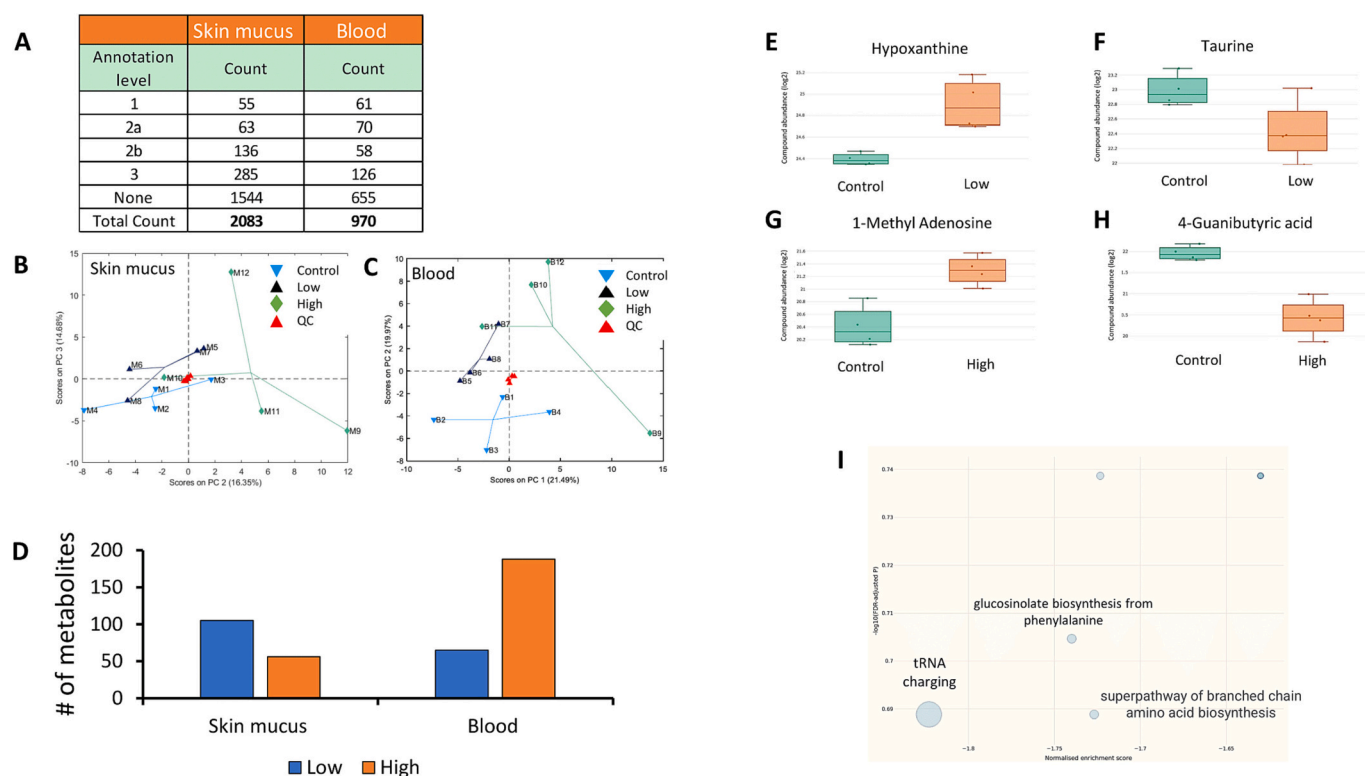


Fig. 6. Mucus cell counts in four mucosal organs of Atlantic salmon transiently exposed to different H<sub>2</sub>S levels: control (0  $\mu$ M), low (0.6  $\mu$ M), and high (1.2  $\mu$ M). Data are presented as mean  $\pm$  standard deviation of 8 individual fish.





**Fig. 7.** Metabolomic changes in the skin mucus and blood of Atlantic salmon transiently exposed to  $H_2S$ . **A)** The total number of compounds identified. **B, C)** Score plot from PCA model calculated on the relative concentrations of the variables in the reduced dataset of **B)** skin mucus and **C)** blood metabolome. Data have been auto-scaled. **D)** Number of metabolites that significantly changed following transient exposure to  $H_2S$  relative to the unexposed control group. Representatives from level 1 metabolites significantly changed in **(E, F)** skin mucus and **(G, H)** blood. Only the representative metabolites from the low group are given in the skin mucus, while only those from the high group are in the blood based on the dose profile in **D**. **I)** Plot of statistical significance of pathway-specific gene set enrichment ( $-\log_{10}$  FDR-adjusted  $p$ -value) against normalised enrichment score (NES). The size of each point reflects the number of compounds evaluated for each pathway. Only pathways with  $P$  (FDR) < 0.25 were included.

et al., 2022), suggesting involvement of this gene in the stress response to potential toxicants.

The upregulation of the two gel-forming mucin genes likely triggers an increased production of mucus on the epidermal surface. Although not statistically significant, the mucus cell number changed following  $H_2S$  exposure in the skin.  $H_2S$  has been identified to have a substantial impact on mucolytic function (Khattak et al., 2021). The increased expressions of *muc5ac* and *muc5b* indicate the protective function of the mucus layer and its associated mucins against  $H_2S$ , hence ensuring mucosal integrity.

Interleukins are cytokines that regulate inflammatory responses by activating immune cells such as lymphocytes, neutrophils, and macrophages as a response to pathogen invasion and stress exposure (Sakai et al., 2021). The *interleukin-8* (*il8*) and *interleukin-1b* (*il1b*) are both well-known proinflammatory cytokines, whereas *interleukin-10* (*il10*) is an anti-inflammatory cytokine and acts as a regulator of processes associated with immunosuppression (Zou and Secombes, 2016). The increase in the transcript level of *il1b* and *il8* in the group transiently exposed to a low level of  $H_2S$  suggest that the induction of proinflammatory response in the skin was not dose-dependent. An increase in cytokine release (*il1b* and *il8*) was also observed in normal human keratinocytes exposed to a carcinogenic sulphur mustard gas (Arroyo et al., 1999).

On the other hand, the expression of *il1b* and *il8* in the olfactory organ demonstrated downregulation, suggesting that the impact of  $H_2S$  on the inflammatory process in Atlantic salmon might be organ dependent. It remains to be proven whether this downregulation was a form of immunosuppression of the nasal immune function. Collectively, the changes in the expression of cytokines in the present study support the potential role of  $H_2S$  as a regulator of inflammation (Whiteman and Winyard, 2011).

#### 4.2. Induction of stress from transient $H_2S$ exposure exhibits distinct profiles between mucosal organs

The antioxidant defence system protects the organism from attack by reactive oxygen species (ROS) which are produced in excess during oxidative stress (Shankar and Mehendale, 2014).  $H_2S$  has been shown to have dual functions in oxidative stress: it can induce oxidative stress but can also protect the host from it, although this duality is primarily governed by the concentration of  $H_2S$  (Xiao et al., 2018).  $H_2S$  upregulates a wide range of enzymes attenuating oxidative stress, such as catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase, and glutathione-S-transferase (Wen et al., 2013). Transient exposure to  $H_2S$  affected the antioxidant defences in the gills and skin, where upregulation of key antioxidant genes was observed, namely *gpx*, *gsta*, *prdx3*, and *cat*. In a previous study it was shown that  $H_2S$  protected neurons from oxidative stress by increasing the glutathione levels (Kimura and Kimura, 2004). On the other hand, these antioxidant gene markers were downregulated in the olfactory organ suggesting potential inhibition of this inherent function to deter the induction of oxidative stress. A similar tendency was documented in liver hepatocytes, where activities of antioxidant enzymes decreased following  $H_2S$  exposure (Guo et al., 2021). These profiles suggest that the skin and gills have a robust ability to respond to  $H_2S$ -triggered oxidative stress, although such a response was likely not executed by the olfactory organ, further demonstrating the differential sensitivity to  $H_2S$ .

Effects of transient exposure to  $H_2S$  on the stress status at the mucosa were further substantiated by the alterations in the expression of heat shock proteins and caspase. Hsp70 and hsp90 are highly conserved intracellular proteins that act as molecular chaperones and mediators for the repair of damaged proteins, maintenance of homeostasis, and active

components of the cytoskeleton, enzymes, and steroid hormone receptors (Iwama et al., 2004). On the other hand, caspase-3, a protease encoded by the *casp3* gene, acts as a key mediator of programmed cell death (apoptosis) (Porter and Jänicke, 1999), in which a disturbed cell commits programmed suicide without affecting its neighbouring normal cells as a response to a threat or stress (Takle and Andersen, 2007). These genes were upregulated in the gills and skin, while their expression was downregulated in the olfactory organ.

The changes in the gills and skin may indicate physiological countermeasures from mucosal immunotoxicity of transient H<sub>2</sub>S exposure. This is a crucial response in maintaining mucosal homeostasis following H<sub>2</sub>S-induced stress. Interestingly, these stress markers were downregulated in the olfactory organ, but only in the low-level group, exhibiting a similar profile as with other oxidative stress biomarkers.

#### 4.3. Exogenous H<sub>2</sub>S exposure minimally alters the histological structures of mucosal organs

Moderate pathological changes were documented in the gills, distal gut, olfactory organ, and skin structures. Structural alterations in the gills associated with transient H<sub>2</sub>S exposure were mainly related to epithelial lifting. Epithelial lifting is a common gill lesion in fish during a fluid penetration and is considered a potential protective response to impede the entry of a toxicant (Wood, 2001). Cases of epithelial lifting observed in the H<sub>2</sub>S-exposed fish suggest a compromised barrier since these lesions can stretch the diffusion distance between the environment and the blood and reduce the interlamellar space (Baskar, 2014; Roberts, 2012; Strzyżewska-Worotyńska et al., 2017; Wood, 2001).

In a previous experiment with acute exposure of Atlantic salmon to H<sub>2</sub>S, gills were found to have severe irreversible damages coupled with secondary lamellar fusion and thickening of the primary lamella, but no epithelial lifting was observed (Kierner et al., 1995). In addition, an increase in the number of mucus cells in the gills exposed to H<sub>2</sub>S was documented previously (Kierner et al., 1995), but not in the current study. This is probably due to the difference in the H<sub>2</sub>S concentrations used (20 vs. 22 µM) and the length of exposure (1 h vs. 14 days), which could have a greater impact on the structure of the gills. Notably, Kierner et al. (1995) did not empirically measure the concentration of H<sub>2</sub>S/sulphide in the water, and the values presented were from theoretical calculations.

The distal gut of H<sub>2</sub>S-exposed fish showed moderate deviations from the normal structures and exhibited a decrease in the length of mucosal folds and widened *lamina propria*. These morphological changes in the distal gut mirrored the response of Atlantic salmon exposed to environmental stress, such as acute hypoxia and crowding stress (Djordjevic et al., 2021), as well as a model toxicant (Sanden and Olsvik, 2009b). Since the exposure time of the fish to H<sub>2</sub>S was transient, these distinct alterations may be influenced by other factors that could not be singled out in the present study. Interestingly, there were cases in which mucus cells increased in number and almost completely covered the surface of the simple folds. However, this increase did not show any statistically significant difference from the control.

The significant changes identified in the olfactory organs after exposure to H<sub>2</sub>S are characterised by rough epithelial surface, denser mucus cells at the mucosal tips, and random lesions such as a disrupted surface surrounded by mucus cells. Despite not being very responsive at the molecular level, the olfactory organ exhibits structural alterations from transient H<sub>2</sub>S exposure. This may indicate that the olfactory organ is sensitive to H<sub>2</sub>S, which was not captured by the targeted molecular profiling included in this study.

Even though the molecular data suggest a strong response from the skin, histological changes were minimal to non-existent. While there were sporadic alterations, including epithelial roughness and an apparent increase in mucus cells in the current study, the changes were not statistically significant. This suggests that the exposure of Atlantic salmon to H<sub>2</sub>S for a short period may not compromise the skin

morphology despite the pronounced molecular alterations. These apparent differences between histology and gene expression warrant in-depth investigation using more advanced tools that can provide better resolution of the response, such as transcriptome-wide analysis. This is currently being done by our group, but on a different experimental H<sub>2</sub>S exposure set-up.

#### 4.4. Mucosal and systemic metabolomic perturbations differ in Atlantic salmon transiently exposed to H<sub>2</sub>S

This is the first study demonstrating the consequences of H<sub>2</sub>S exposure in fish skin mucus and blood metabolomes. The two biological matrices showed different metabolomic responses to transient exposure to H<sub>2</sub>S. The magnitude of response was higher in the blood than the skin, as indicated by the number of significantly affected metabolites, which was 2 times higher in the former than the latter. The metabolomes of the two matrices also revealed that the low level had more impact on the skin mucus while the high level elicited more alterations in the blood. However, functional inferences of affected pathways indicate that metabolomic perturbations were more pronounced in the skin mucus, further indicating that H<sub>2</sub>S affected the mucosal more than the systemic metabolome of Atlantic salmon.

We next focus on the representative metabolites that were affected in the low-exposure group in the skin mucus and the high-exposure group in the blood. Hypoxanthine and taurine are two of the level 1 metabolites that were significantly affected by the low dose of H<sub>2</sub>S in skin mucus. Hypoxanthine is a naturally occurring purine derivative and a reaction intermediate in the metabolism of adenosine and the formation of nucleic acids by the salvage pathway (Hayman and Oxenham, 2016). In HT29 cells, hypoxanthine accumulates in response to H<sub>2</sub>S (Kumar et al., 2022) and is considered a checkpoint stress metabolite (Lee et al., 2018). Its increase may indicate a potential involvement in the physiological recovery from the stressful episode of transient H<sub>2</sub>S exposure.

Taurine has been identified to have an anti-stress function in fish (Mezzomo et al., 2019), but it remains to be functionally verified whether its reduction in the skin mucus of fish exposed to low H<sub>2</sub>S has implications in the stress response. On the other hand, 1-methyl adenosine and 4-guanibutyric acid are two level 1 metabolites that were significantly changed in the blood of the high H<sub>2</sub>S-exposed group. Both of these compounds are products of arginine and urea metabolism in fish (Kaushik et al., 1988; Mommsen and Walsh, 1992). Both of these metabolic processes have been shown to be regulated by H<sub>2</sub>S in mammalian models (Geng et al., 2007; Seifi et al., 2019) and may likely have the same regulatory function in salmon.

To further uncover the extent of the H<sub>2</sub>S-induced effects on the salmon metabolome, the functional analysis showed pronounced perturbations in skin mucus, which specifically affected tRNA charging, the superpathway of branched chain amino acid biosynthesis, and glucosylate biosynthesis from phenylalanine. Aminoacyl-transfer RNA (tRNA) synthetases define the genetic code by correctly pairing amino acids with their cognate tRNAs, and the faithful translation of the genetic code during protein synthesis is critical to the growth, development, and function of a living organism (Wang and Pan, 2016). Tailoring tRNA abundance is necessary to selectively regulate protein synthesis during stressful conditions (Torrent et al., 2018). Moreover, sulphur species have been shown to regulate tRNAs, and this interaction has implications for mitochondrial bioenergetics (Akaike et al., 2017; Takahashi et al., 2017).

We speculate that transient exposure to H<sub>2</sub>S subjected salmon to a stressful event affected protein synthesis at the skin mucosa, which was counteracted by activation of tRNAs to ensure the correct translation of genetic code. The branched-chain amino acids (BCAAs) valine, leucine, and isoleucine are essential substrates and important regulators in the synthesis of body proteins, substrates for energy production, and precursors for the formation of other amino acids (Holecsek et al., 2016). In rats, H<sub>2</sub>S plays a regulatory function in BCAA catabolism and mediates

critical cardiovascular protection in heart failure (Li et al., 2022). The significant impact in the superpathway of BCAA biosynthesis following transient H<sub>2</sub>S exposure can be explained by the previous observations linking the role of H<sub>2</sub>S in BCCAs, which is likely necessary for mucosal adaptation and recovery following H<sub>2</sub>S exposure.

#### 4.5. Conclusions

This study demonstrated that transient exposure to H<sub>2</sub>S influenced the key defence mechanisms of Atlantic salmon mucosa. Transient exposure to H<sub>2</sub>S induced molecular and histological changes in the four mucosal organs, but the latter were marginal. H<sub>2</sub>S affected the expression of the genes coding for sulphide-detoxification, mucins, cytokines, antioxidants, and apoptosis. While the distal gut was relatively unaffected, the gills and skin generally exhibited gene upregulation, whereas the olfactory organ displayed a predominant downregulation. These distinct responses imply a diverse and likely tissue-specific response as an adaptation and recovery strategy following H<sub>2</sub>S exposure.

Metabolomics revealed that transient exposure to H<sub>2</sub>S resulted in more mucosal than systemic perturbations. Overall, H<sub>2</sub>S interferes with mucosal and systemic responses in Atlantic salmon. These results offer insights into the physiological consequences of H<sub>2</sub>S in Atlantic salmon and contribute to resolving the biological underpinnings of the mucosal immunotoxicity of H<sub>2</sub>S in teleosts. In a broader context, the results are expected to contribute to addressing the H<sub>2</sub>S-related mortality events in Atlantic salmon by offering potential biomarkers for sulphidic response and opportunities for risk assessments.

The study provided a short-term single timepoint recovery response profiling of the mucosal organs to H<sub>2</sub>S, thus, revealing only a snapshot of physiological changes during recovery following H<sub>2</sub>S exposure. It is important in the future to investigate the temporal recovery response over a period to uncover whether exposure dose dictates the kinetics of physiological recovery from H<sub>2</sub>S exposure.

#### Author contributions

C.C.L. prepared the original project concept. J.H.B. and H.R.A. formulated the study design. J.H.B. and H.R.A. performed the trial and collected the samples. H.R.A. conducted the lab analyses and data visualisation. H.R.A., J.H.B., and C.C.L. interpreted the data. C.C.L. and J.H.B. supervised the student. H.R.A. wrote the first manuscript draft. All authors contributed to the article and approved the submitted version.

#### Declaration of Competing Interest

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

#### Data availability

Data will be made available on request.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aquaculture.2023.739595>.

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