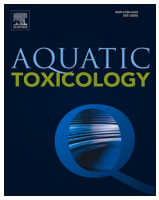
Aquatic Toxicology 260 (2023) 106574

Contents lists available at ScienceDirect

Aquatic Toxicology

journal homepage: www.elsevier.com/locate/aqtox



Fast and slow releasing sulphide donors engender distinct transcriptomic alterations in Atlantic salmon hepatocytes

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ARTICLE INFO

Keywords:
Cellular stress
GYY4137
Hydrogen sulphide toxicity
Fish health
Immunity

ABSTRACT

Hydrogen sulphide (H_2S) is a naturally occurring compound generated either endogenously or exogenously and serves both as a gaseous signalling molecule and an environmental toxicant. Though it has been extensively investigated in mammalian systems, the biological function of H_2S in teleost fish is poorly identified. Here we demonstrate how exogenous H_2S regulates cellular and molecular processes in Atlantic salmon (*Salmo salar*) using a primary hepatocyte culture as a model. We employed two forms of sulphide donors: the fast-releasing salt form, sodium hydrosulphide (NaHS) and the slow-releasing organic analogue, morpholin-4-ium 4-methoxyphenyl(morpholino) phosphinodithioate (GYY4137). Hepatocytes were exposed to either a low (LD, 20 μg/L) or high (HD, 100 μg/L) dose of the sulphide donors for 24 hrs, and the expression of key sulphide detoxification and antioxidant defence genes were quantified by qPCR. The key sulphide detoxification genes *sulfite oxidase 1* (*soux*) and the *sulfide: quinone oxidoreductase 1* and *2* (*sqor*) paralogs in salmon showed pronounced expression in the liver and likewise responsive to the sulphide donors in the hepatocyte culture. These genes were ubiquitously expressed in different organs of salmon as well. HD-GYY4137 upregulated the expression of antioxidant defence genes, particularly *glutathione peroxidase, glutathione reductase* and *catalase*, in the hepatocyte culture. To explore the influence of exposure duration, hepatocytes were exposed to the sulphide donors (i.e., LD versus HD) either transient (1h) or prolonged (24h). Prolonged but not transient exposure significantly reduced hepatocyte viability, and the effects were not dependent on concentration or form. The proliferative potential of the hepatocytes was only affected by prolonged NaHS exposure, and the impact was not concentration dependent. Microarray analysis revealed that GYY4137 caused more substantial transcriptomic changes than NaHS. Moreover, transcriptomic alterations were more marked following prolonged exposure. Genes involved in mitochondrial metabolism were downregulated by the sulphide donors, primarily in NaHS-exposed cells. Both sulphide donors influenced the immune functions of hepatocytes: genes involved in lymphocyte-mediated response were affected by NaHS, whereas inflammatory response was targeted by GYY4137. In summary, the two sulphide donors impacted the cellular and molecular processes of teleost hepatocytes, offering new insights into the mechanisms underlying H_2S interactions in fish.

1. Introduction

Hydrogen sulphide (H_2S), accompanying nitric oxide and carbon monoxide, belongs to a family of labile biological mediators termed gasotransmitters and plays essential roles in a wide array of physiological and pathological processes in many organisms (Mani et al., 2014; Tobler et al., 2016; Wu et al., 2018). H_2S can be generated either endogenously or exogenously. Both forms can have beneficial (i.e., signalling molecule, cytoprotection) and detrimental (i.e., cytotoxicity) consequences depending on several factors, including concentration, duration, and frequency of exposure. A typical characteristic of this flammable and colourless gas is a "rotten egg" odour that has long been known as an environmental toxicant (Xiao et al., 2018). It is produced mainly byanaerobic bacterial decomposition that utilises SO^{4-} to oxidise organic matter. On the other hand, low concentrations of H_2S are produced in multiple organs (including the gut, heart, brain, kidney, and aorta) (Linden et al., 2010; Olas, 2014), and has been shown to be a regulator of various biological systems, such as angiogenesis, immunity and redox homeostasis (Dilek et al., 2020; Xie et al., 2016; Zhou et al., 2016).

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https://doi.org/10.1016/j.aquatox.2023.106574
Received 26 August 2022; Received in revised form 8 May 2023; Accepted 11 May 2023
Available online 13 May 2023

Despite the limited number of published studies exploring the physiological functions of H_2S in fish, available knowledge offered insights into its ecotoxicity and regulatory function. Majority of the earlier studies demonstrated the toxicity of H_2S focusing mainly on its implications in fish farming, as H_2S-related mortality was a significant problem that entailed high economic cost (Black et al., 1994; Kiemer et al., 1995; Torrans and Clemens, 1982). H_2S exerts a toxic effect through the inhibition of oxidative phosphorylation, leading to failure of the proton transport across the mitochondrial membrane and impairing the proton-driven ATP synthase (Jiang et al., 2016). Key adaptation strategies to a toxic environment have been extensively investigated in the H_2S-tolerant shortfin molly (*Poecilia mexicana*) (Kelley et al., 2016; Pfenninger et al., 2014). Transcriptome-wide analysis revealed the involvement of genes associated with enzymatic H_2S detoxification and transport of oxidised sulphur species, oxidative phosphorylation, energy metabolism, and pathways involved in responses to oxidative stress in the adaptation to a sulphidic environment (Kelley et al., 2016). In recent years, novel insights into the biological functions of H_2S in rainbow trout (*Oncorhynchus mykiss*) and zebrafish (*Danio rerio*) have revealed that H_2S serves as an oxygen sensor in the gill chemoreceptors (Olson et al., 2008; Olsson, 2015). Endogenous production of H_2S in vertebrates is regulated by the enzymes *cystathionine β-synthase* (CBS) and *cystathionine γ-lyase* (CSE), and inhibition of both enzymes with aminooxyacetate (AOA) and propargyl glycine (PPG) negatively affected hypoxic hyperventilation (Porteus et al., 2014).

Sulphide salts such as sodium hydrosulphide (NaHS) and sodium sulphide (Na_2S) have been widely used to investigate the biological effects of H_2S in many cells, tissues and animals (Lee et al., 2011). Upon contact with water, these sulphide salts release H_2S instantaneously into an aqueous solution, thus often used to investigate acute exposure responses to H_2S. However, there are instances when cells are exposed gradually to H_2S, and these salts are not the ideal sulphide donors. Morpholin-4-ium 4-methoxyphenyl(morpholino) phosphinodithioate (GYY4137) is an organic molecule capable of releasing H_2S over extended periods of time (Li et al., 2008) that makes it an appropriate sulphide donor for exposing a biological system progressively to H_2S. Whereas the release of H_2S from NaHS in the culture medium was rapid, peaking at or before 20 min and almost undetectable by 48 h, H_2S release from GYY4137 was at a much lower rate, roughly 3x slower than NaHS, but the level could be sustained for up to 72 h (Lee et al., 2011; Sakuma et al., 2019).

Here we report the regulatory function of H_2S in Atlantic salmon, focusing on its consequences on liver hepatocytes. We employed two sulphide donors, NaHS and GYY4137, to reveal whether H_2S-releasing kinetics could impact hepatic responses. Regulation of genes involved in sulphide detoxification and oxidative stress following exposure to two concentrations of the sulphide donors was determined first, followed by a transcriptome-wide analysis of responses to either transient or prolonged exposure. The cellular and molecular responses of hepatocytes offered insights into the different physiological mechanisms that Atlantic salmon mount to sulphidic stress.

2. Materials and methods

2.1. Ethical statement

We confirm that the experimental protocols used for handling live fish were based on the Norwegian Animal Welfare Act (https://www.regjeringen.no/en/dokumenter/animal-welfare-act/id571188/) and performed in accordance with relevant guidelines set by the Norwegian Animal Research Authority. Key personnel in the trial possess a FELASA C certificate. Smolts (300 – 500g, 35 ppt reared in a flow-through system) were purchased from the Norwegian Institute for Water Research (NIVA) in Drøbak, Norway. Experimental fish did not show any signs of severe disorders based on the FishWell standard, though infrequent cases of mild fin damages were observed in some specimens.

2.2. Sulphide donors

Two sulphide donors (SD) with different theoretical release kinetics were used in the present study. The salt form, sodium hydrosulphide (NaHS), releases H_2S instantaneously into an aqueous solution and is considered a fast-release sulphide donor (Wu et al., 2017). The second sulphide donor was a slow-releasing H_2S donor, the organic analogue morpholin-4-ium 4-methoxyphenyl(morpholino) phosphinodithioate (GYY4137) (Li et al., 2008). We have earlier used these sulphide donors in Atlantic salmon nasal leucocytes (Cabillon and Lazado, 2022).

2.3. Identification of H2S detoxification genes

Three genes with a key function in H_2S detoxification were identified by searching the published genome of Atlantic salmon (*Salmo salar*) (ICSASG_v2): *sulfite oxidase* (*suox*) and two *sulfide: quinone oxidoreductase* paralogs *sqor1* and *sqor2* (accession nos XP_014022726.1, XP_014025721.1 and XP_013981468.1). Gene sequences were retrieved, and qPCR primers were designed with PerlPrimer (www.perlprimer.sourceforge.net), crossing intron/exon borders to avoid amplification of contaminating genomic DNA. The tissue expression of these genes was studied in eight individual salmon from the same cohort as with the fish used in the hepatocyte isolation. Six fish weighing approximately 350-400 g were supplied by NIVA and upon arrival at laboratory, they were euthanised by an overdose of isoeugenol (Aqui-S vet., MSD Animal Health Intervet International B.V., Netherlands). Thereafter, samples were taken from the liver, gills (second gill arch), skin (below the dorsal fin), olfactory organ (left), posterior intestine, eyes (left), stomach, muscle (same region as the skin sample), forebrain, spleen and head kidney. Samples were preserved in RNAlater™ (Thermo Fischer Scientific, 5x volume was used per tissue/organ) and kept at -80°C prior to RNA isolation. The RNAs and cDNAs were prepared, and expression profiling in different organs/tissues were conducted, as detailed in Section 2.7.

2.4. Isolation and culture of fish hepatocytes

Six fish from the same supplier were used at each isolation event. Hepatocytes were isolated by a two-step perfusion method earlier described (Dannevig and Berg, 1985; Seglen, 1976). Immediately following euthanasia by isoeugenol overdose, the liver was cleared of blood by perfusion of 20 mM ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich, Germany; suspended in a solution containing 1.43 mM sodium chloride [NaCl], 6.7 μM potassium chloride [KCl] and 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES]) via the hepatic vein followed by digestion with collagenase type 1 (1 mg/mL) (Worthington Biochemical Corp., suspended in a similar diluent as EDTA but added with calcium chloride [$CaCl_2$] at a final concentration of 1μM) for around 20-25 mins. The digested liver was carefully dissected out from the fish and placed in sterile L-15 medium. Cells were carefully dissociated from the enzymatically digested liver by gentle agitation using tweezers. The dissociated cell-tissue suspension was consequently filtered through 100 μm cell strainer to collect the cell solution. Thereafter, the cell suspensions were centrifuged at 120 x *g* for 2 min and the supernatant was removed. The cells were washed twice: first with L-15 and the second washing with 10% growth media (GM; Leibovitz's L-15 GlutaMAX Supplement (Gibco, ThermoFisher) with 10% foetal bovine serum (Sigma-Aldrich), 10 mM HEPES (Sigma-Aldrich), 1% Antibiotic-Antimycotic Solution (100x) (Sigma-Aldrich) and 0.5% sodium bicarbonate solution (7.5%) (Sigma-Aldrich)) following a similar centrifugation protocol. The collected cells were resuspended in 10% GM. The viability of cells was determined by the Trypan Blue exclusion method and the cell density was further determined in a haemocytometer. Cells were plated on 24 well culture plates (Nunc™ ThermoFisher) previously coated with ECL cell attachment matrix (Sigma-Aldrich) at a density for monolayer cells of 0.75×10^6 cells/well

in 1 mL 10% GM. The cells were cultured at 12°C and washed and after 20 h. Exposure to sulphide donor was performed 40 h after seeding.

2.5. Exposure to sulphide donors

Two independent *in vitro* exposure trials were performed and, on each occasion, six fish with an approximate weight of 300 g were used for hepatocyte isolation. In all exposure trials, solutions with sulphide donors were prepared just before their addition to the hepatocyte culture and were protected from light. In Trial 1, the hepatocytes were exposed to low (20 μg/L, LD) and high (100 μg/L, HD) doses of the sulphide donors and incubated for 20 h at 12°C. The doses were selected based on preliminary trials that these concentrations were physiologically active but not toxic to the hepatocytes. We aimed to trigger a response but also ensured that the stimuli were not toxic to the cells. One 24-well plate was dedicated for each sulphide donor (i.e., 6 wells = control, 6 wells = LD and 6 wells = HD). After the exposure period, the media was discarded, cells were washed once with sterile phosphate-buffered saline (PBS, Sigma-Aldrich), lysis buffer (Agencourt RNAdvance™ TissueTotal RNA Purification Kit, USA) was added, and the cells were scrapped. The suspended cells were transferred to a new sterile centrifuge tube and stored at -80°C until further processing as detailed in Section 2.7. Trial 1 focused on the expression of sulphide detoxification and antioxidant defence genes.

Trial 2 explored whether exposure duration could impact the global molecular responses of hepatocytes to the sulphide donor. Cells were isolated and cultured as described in Section 2.4. The approach on plate allocation for each sulphide donor was similar to Trial 1. In the transiently exposed group, cells were exposed to LD and HD of the sulphide donors for 1 hr. Thereafter, the solution-media was discarded, cells were washed once with sterile PBS and a new 10% GM was added. After 24 hrs, cells were collected for RNA processing as described earlier in this section. For group subjected to prolonged exposure, cells were treated and handled in a similar manner as in Trial 1, except that the cells were incubated with sulphide donors for 24 hr. Prior to collection, cells were washed once with sterile PBS and thereafter suspended in cell lysis buffer. Cell suspensions from Trial 2 were likewise kept at -80°C until RNA extraction. In both *in vitro* exposure studies, hepatocytes from 6 individual fish were used as biological replicates.

2.6. Effects of the sulphide donors on cell viability and proliferation

Hepatocytes were collected as described in Section 2.4 and cultured in 96-well ECL-coated plate at a seeding density of 6.25×10^4 cells/well. Cells were exposed to a similar manner as in Trial 2 detailed in Section 2.5. Cell viability was measured by CellTiter-Glo® Luminescent Cell Viability Assay (Promega). Cell proliferation was measured using the CyQUANT Proliferation Assay Kit (Thermo Fisher). Cells from each individual fish were seeded in duplicate wells, for a total of 12 wells for each treatment group and unexposed control group. Percentage viability and rate of proliferation was expressed relative to the control group.

2.7. RNA isolation and cDNA synthesis

RNA from the tissues and cells were isolated using Agencourt RNAdvance™ Tissue Total RNA Purification Kit (Beckman Coulter Inc., CA, USA) using the Biomek 4000 Benchtop Workstation. RNAs were quantified by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, DE, USA), and the quality was further assessed using an Agilent® 2100 Bioanalyzer™ RNA 6000 Nano kit (Agilent Technology Inc., Santa Clara, CA, USA). Samples had RIN values above 8.0. For RNA samples for quantitative real-time PCR (qRT-PCR), first-strand synthesis was performed from 300 ng RNA input using the High-Capacity RNA-to-cDNA Reverse Transcription kit (Applied Biosystems, USA) following a synthesis protocol of 25°C for 10 min, followed by 37°C for 60 min and then 5 min at 85°C. The expression of selected H_2S-detoxifying and antioxidant defence genes (primers are provided in **Supplementary File 1**) was quantified using a QuantStudio5 real-time quantitative PCR system (Applied Biosystems). The qPCR reaction mixture included 4 μL of diluted cDNA, 5 μL SYBR™ Green Master Mix (Applied Biosystems), and 1 μL of the forward and reverse primer. All samples were run in duplicate, including minus reverse transcriptase and no-template controls. The thermocycling protocol included pre-incubation at 95°C for 2 min, amplification with 40 cycles at 95°C for 1 s and 60°C for 30 s, and a dissociation step series of 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. Amplification efficiency was calculated from a five-point standard curve of 2-fold dilution series of pooled cDNA. The expression of the target genes was normalised using geometric average of the two-three reference genes, namely *elongation factor 1a* (*eef1a*) and *acidic ribosomal protein* (*arp*) for tissue distribution, while *eef1a, arp*, and *β-actin* (*actb*) for qRT-PCR in the hepatocytes (Nagasawa et al., 2012).

2.8. Microarray

Microarray analysis was performed to study the global transcriptomic responses of hepatocytes in Trial 2 using a custom-designed 15K Atlantic salmon DNA oligonucleotide microarray (Agilent Array, ICSASG_v2, all reagents used were from Agilent Technologies). Briefly, the One-Color Quick Amp Labelling Kit was employed for RNA amplification and Cy3 labelling using a 110-ng RNA template per reaction. Gene Expression Hybridization Kits were used for the fragmentation of labelled RNA. A 15-h hybridisation followed this in an oven held at 65°C and a constant rotational speed of 10 rpm. Thereafter, the arrays were washed successively with Gene Expression Wash Buffers 1 and 2 and were scanned using an Agilent SureScan Microarray Scanner. Preprocessing was carried out in Nofima's bioinformatics package STARS (Salmon and Trout Annotated Reference Sequences) (Krasnov et al., 2011).

2.9. Data analysis

For targeted qPCR and cellular assays, statistical evaluations were performed in Sigmaplot 14.0 Statistical Software (Systat Software Inc., London, UK). The data set was subjected to the Shapiro-Wilk test to evaluate normality and the Brown-Forsyth test to check for equal variance requirements for ANOVA. One-way ANOVA followed by the Holm-Sidak test was used to identify significant differences among the treatment groups in a particular sulphide donor in the expression of sulphide detoxification and antioxidant defence genes. For cell viability and proliferation, three-way ANOVA followed by multiple pairwise comparisons by Holm-Sidak test to identify differences between the sulphide donor types, among treatment doses and exposure duration, as well as the interaction of SDF types, treatment doses and exposure time, were performed. The level of statistical significance was set at $P < 0.05$.

Differentially expressed gene (DEGs) for the transcriptomic (microarray) results were defined by two thresholds: (1) t-test P-value < 0.05 between groups and (2) log2 expression difference between groups $> |0.8|$. This resulted in 456 DEGs for the GYY4137 treatment groups and 276 genes for the NaHS treatment groups. Both sets were filtered for existing functional annotation categories and combined to a single data set with 562 genes. The group means for each DEG were then clustered according to their Euclidean distance and by complete linkage in R (https://www.r-project.org, version 4.0.2, stats package, hclust() and dist() functions). From these results, a heatmap was created (heatmap.2 (), gplots package) and statistical overrepresentation of functional categories were tested by one-sided Fisher-tests ($P<0.05$, fisher.test(alterantive = "greater"), stats package).

3. Results and discussion

Hydrogen sulphide is a Janus-faced molecule – it can play as an important gaseous messenger molecule involved in several signalling

processes, but at certain levels, it is highly toxic to many physiological systems. Here we present evidence that key molecules for sulphide detoxification are expressed in many organs and tissues of Atlantic salmon, and by using a fish hepatocyte model, we revealed the cellular and molecular processes affected by the sulphide donors with different theoretical release kinetics.

3.1. *Essential H_2S-detoxifying genes are expressed in different organs and their expression is affected by the sulphide donors in hepatocytes*

Many organisms, including fish, are known to have a system for sulphide detoxification, keeping the balance and toxicity of H_2S under control (Tobler et al., 2016). We first asked the question of whether Atlantic salmon organs and tissues (i.e., liver, gills, skin, olfactory organ, posterior intestine, eyes, stomach, muscle, forebrain, spleen and head kidney) express some crucial molecules for sulphide detoxification. Based on known functions, we focused on the *sulfite oxidase* (*suox*) and the two salmon paralogs of *sulfide quinone oxidoreductase* (*sqor*) of which we successfully managed to design gene specific primers. *Sulfite oxidase* is a molybdenum-containing enzyme located in the intermembranous space of the mitochondria which oxidises sulphite to sulphate in a two-electron oxidation. It protects the cells from sulphite toxicity (Cohen et al., 1971). On the other hand, *sulfide:quinone oxidoreductase* is a catalytic enzyme in the first step of mammalian H_2S metabolism (Jackson et al., 2015). This enzymatic oxidation will subsequently lead to the excretion of oxidised sulphur species (Hildebrandt and Grieshaber, 2008; Lagoutte et al., 2010).

The majority of the tissues and organs analysed expressed the three sulphide detoxification genes, except for the spleen and head kidney, while the expression in muscle and forebrain was almost negligible. (Fig. 1A). The genes examined were highly expressed in the liver and eyes, which is not surprising since liver and eyes have been identified to be involved in both endogenous and exogenous sulphide metabolism (Allore et al., 2021; Wu et al., 2019). Mucosal organs, i.e., gills, skin, olfactory rosette, posterior intestine and stomach, likewise demonstrated pronounced expression of these genes, particularly the two *sqor* paralogues. In mammalian systems, the mucosa is exposed to H_2S either from the bacteria residing on the surfaces (Levitt et al., 1999; Wallace, 2012) or from the environment (Haouzi et al., 2016). Earlier, we have shown that leukocytes from the olfactory organ of Atlantic salmon responded to sulphide donors by activating an array of genes including those involved VEGF ligand-receptor interactions, oxidative stress, innate and adaptive and adaptive immunity, and interleukin signalling (Cabillon and Lazado, 2022). It was also reported that *soux* is critical in the gills of the shortfin molly thriving in a sulphidic environment (Tobler et al., 2014). Low to undetectable basal expression of the three genes was documented in the muscle, forebrain, spleen and head kidney, and it remains to be elucidated whether the expression is generally low in these tissues, or it is stimulation dependent. Nonetheless, evidence in other animal models suggests that H_2S is a regulatory molecule in homeostasis, pathophysiological changes and therapeutic interventions in these organs (Chi et al., 2019; Veeranki and Tyagi, 2015; Zhang et al., 2017).

After identifying that the liver pronouncedly expressed these key genes (Fig. 1A), we isolated hepatocytes and exposed them to either a fast-release sulphide donor (i.e., NaHS) or a slow-release sulphide donor (i.e. GY4137) to investigate whether these marker genes were responsive to sulphide stimuli (Fig. 1B). Though *soux* expression appeared to be affected by the two sulphide donors at the higher dose, the change was not statistically significant to the unexposed cells. The two *sqor* genes, particularly *sqor1,* were responsive to the sulphide donors. The expression of *sqor1* in hepatocytes was significantly elevated relative to the control group after exposure to NaHS, and the effect was not dose dependent. For GYY4137, a dose-dependent response was identified where *sqor1* expression was significantly higher in the 100μg/L group than in the control and 20μg/L groups. Expression of *sqor2* was only

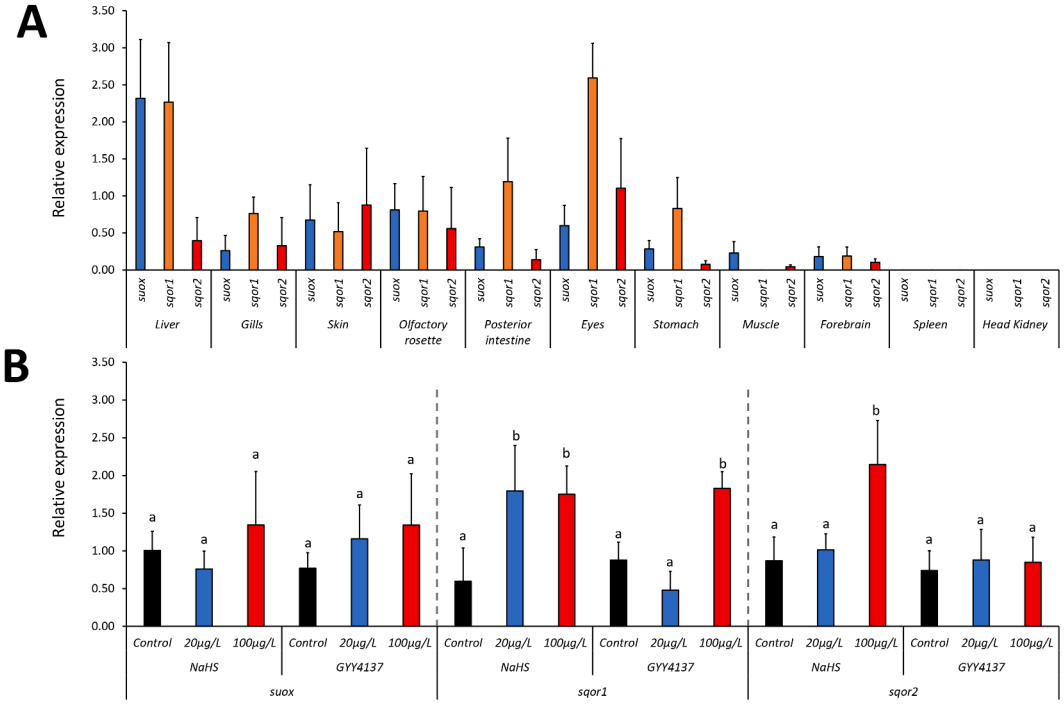

Fig. 1. Tissues distribution of key H_2S-detoxifying genes and their regulation in hepatocytes by two sulphide donors. A) Expression of *sulfite oxidase* (*suox*) and *sulfide: quinone oxidoreductase* (*sqor*) in different tissues of Atlantic salmon smolts. Values are presented as mean ± standard deviation (SD) of 6 individual (n=6) specimens weighing approximately 350 g. **B)** Expression of *soux* and *sqor1 and 2* in Atlantic salmon hepatocytes stimulated with either sodium hydrosulfide (NaHS) or morpholin-4-ium 4-methoxyphenyl(morpholino) phosphinodithioate (GYY4137). The hepatocytes were treated with sulphide donors at two concentrations for 24 h before cells were collected for gene expression analysis. Values are presented as mean ± SD of 6 individual fish (n=6). Different letter notations indicate that the gene expression among the treatment groups in a particular sulphide donor is statistically significant at $P<0.05$.

affected by NaHS, and a significantly elevated transcript level was noted in the 100μg/L group compared with the two other groups. These results indicate that *sqor* likely has a crucial H_2S detoxifying function in salmon hepatocytes, confirming earlier evidence of its role in sulphide response in the shortfin molly (Tobler et al., 2014). Moreover, both sulphide donors modulated the expression of these marker genes in a predominantly dose-dependent manner.

3.2. Sulphide donors induce changes in the expression of antioxidant defence genes in hepatocytes

One of the known physiological consequences of many toxicants is oxidative stress, a state where reactive oxygen species (ROS) production exceeds the buffering (e.g. neutralisation, sequestration) capacity of the antioxidant defence systems that may lead to pathophysiological abnormalities when not controlled (Slaninova et al., 2009). Some antioxidant defences include superoxide dismutases, peroxidases, glutathiones and catalases (Martínez-Álvarez et al., 2005). H_2S could trigger oxidative stress in mammalian hepatocytes (Guo et al., 2021; Xiao et al., 2018), but there is no clear evidence of such a consequence in fish liver. We have earlier identified that the sulphide donors could alter the oxidative stress response pathway in salmon nasal leukocytes (Cabillon and Lazado, 2022), and we believe that such a mechanism may likely be at play in hepatocytes. This hypothesis is supported by a study in shortfin molly showing that the oxidative stress pathway is crucial for adaptation in sulphidic environments (Kelley et al., 2016). Here we have identified that antioxidant defences in Atlantic salmon hepatocytes responded to the sulphide donors (Fig. 2). Except for *gsta* and *mnsod*, the expression of antioxidant genes was significantly affected by the sulphide donors in a dose-dependent regulation; GYY4137-exposed cells exhibited pronouncedly such profile. The expression of *gr* (Fig. 2A) and *cat* (Fig. 2B) was significantly affected by GY4137 at the highest dose but not by NaHS. On the other hand, *cu/zn sod* transcript level was significantly elevated by the highest dose of NaHS, but no such response was elicited by GYY4137 (Fig. 2B). Both sulphide donors significantly elevated the expression of *gpx*, where both doses of GYY4137 demonstrated such a consequence (Fig. 2A). The increased expression of these antioxidant markers suggests that oxidative stress might have been triggered by the sulphide donors, likely by inducing ROS imbalance. The antioxidant system is involved in detoxifying ROS through a series of enzymatic pathways, thus providing a protective mechanism to the cells. Glutathione has been shown to increase in the presence of H_2S, and such elevation suppresses oxidative stress in mitochondria in mammalian models (Yuka Kimura et al., 2010). The expression profile demonstrated that the slow-release sulphide donor had a more considerable impact on the expression of antioxidant defence in salmon hepatocytes. The slow release could have progressively triggered oxidative stress but at the same time allowed the cells to muster a robust response to counteract the stress stimuli. The release kinetics of these two sulphide donors appear to play an important regulatory factor in the expression of these antioxidant defence genes. In hypertensive pregnancy complicated by fetoplacental growth restriction, GYY4137 reduced placental oxidative stress caused by hypertension, while more significant increases in oxidative stress were found in NaHS group (Zochio et al., 2019). In another mammalian study, regulation of oxidative stress in the muscle by H_2S did not reveal clear distinction between the two sulphide donors (Xu et al., 2022).

3.3. Cellular viability and proliferation are affected by the duration of exposure

Transient exposure to the sulphide donors did not significantly affect the viability and proliferation of hepatocytes (Fig. 3). However, prolonged exposure significantly impacted the viability and proliferation of hepatocytes, where a significant reduction was observed relative to the transiently exposed cells, but only in the groups exposed to NaHS and not to GYY4137. In addition, in the prolonged exposure cells, hepatocytes exposed to NaHS showed a significantly lower proliferation rate than the GYY4137-exposed cells. Moreover, concentration did not play a significant role in cell viability and proliferation under transient or prolonged exposure. It has been reported earlier that H_2S could have a dual role in the growth and migration of human hepatocytes by stimulating or suppressing growth, migration and proliferation via the EGFR/ERK/MMP-2 signalling pathway (Wu et al., 2017). The EGFR/ERK/MMP-2 pathway is a fundamental mitochondrial process in the proliferation, migration, and invasion of hepatocellular carcinoma

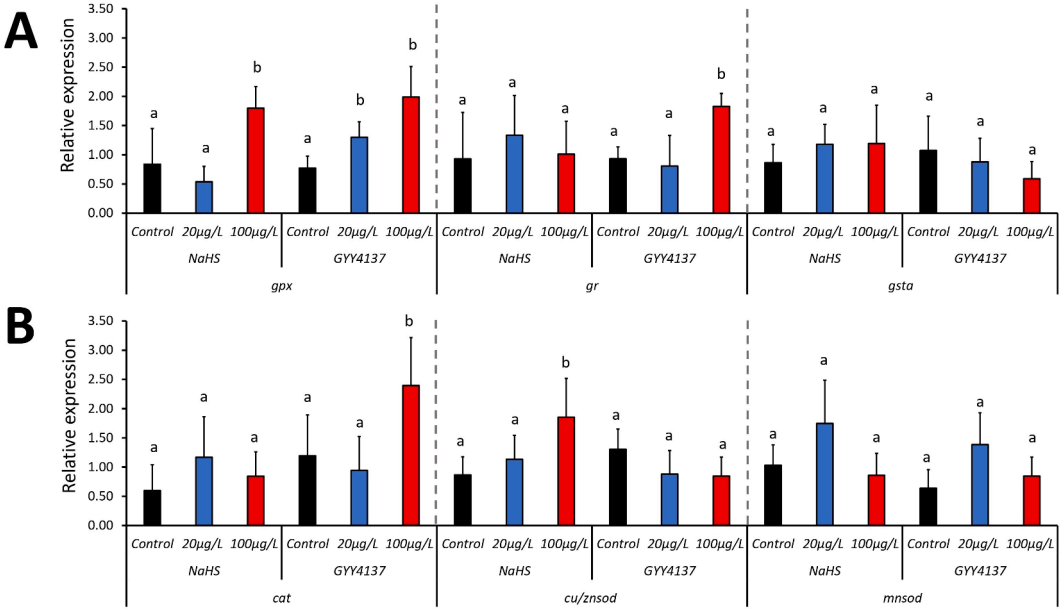

Fig. 2. Expression of key antioxidant defence genes in Atlantic salmon hepatocytes stimulated with either NaHS or GYY4137 for 24 hr. Six antioxidant genes were studied: **A)** *glutathione peroxidase* (gpx), *glutathione reductase* (*gr*), *glutathione S-transferase* (*gsta*), **B)** *catalase* (cat), *copper/zinc superoxide dismutase* (*cu/znsod*), *manganese superoxide dismutase* (*mnsod*). Values are presented as mean ± SD of 6 individual fish (n=6). Different letter notations indicate that the gene expression among the treatment groups in a particular sulphide donor is statistically significant at $P<0.05$.

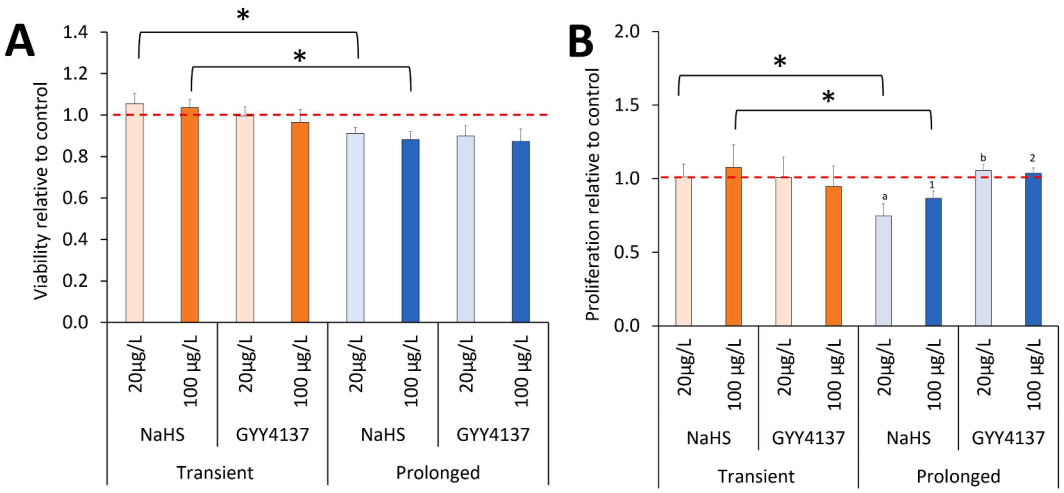

Fig. 3. A) Viability and B) proliferation of Atlantic salmon hepatocytes stimulated with the two sulphide donors either to transient or prolonged exposure duration. A) viability and B) proliferation are expressed relative to the control, unexposed cells (1.0), designated by the red dashed line. Values are presented as mean ± SD of 6 individual fish (n=6). Asterisk (*) indicates that viability/proliferation significantly differed between the transient and prolonged exposed group, particularly sulphide donor and concentration. Different letter/number notations indicate that the proliferation rate significantly differs between the two sulphide donors in a particular dose. Statistical significance was set at $P<0.05$.

(Tomas et al., 2014). This pathway may likely be involved in the cellular changes in the salmon hepatocytes exposed to H_2S as one of the gene clusters substantially affected in the NaHS-prolonged-exposed group was mitochondrial metabolism (Fig. 6). Overall, cell viability and proliferation results indicate that prolonged exposure to the sulphide donors interfered with these fundamental cellular processes in hepatocytes.

3.4. Hepatocytes exhibit distinct transcriptomic profiles between the two sulphide donors

To provide a broader understanding of how the sulphide donors affected salmon hepatocytes, microarray transcriptomic analysis was performed in a group of cells exposed to the sulphide donors, either transient or prolonged, at two doses. Overall, hepatocytes exposed to GYY4137 elicited a higher number of differentially expressed genes (DEGs) than NaHS-exposed cells (Fig. 4), indicating that gradual rather than instantaneous release of H_2S could trigger more transcriptomic alterations in hepatocytes. The DEG set for GYY4137 contained 456 genes (389 unique genes), and the set for NaHS contained 276 genes (241 unique genes). Each gene in the two sets had at least one significant regulation in one of the four treatment groups (transient or prolonged treatment, high or low dose). Duration of exposure significantly impacted the regulation of hepatocyte transcriptome, as groups under prolonged exposure had a higher number of DEGs than transiently exposed cells, regardless of the sulphide donor. For GYY4137-exposed

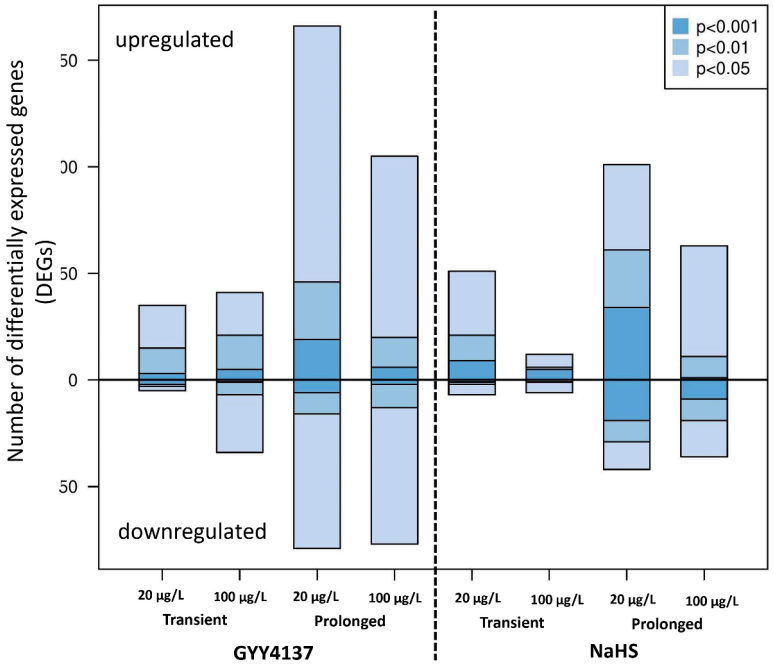

Fig. 4. The number of differentially expressed genes (DEGs) in Atlantic salmon hepatocytes exposed to sulphide donors. The four bars to the left show numbers for GYY, and the four bars to the right, are numbers for NaHS. The four bars represent the four treatment groups (short or long treatment, high or low dose). Each bar was split into three P-value levels, represented by shades of blue.

cells, a high dose elicited a higher number of DEGs than low following transient exposure; however, this was reversed following prolonged exposure. For NaHS, on the other hand, the low dose had a higher number of DEGs for both transient and prolonged exposed groups.

The DEGs in the two sets (i.e., YY4137 and NaHS) were split for up- or downregulation, filtered for unique entries and genes significantly regulated in any of the four treatment groups. The Venn diagram shows that the GYY4137 and NAHS data sets were largely different from each other (Fig. 5A). Seven genes were upregulated in both sets (e.g., *cadherin 2, neuronal*). Cadherin is a large class of type-1 transmembrane proteins important for cell-cell adhesion. E-cadherin level has been shown to increase when TGF-beta1-treated A549 cells were exposed to exogenous H_2S, where it was identified as a critical regulator in preserving epithelial phenotype (Chen et al., 2021); a related role might likely play in salmon hepatocyte. In both sets, a similar number of genes was down-regulated (e.g., glutathione reductase, mitochondrial, apoptosis-inducing factor). This result did not correspond to the targeted qPCR in Fig. 2, where *gr* was shown to be upregulated in GYY4137. The discrepancy could be related to variability in cellular response of different fish batches for cell studies. H_2S has a therapeutic role in some cases; for instance, it can inhibit apoptosis in the mammalian liver with pathological anomalies (Chen et al., 2021; Wu et al., 2020). The downregulation of *apoptosis-inducing factors* could be involved in such a process in the salmon liver, though it remains to be functionally validated. Eight genes were down-regulated in GYY4137 but upregulated in NaHS, including *interleukin-6 (il6),* which is a pleiotropic cytokine that controls haematopoiesis, inflammation, and several other immune processes in mammals. In salmonids, *il6* has been shown to regulate patrolling macrophages and reduce iron availability, thus limiting the infection spread (Costa et al., 2011). In the mammalian model, H_2S protects from methotrexate hepatotoxicity by modulates the IL-6/STAT3 pathway (Fouad et al., 2020). We argue that its regulatory role in salmon hepatocytes is influenced by the sulphide donors, and the kinetics of release induced different *il6* regulation to resolve the impact of H_2S.

Since the two sulphide donor data sets were very apparently different from each other, we examined the groups separately (Fig. 5B, C). The association of the numbers of DEGs from the four treatment groups in the GYY4137 data set are shown in Fig. 5B. It is evident that the duration (transient versus prolonged) and not the dose caused substantial effects on the hepatocytes. Like the GYY4137 treatment, NaHS-treated cells showed that the treatment duration had a stronger effect than the dose (Fig. 5C).

3.5. Sulphide donors affect pathways involved in mitochondrial metabolism and immunity in hepatocytes

The DEGs were grouped based on their functional categories using the STARS annotation pipeline developed in-house (Krasnov et al., 2011). The functional groupings generated six distinct clusters (Fig. 6, Supplementary File 2). Cluster 1 is the second-largest cluster with 182 genes and is mainly represented by molecules involved in immune T-cells and sugar metabolism. The two sulphide-exposed groups showed different profiles: in the GYY4137-exposed groups, transient exposure resulted in a negligible response, while prolonged exposure caused substantial downregulation, and these responses were not dose-dependent. NaHS-exposed cells showed upregulation which was not affected by dose and exposure duration. Cluster 2 comprised 44 transcripts, and downregulation was a typical response, where such a profile was more pronounced following transient exposure to GYY4137 but in prolonged exposure for NaHS. The magnitude of response was higher in NaHS-exposed cells. Many of the genes identified affected have vital functions in mitochondrial metabolism. Cluster 3 is a group of 11 genes mainly involved in cellular exocytosis. Upregulation was predominantly exhibited by cells transiently exposed to the sulphide donors, while downregulation was characterised following prolonged exposure. Cluster 4 is the third largest cluster with 119 genes, where upregulation was a common response between the two sulphide donors. The response magnitude was highest in cells following prolonged exposure to NaHS, though the response was not dose dependent. Most of these genes are involved in the immune response. Cluster 5 is the most

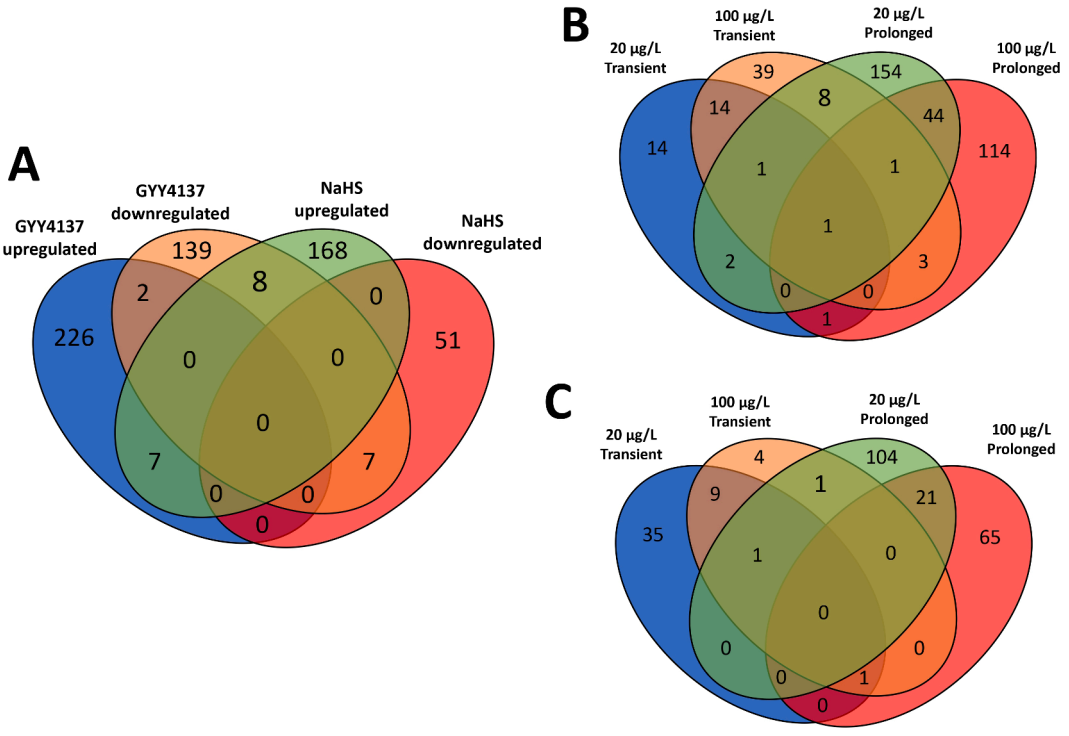

Fig. 5. Venn diagrams of different comparisons of DEGs in Atlantic salmon exposed to sulphide donors. A) Visualisation of numbers of unique and shared DEGs in the two sulphide donors. **B)** Venn diagram for the DEGs in the four GYY4137 treatment groups (i.e., two doses, two exposure durations). **C)** Venn diagram for the DEGs in the four NaHS treatment groups.

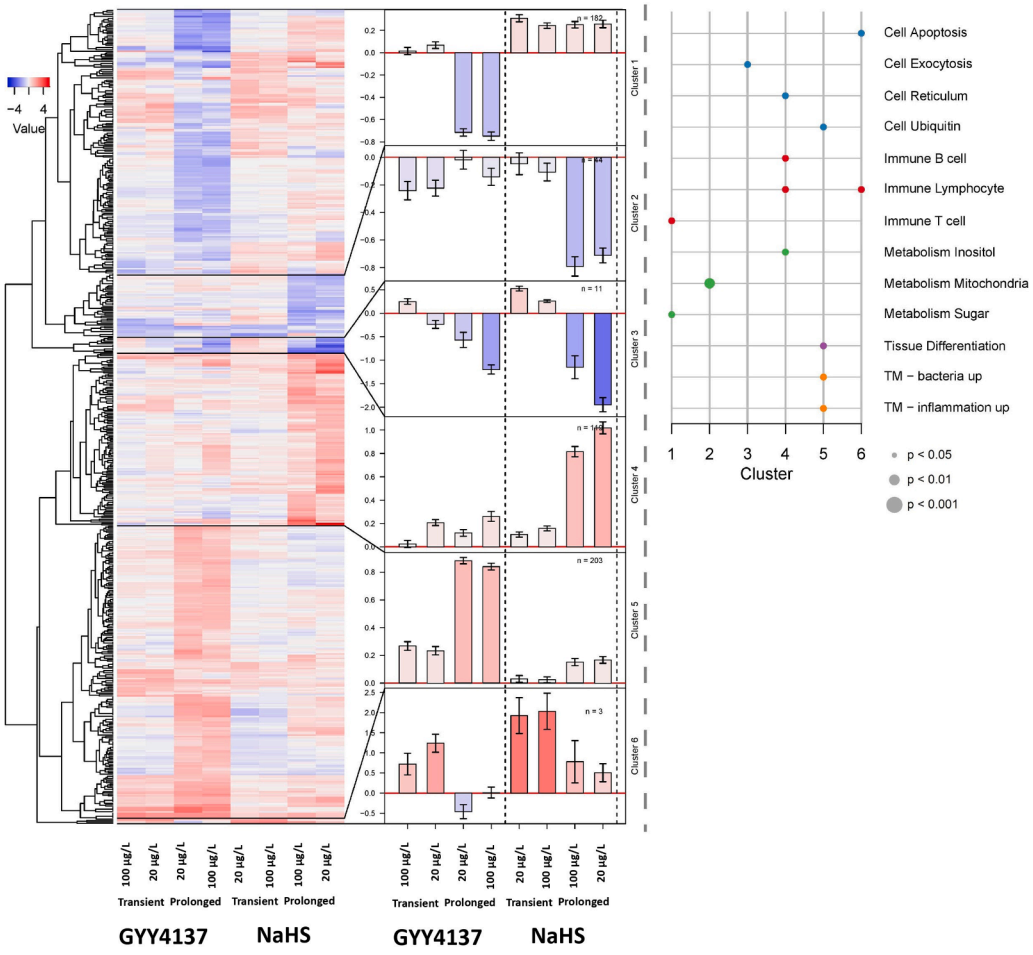

Fig. 6. Transcriptomic changes in Atlantic salmon hepatocytes engendered by two sulphide donors, NaHS and GYY4137. The sulphide donors were provided in two doses (i.e., 20 and 100 µg/L) and by two exposure durations (i.e., transient, or prolonged). **A)** The heatmap on the left shows the down- and upregulation of DEGs in a colour gradient from blue to red. The dendrogram was split into 6 clusters, and the mean values for genes within these clusters are represented in bar plots (error bars show +/- standard error of the mean) in the centre. B) Functional gene categories of the 6 clusters. The size of the dot represents the P-value according to the Fischer's Exact Test.

prominent gene cluster, with 203 transcripts displaying upregulation following exposure to the sulphide donor. The cluster shows an opposite profile compared with cluster 4, where the magnitude of response was pronounced in GYY4137-exposed cells under prolonged exposure. This response was not-dose dependent. Many of the genes in this cluster are involved in tissue differentiation and inflammation. The last cluster is Cluster 6, with only three genes involved in lymphocyte response.

At lower physiological concentrations, H_2S confers protection against cellular stress by promoting mitochondrial biogenesis and function (Murphy et al., 2019). The cellular phenotypic response indicates that the level used in the current trial was moderately toxic for the hepatocytes, particularly reducing viability and proliferation potential following prolonged exposure (Fig. 3). This moderate toxicity can be partly explained by reversible inhibition of electron transport and ATP production in mitochondria (Borisov and Forte, 2021). This relationship was mirrored by the transcriptomic responses where the sulphide donors downregulated most of the genes involved in mitochondrial metabolism, including *electron transfer flavoprotein-ubiquinone oxidoreductase, mitochondrial,* and *ATP synthase epsilon chain, mitochondrial*. Further, the fast release (i.e., NaHS) of H_2S resulted in substantial changes in these groups of genes than by the slow-release donor (i.e., GYY4137). Therefore, fast release and prolonged exposure period caused a metabolic solid impact on the hepatocytes, thereby contributing to its cytotoxic effect. We speculate that the gradual exposure to the sulphide donors may have allowed the hepatocytes to adapt to a sulphide-rich microenvironment metabolically, and thus, the downregulation was not as strong as with NaHS-exposed cells. The strong metabolic inhibition by NaHS somehow corroborated the proliferation data, where there was an observed slight reduction under prolonged exposure (Fig. 3).

H_2S, as a gasotransmitter, has been shown as an endogenous regulator of the mammalian immune system (Dilek et al., 2020). It can regulate the functions of different cell types associated with innate (e.g., neutrophils, macrophages, dendritic cells, natural killer cells, mast cells, basophils, and eosinophils) and adaptive immunity (e.g., T and B lymphocytes). We have recently demonstrated that such a function is also present in fish, as our group had shown that both forms of the sulphide donors used in the present study affected the immunological properties of Atlantic salmon nasal leukocytes (Cabillon and Lazado, 2022). The liver is a least explored organ involved in immunity, but a growing body of evidence demonstrates its crucial function as an immunological organ both in mammals and fish, which is characterised by the presence of hepatic immune cells (e.g. Kupffer cells, neutrophils) and constant exposure to circulating antigens and endotoxins (Robinson et al., 2016; Wu et al., 2016). Hepatocytes are central drivers of the systemic acute-phase proteins in response to infection and inflammation (Crispe, 2016). We showed that the two sulphide donors modulated the immunological properties of salmon hepatocytes differently – NaHS mainly targeted the pathways of immune cells and their associated effector molecules while GYY4137 targeted the transcriptional modules of inflammation, though both in agreement that prolonged exposure carried a more robust response. To our knowledge, this is the first evidence in fish that H_2S regulates hepatic immune functions, and these transcriptional changes further support the immunomodulatory role of H_2S.

4 Conclusions

The study revealed that Atlantic salmon ubiquitously express genes

involved in sulphide detoxification which are crucial regulatory molecules of response to H_2S. Liver and mucosal organs demonstrated higher expression of these sulphide detoxification genes. The pronounced expression in this group of genes in mucosal organs is likely related to their spatial feature where H_2S-producing bacteria reside on these surfaces, and they are likewise exposed continuously to the external environment where exogenously generated H_2S can be found. The liver is a key organ for xenobiotic metabolism and, as such, plays a role in H_2S response. Targeted gene expression profiling showed that the sulphide donors, NaHS and GYY4137, altered the expression of sulphide detoxification and antioxidant defence genes in hepatocytes.

Moreover, transcriptome-wide profiling of the hepatocytes exposed to the two sulphide donors demonstrated that they were not heavily influenced by dose but rather by exposure duration. The inherent feature of NaHS and GYY4137, which governs their H_2S-releasing pattern, contributes to the magnitude of responses provoked by the hepatocytes. The slow release of H_2S from GYY4137 triggered more substantial transcriptomic changes in hepatocytes than the slow release from NaHS. Such a distinct response must be considered in future trials using these compounds as sulphide donors. Inhibition of mitochondrial metabolism was evident in the transcriptomic response, which supported the moderate cytotoxicity of H_2S in hepatocytes. Nonetheless, the exposure set-up described in this study was not highly toxic to the hepatocytes but only enough to trigger physiological changes. The transcriptomic alterations revealed that H_2S is a potent modulator of the immune functions in hepatocytes. Future studies must explore how different *in vitro* conditions influence the release kinetics and dissolution of H_2S in the aqueous solution. The results provided evidence of the cellular and molecular mechanisms Atlantic salmon hepatocytes employed to respond to sulphide donors with different H_2S-releasing patterns, thus advancing our understanding of the regulatory functions of H_2S in fish.

Authors' contributions

- CCL and ØA conceived the research idea.
- CCL and ØA designed the trial.
- CCL and VV performed the fish trial.
- CCL and VV collected the samples.
- CCL and VV performed the lab analyses.
- CCL and GT handled and processed the data.
- GT visualised the data.
- CCL, GT and ØA interpreted the data.
- CCL wrote the first draft of the manuscript.
- All authors contributed to the writing and review of the final version of the manuscript.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

Acknowledgment

The Research Council of Norway financed the study (H2Salar, ref. 300825). We thank Bente Ruyter for her assistance and advice on hepatocyte isolation and the personnel of the Norwegian Institute for Water Research in Solbergstrand for facilitating the fish used in the trial.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.aquatox.2023.106574.

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