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Exogenous sulphide donors modify the gene expression patterns of Atlantic salmon nasal leukocytes

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ABSTRACT

Hydrogen sulphide (H_2S) is a known mediator of immunity, but the regulatory function of its exogenous form is not well understood in fish particularly in the mucosa. Here we report transcriptomic changes in the nasal leukocytes of Atlantic salmon (*Salmo salar*) following exposure to two forms of H_2S donors – the salt sodium hydrosulfide (NaHS) and the organic analogue morpholin-4-ium 4-methoxyphenyl (morpholino) phosphinodithioate (GYY4137). Nasal leukocytes were exposed to three concentrations (1, 10 and 100 μM) of either of the two H_2S forms for 24 h before the cells were checked for viability and collected for microarray analysis. Though cellular viability was minimally affected by the exposure to two H_2S donors, GYY4137-exposed cells exhibited reduced viability compared with the NaHS group at the highest dose. The H_2S-induced transcriptomic changes in the nasal leukocytes were concentration-dependent regardless of the sulphide forms. However, a larger number of differentially expressed genes (DEGs) were identified in the NaHS-exposed versus GYY4137-exposed groups across concentrations. In all comparisons, at least 53% of the DEGs identified were significantly upregulated. Gene ontology (GO) terms enriched in the lists of upregulated DEGs at higher concentrations included ferric iron binding. A comparison of the two H_2S forms showed a clear grouping of different GO terms relative to concentrations. Pathway enrichment analysis revealed a significant influence in VEGF ligand-receptor interactions, oxidative stress, innate and adaptive immunity, and interleukin signalling especially at higher concentrations. Congruence analysis demonstrated that there were 16 GO terms overlapping; of these, 12 were upregulated by both sulphide donors including several involving iron binding and transport. The study offers the first molecular insights into how fish nasal leukocytes respond to exogenous H_2S, and the results will be vital in resolving the regulatory function of H_2S on mucosal immunity in fish.

1. Introduction

Olfaction is one of the oldest senses in the animal kingdom and facilitates odour-based identification of food, potential mating partners, dangers, and enemies; hence, it is often the most important way of interacting with the environment [1]. In land animals, the olfactory system detects low concentrations of airborne chemical substances while aquatic vertebrates such as teleost fish detect waterborne odorants, chemicals, or pollutants [2]. Besides its role in olfaction, the olfactory organ has a fundamental defence function as characterised by a mucosal microenvironment with a myriad of molecular and cellular immune components [2,3]. The nasopharynx-associated lymphoid tissue possesses B-, T-, and myeloid cells in addition to an array of innate and adaptive immune molecules [4]. These intricate layers of defence protect and maintain the balance in the nasal microenvironment, which is crucial because the olfactory organ is a portal of entry for several pathogens and is attacked by waterborne chemical stressors [3,4].

Hydrogen sulphide (H_2S) is a naturally occurring compound that is generated either endogenously or exogenously and is common in many physical and biological systems [5]. Substantial evidence has elucidated the role of endogenously generated H_2S as a gasotransmitter and a redox-active sulphur species that acts as an antioxidant and signalling molecule to support cellular functions [6–8]. On the other hand, the exogenously produced counterpart is a water-soluble and colourless gas with the distinct odour of rotten eggs. It is a potentially toxic gas produced by anaerobic bacterial decomposition of protein and other sulphur-containing organic matter [9,10]. Its toxicity mechanism is via interruption of mitochondrial cellular respiration through binding to

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and inhibiting cytochrome C oxidase in complex IV of the electron transport chain (the last complex in the chain before ATP synthesis) [11].

H_2S is a known regulator of immunity both at steady and stimulated states [6,12]. The pharmacological duality of H_2S function in host immunity has been demonstrated. It exhibits physiological, regulatory, and/or modulatory effects. It is a cytoprotective, antioxidant, and anti-inflammatory agent, but it may pose detrimental consequences as well including pro-oxidant effects and cytostatic or cytotoxic responses. These functions are controlled by an optimal H_2S zone dictated by its level during biosynthesis or external sulphide donation [6]. Through these processes, H_2S participates in the maintenance, surveillance, and activation of immune functions. Immune cells have been shown to be targets of H_2S-mediated regulation. For example, oxidants and free radicals are produced in activated neutrophils and react with H_2S or polysulfides to produce secondary and tertiary reactive species that may augment the defence mechanism [6,13]. Moreover, sulphite may directly activate neutrophil functions including cytokine production, adhesion, and chemotaxis [14]. The duality of H_2S function is well described in the complex feedback loop mechanisms in macrophages where H_2S promotes activation, polarisation, and inflammasome formation; the activated macrophages, in turn, influence the intrinsic H_2S synthetic machinery following external stimuli [12]. These interactions have not been demonstrated in fish despite the numerous evidence that H_2S plays a crucial physiological function in many biological processes in fish including oxygen sensing, chemoreception, and energy metabolism [15–17]. Moreover, most of the available knowledge of H_2S in fish focuses on the endogenous form; little is known about how the exogenous counterpart impacts physiological processes including immunity.

H_2S attacks the olfactory organ of humans [7,18], and we expect that such a mechanism is at play in waterborne H_2S for fish. Using the primary culture of the nasal leukocytes, this study elucidated the immunological effects of exogenous H_2S through two sulphide donors: sodium hydrosulfide (NaHS) and morpholin-4-ium 4-methoxyphenyl (morpholino) phosphinodithioate (GYY4137). We used them to study the transcriptomic landscape of the olfactory mucosa of a teleost fish. NaHS releases H_2S instantaneously into an aqueous solution while GYY4137 is a slow-releasing H_2S donor [19]. We employed different association tests and computational-based functional analyses to identify genes that were H_2S-sensitive and biological processes that were altered as an adaptive and protective strategy towards H_2S immunotoxicity. The use of Atlantic salmon (*Salmo salar*) as a model in this study has applied importance since H_2S-related mortality is prevalent in recent years [10]. Biological knowledge of this interaction is scarce in this species thereby posing a significant challenge in understanding the threats and developing preventive measures. The H_2S-responsive molecules identified in this study are potential biomarkers for early warning signal of H_2S levels in production systems.

2. Materials and methods

2.1. Isolation of leukocytes from olfactory organs

Leukocytes from the olfactory organ of both sides of the nares were isolated from salmon smolts (ca 80–90 g) with a similar genetic background following a method described earlier [3]. Briefly, fish were humanely euthanised with an anaesthetic overdose (Aqui-S, MSD Animal Health, Norway), and the olfactory organs were dissected and immediately placed in a modified L-15 on ice (supplemented with 5% foetal bovine serum, 1% Penstrep, 1% HEPES, NaCl). The tissues were cut into small pieces (0.5–1 cm) followed by mechanical dissociated via constant agitation at 4 °C for 30 min. The cell supernatant was collected, and the remaining tissue fragments were suspended in a modified L-15 medium. The mechanical dissociation step was repeated four times. The supernatant was collected at each repetition. After that, the sample was combined with previous collections and stored temporarily at 4 °C. The remaining tissue fragments were treated with EDTA (1 mM) and DTT (0.9 mM) in phosphate-buffered saline (PBS) at 4 °C for 30 min with constant gentle agitation. After the PBS supernatant was discarded, the remaining tissue fragments were subjected to enzymatic digestion in collagenase solution (0.15 mg/mL in L-15, with 1% Penstrep) for 2 h at room temperature (20 °C) with agitation. The supernatants from mechanical dissociations and enzymatic digestion were combined and passed through a 100-μm filter. The cells were then pelleted by centrifugation at 300*g* for 10 min. The washed cell pellet was suspended in modified L-15 and subjected to density gradient separation in 34%/51% Percoll® (Sigma-Aldrich, Norway) for 30 min at 400 g at 4 °C. The cells were collected, suspended in a modified L-15 medium, centrifuged for 10 min at 400 g at 4 °C, and finally suspended in a new modified L-15 medium. Cell viability and number were determined by CellCountess™ II (Thermo Fisher). All cells used in the study had a viability of >80%. Cells intended for CellTiter-Glo® viability assessment were seeded out onto 96-well plate at ca 10^4 cell per well while cells for microarray analysis were seeded out onto a 12-well plate (Corning® CellBIND® Surface, Sigma-Aldrich) at a density of 10^5 cells per well and incubated at 13 °C.

2.2. Exposure to sulphide donors

The H_2S exposure trial was performed after allowing the cells to recover for 35 h. The media were pipetted out, and the cells were washed with sterile PBS once. Thereafter, new media was added containing different concentrations based on earlier studies (1, 10, 100 μM) of either of the two sulphide donors NaHS and GYY4137 [19,20]. Cells handled similarly but without any H_2S addition served as the untreated control. The plates were incubated for 24 h at 13 °C.

2.3. Cell viability post-H_2S exposure

The effects of H_2S exposure on cellular viability (n = 6 fish) was assessed by CellTiter-Glo® Luminescent Cell Viability Assay following the manufacturer's protocol (Promega, WI, USA). Briefly, an equal volume of CellTiter-Glo® Reagent was added to the cell culture medium in each well and allowed to incubate for 2 min on an orbital shaker to facilitate lysis. The luminescent signal was stabilised for 10 min at room temperature before luminescence was measured. Readings from wells with cells containing media only served as background correction value. Viability was expressed as relative light units (RLU).

2.4. RNA isolation and quality control

After 24 h of exposure, cells (n = 4 fish) were collected by removing the media followed by a single wash with PBS. The cells were scraped and suspended in lysis buffer (ZYMO Quick-RNA™ Microprep kit, USA), and the cell suspension was stored at 70 °C until RNA isolation.

The total RNA from the nasal leukocytes were isolated using a commercially available kit (Quick-RNA™ Microprep kit). RNA concentration was quantified in a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, DE, USA), and the quality of the samples was assessed using an Agilent® 2100 Bioanalyzer™ RNA 6000 Nano kit (Agilent Technology Inc., Santa Clara, CA, USA). All samples had an RNA Integrity Value higher than 9.0.

2.5. Microarray analysis

Transcriptomic responses in the nasal leukocytes following H_2S exposure were evaluated using custom-made Atlantic salmon DNA oligonucleotide microarray SIQ-6 (custom design, GPL16555), which contains 15 K probes for protein-coding genes involved in immunity, cell communication and junctions, tissue architecture, integrity and functions, and extracellular matrix. Microarrays were manufactured by

Agilent Technologies (Santa Clara, CA, United States), and unless indicated otherwise, the reagents and equipment were purchased from the same supplier. Using an input of 110 ng, RNA amplification and labelling was carried out using a One-Colour Quick Amp Labelling Kit. The fragmentation of labelled RNA was performed using a Gene Expression Hybridization kit. The arrays were allowed to hybridise overnight in an oven (ca 17 h, 65 °C, rotation speed 0.01 *g*) washed with gene expression wash buffers and scanned through an Agilent SureScan microarray scanner. Data pre-processing used Nofima's bioinformatics package STARS.

2.6. Data treatment

The cell viability data after H_2S exposure was tested for normality (Shapiro-Wilk test) and for equal variance (Brown-Forsyth test) before inter-treatment evaluations. Differences among treatment groups were identified using a two-way ANOVA followed by multiple pairwise comparisons via the Holm-Sidak test. All statistical tests were performed in Sigmaplot 14.0 Statistical Software (Systat Software Inc., London, UK); significance was set at $P < 0.05$.

Normalised microarray data were subjected to statistical comparisons 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., concentration contrasts or H_2S forms contrasts or control contrasts), a positive log2 (fold change) indicates up-regulation in group A relative to group B (e.g., NaHS 10 μM versus NaHS 1 μM). A hypergeometric test was used to identify Gene Ontology (GO) terms in which significant features (defined using p-value < 0.01) were over-represented. This was carried out for all three ontologies (biological process, molecular function, and cellular compartment). Significant genes (defined using p-value < 0.01) from each comparison were analysed for enrichment of Reactome pathway membership using a hypergeometric test. Functional enrichment analysis was performed for the genes identified as significantly differentially expressed in association tests.

Atlantic salmon genes in the microarray were mapped to zebrafish orthologues to enable the functional assessment of differentially expressed genes (DEGs) using the Reactome pathway database. Enrichment ($p < 0.05$) was assessed for up- and down-regulated genes separately. Congruence analysis was performed to compare the two sulphide donors for equivalent concentration contrasts against control with the aim of identifying the genes and pathways impacted by both H_2S donors. The intersection for DEGs (using threshold $p < 0.01$) and enriched pathways was assessed for the two donors at each of the three concentration levels (the "control contrasts" for each donor at 1 μM, 10 μM, and 100 μM). Congruence for pathways was assessed separately for pathways enriched for upregulated and down-regulated genes, and "all" giving the combination of the up- and down-regulated gene enriched pathways.

3. Results

3.1. H_2S has minimal impacts on nasal leukocyte viability

The viability of GYY4137-exposed cells did not significantly vary from the unexposed control group regardless of the concentrations (Fig. 1). For NaHS group, a significant viability reduction was observed in 1 μM-exposed group versus the unexposed control. Such a change was not observed at higher concentrations. In addition, there was no significant group-wise differences amongst NaHS-exposed cells regardless of concentrations. A significant difference was identified between the two sulphide forms at 100 μM where cellular viability in the GYY4137 group was significantly lower than the NaHS group.

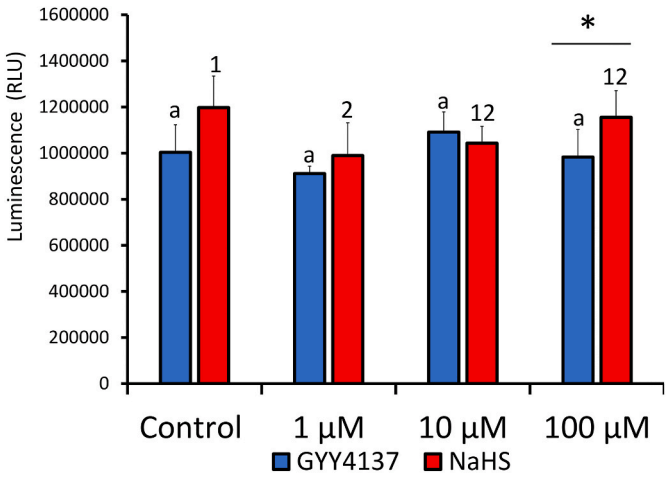

Fig. 1. Viability of Atlantic salmon nasal leukocytes following a 24-h exposure to different concentrations (1, 10, and 100 μM) of sulphide donors GYY4137 and NaHS. Different letters indicate significant difference amongst the groups in GYY4137-exposed cells while different numbers denote significant differences in NaHS-exposed group. An asterisk (*) indicates that a significant difference exists between GYY4137- and NaHS-exposed groups at that particular exposure concentration. Values are from mean ± SD of 6 fish.

3.2. Differentially expressed genes in H_2S-stimulated leukocytes follow a dose-dependent tendency

Differentially expressed genes (DEGs) were identified under different association tests (Table 1). Differential expression analysis was performed between concentration levels for each sulphide donor to assess whether H_2S response was dependent on the concentration to which the leukocytes were exposed. For each NaHS and GYY4137, tests were performed comparing samples exposed at 1 μM, 10 μM, and 100 μM; the results revealed a dose-dependent response. Little change was observed between 1 μM and 10 μM for either NaHS or GYY4137 (1 and 0 DEGs at adjusted $P < 0.05$, respectively). Comparing 10 μM versus 100 μM, DEGs increased to 40 in GYY4137 and 173 DEGs in NaHS (adjusted $P < 0.05$). Some of the genes that substantially changed between 10 μM and 100 μM include *heat shock cognate 70* (Fold change = 2.588), *neutrophil cytosolic factor 1* (FC = 1.849), *vps13a protein* (FC = −3.459) and *pi-plc x domain-containing protein 1* (FC = −2.736) for NaHS. The *hexim protein* (FC = 1.977), *dynein, light chain, LC8-type 1* (FC = 1.959), *puromycin-sensitive aminopeptidase* (FC = −1.702) and *myocyte enhancer factor 2 cb* (FC = −1.470) were changed for GYY4137. For NaHS 100 μM versus 10 μM, 68% of the DEGs were upregulated while 32% were downregulated.

Table 1
Summary of DEGs based on different association tests.

Association test	Contrast	Number of DEG P (adj) < 0.05
Concentration	NaHS 10 μM vs 1 μM	1
	NaHS 100 μM vs 1 μM	98
	NaHS 100 μM vs 10 μM	173
	GYY4137 10 μM vs 1 μM	0
	GYY4137 100 μM vs 1 μM	53
	GYY4137 100 μM vs 10 μM	40
Sulphide donor	NaHS vs GYY4137 at 1 μM	27
	NaHS vs GYY4137 at 10 μM	363
	NaHS vs GYY4137 at 100 μM	466
Control	NaHS 1 μM vs control	66
	NaHS 10 μM vs control	1021
	NaHS 100 μM vs control	265
	GYY4137 1 μM vs control	1
	GYY4137 10 μM vs control	3
	GYY4137 100 μM vs control	113

On the other hand, GYY4137 100 μM versus 10 μM showed around 54% upregulated and 47% downregulated (Fig. 2).

To investigate differences between the two sulphide donors (NaHS versus GYY4137), the two treatment groups were compared at each concentration level (Table 1). The number of DEGs between NaHS and GYY4137 increased with concentration (27, 363, and 466 genes in the 1 μM, 10 μM, and 100 μM comparisons, respectively, at adjusted $P < 0.05$). DEGs in 100 μM NaHS versus GYY4137 are represented by 60.4% upregulated and 39.4% downregulated genes (Fig. 2) including substantial changes in *DNA replication complex GINS protein PSF3* (FC = 35.039), *zinc finger protein 37-like* (FC = 6.682), *dihydrolipoyl dehydrogenase, mitochondrial* (FC = −3.141), and *short-chain dehydrogenase/reductase 3* (FC = −3.079) expression.

Contrasts between each treatment group and the control samples were performed to identify potential H_2S responsive genes (Table 1). All groups showed at least one DEG at adjusted $P < 0.05$ (between one and 1021 genes across contrasts). Across concentrations, a larger number of DEGs were identified in the NaHS contrasts than the GYY4137 contrasts where the former showed 11-fold higher total DEGs than the latter. The lists of DEGs identified by the treatment vs control contrasts were highly overlapping particularly between treatment groups for the same sulphide donor. *Redox-regulatory protein FAM213A* (FC = −1.506 in NaHS, −1.436 in GYY4137), *complement c3* (FC = 1.229 in NaHS, 1.21 in GYY4137), *ferritin heavy polypeptide 1b* (FC = −1.785 in NaHS, 1.706 in GYY4137) were some of the genes found in the two groups that were changed considerably and with almost similar response pattern. Comparisons at 100 μM revealed a contrasting pattern between the two forms: 63.3%:36.7% was the ratio of upregulated to downregulated DEGs in NaHS; this was 43:57% in GYY4137 (Fig. 2).

3.3. Enriched GO terms and biological pathways affected by H_2S

Functional enrichment analysis was performed for genes differentially expressed between different concentration levels (Fig. 3–5). While a clear grouping of GO terms relative to the concentrations was observed in NaHS-exposed groups, such a pattern was not evident in GYY4137 (Fig. 3A). Oxygen carrier activity was enriched at comparisons of 1 and 10 μM while isomerase activity at 10 μM vs 100 μM was seen for both forms. GO terms ferric iron binding and iron ion transport were enriched at higher doses regardless of the H_2S donors. At higher doses, the GO terms spliceosomal snRNP assembly and mRNA splicing via the spliceosome were significantly enriched in NaHS while most of substantially enriched GO terms in GYY4137 included nuclear envelope and regulation of DNA replication besides the two iron-related GO terms mentioned above. In the 1 and 10 μM comparison, signalling by interleukins and by the B-cell receptor were the enriched pathways in NaHS while signalling by GPCR was identified in GYY4137 (Fig. 3B). The VEGF ligand-receptor interaction pathway was enriched in NaHS, and beta-catenin degradation was enriched in GYY4137 when H_2S donors were compared at 10 μM vs 100 μM. In the 10 μM vs 100 μM study, we found that NaHS affected transcriptional activation of mitochondrial biogenesis and VEGFR2 mediated vascular permeability while pathways related to LAT2/NTAL/LAB on calcium mobilisation and DNA replication were enriched in GYY4137.

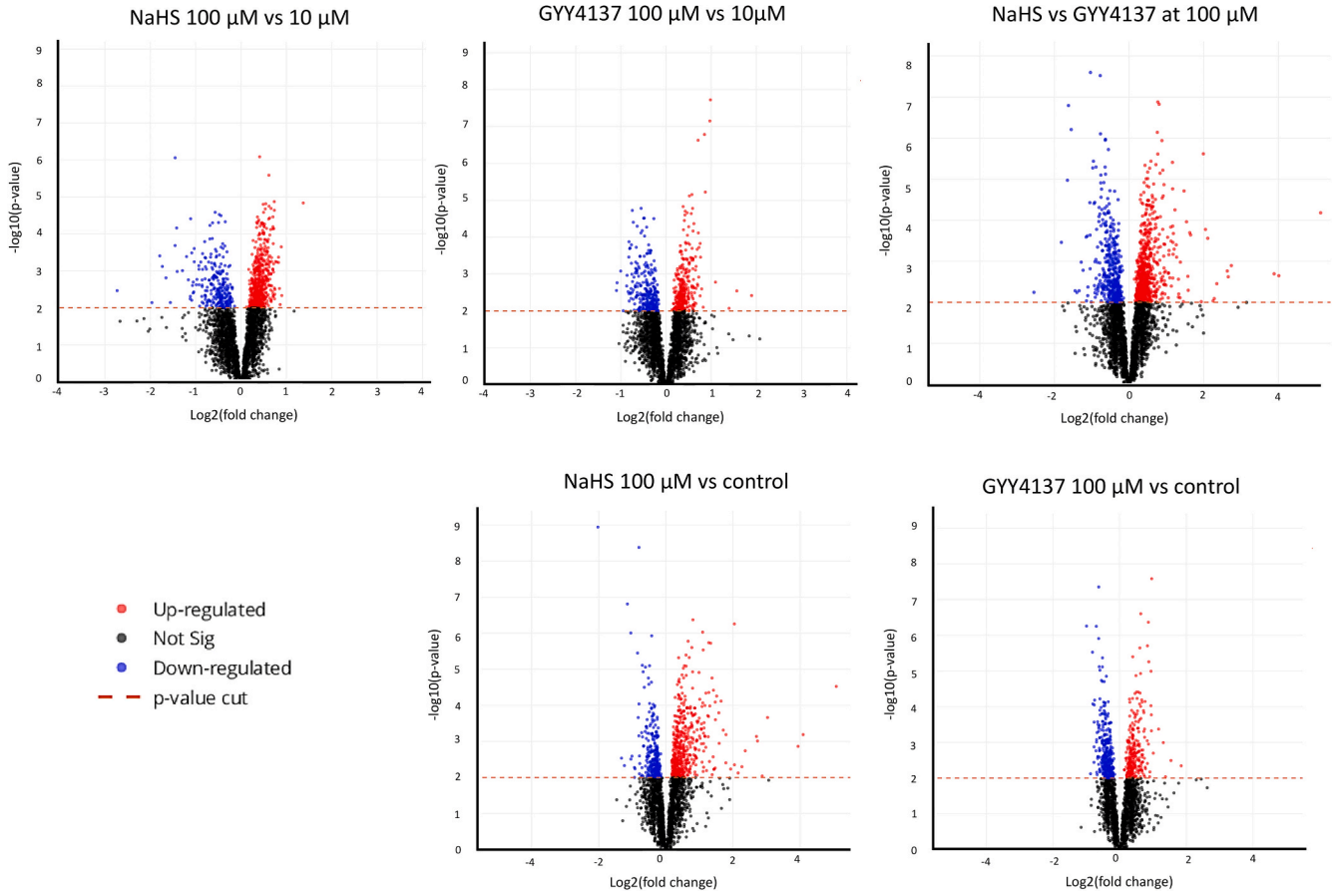

Fig. 2. Volcano plots for representative comparisons showing significance (as -log10 transformed p-values) against magnitude (log2(fold change)). Features identified as having different levels between samples are represented as red (upregulated) or blue (downregulated) dots. To improve performance when there are tens or hundreds of thousands of features, the non-significant features (black) displayed are a representative subsample of the full dataset. The horizontal orange lines represent the applied p-value threshold. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

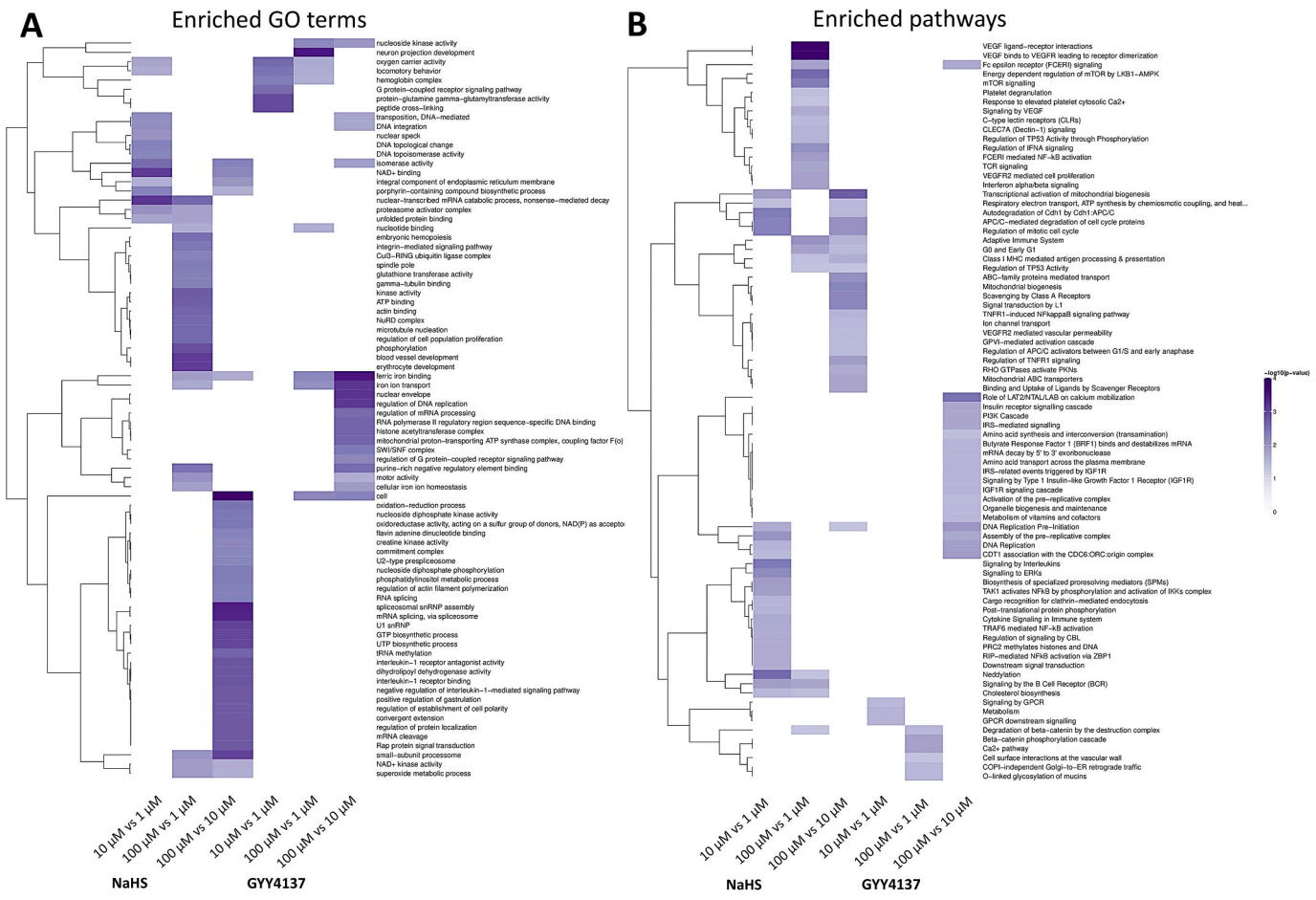

Fig. 3. Heatmaps of significantly enriched (A) GO terms and (B) Reactome pathways for concentration contrasts. Comparisons are shown on the X axis with GO terms/Reactome pathways on the Y axis. When performing tests for enrichment, terms/pathways were restricted to include only those with 2 or more genes. Note that for the purposes of display, only GO terms/Reactome pathways with an enrichment p-value less than 0.05, were included. Furthermore, only the top 80 terms/pathways are displayed. Results are shown for the union of significant up- and down-regulated features. Note that different genes of a given GO term/Reactome pathway may be both up- and down-regulated within a single comparison. Colour purple is assigned based on the -log10 (enrichment p-value) with lighter colours implying less significant enrichment. Hierarchical clustering was applied to terms (rows). The most significant terms/pathways were clustered according to Euclidean distance using the complete linkage method. (*Figure was inserted in the text to facilitate review. HD version is provided separately in the submission*). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Comparing the two H_2S donors at each concentration level revealed evident clusters of GO terms (Fig. 4A). Hemopoiesis, RNA metabolic processes, and autophagy were among the enriched GO terms at 1 µM NaHS vs GYY4137. Negative regulation of transcription, integrator complex, and heme binding were enriched at 10 µM NaHS vs GYY4137 while regulation of DNA replication and Rap protein signal transduction were identified at 100 µM NaHS vs GYY4137. Oxidoreductase activity was a common enriched GO term in 1 and 10 µM NaHS vs GYY4137. On the other hand, heme binding and negative regulation of transcription were common enriched GO terms between 10 and 100 µM. Three main clusters were likewise identified when pathway analysis was performed (Fig. 4B). At the lowest dose, pathways related to the metabolism of glucose, steroids, and cholesterol were significantly enriched. Upon increasing the concentration 10× higher, we observed that most of the pathways affected were associated with the RNA transcription processes including RNA polymerase II transcription. At the highest dose, we observed many pathways related to immune mechanisms (e.g., neutrophil degranulation, interferon signalling) and cell surface interactions affected (e.g., vitamin transport, DSCAM interactions).

We then explored GO terms and pathways affected by the H_2S donors relative to the control group at a specific exposure concentration (Fig. 5A). For NaHS, GO terms related to enzymatic activity (e.g., cytochrome oxidase, carbonate dehydratase, alpha-mannosidase) were significantly enriched at 1 µM. On the other hand, enriched GO terms related to redox physiology were represented substantially in GYY4137. At 10 µM, the integrator complex, iron ion haemostasis, and haemoglobin biosynthetic process were amongst the enriched GO terms in NaHS. GO terms related to microtubules and energy metabolism were enriched in GYY4137 at the same dose. Extracellular matrix, RNA processing, and iron homeostasis were enriched at the highest dose for both H_2S donors. Regardless of the exposure dose, cysteine-type endopeptidase inhibitor activity and glutathione transferase activity were enriched in NaHS and GYY4137, respectively. Pathways related to DNA replication, cell communication and cell cycle were enriched in NaHS at 1 and 10 µM (Fig. 5B). Immune-related signalling pathways such as the regulation of TNFR1, interferon gamma, and NOD1/2 were significantly enriched in NaHS at the highest dose. Affected pathways in GYY4137 showed three distinct clusters. Intracellular processes including mitochondrial and lysosomal-related pathways enriched at 1 µM; cell cycle and metabolism represented the enriched pathways at 10 µM; and cellular communications and small molecule metabolism including VEGF ligand interactions and amino acid and vitamin metabolism were significantly enriched at the highest dose.

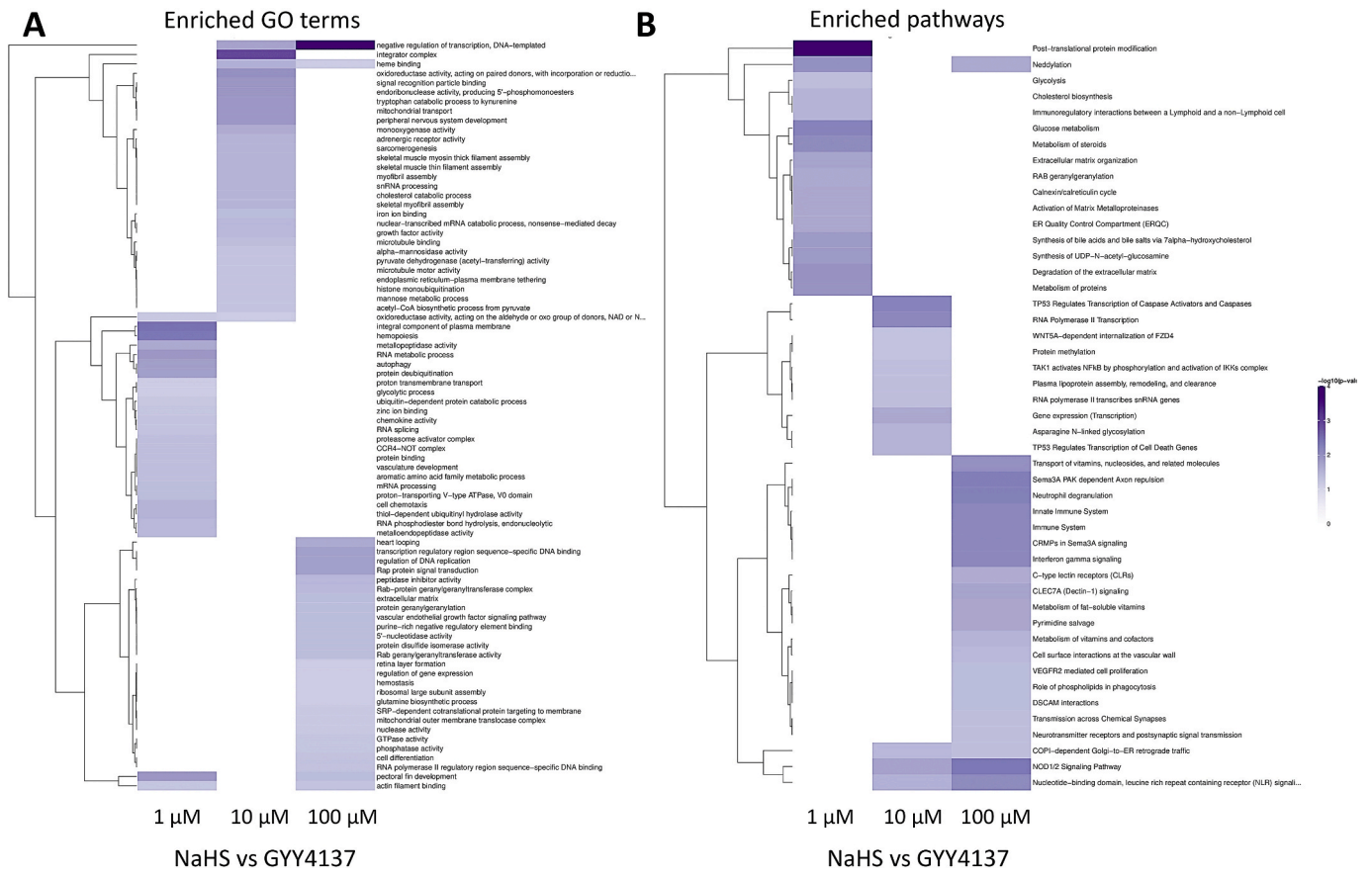

Fig. 4. Heatmaps of significantly enriched (A) GO terms and (B) Reactome pathways for sulphide donor contrasts. For additional information, refer to Fig. 3.

3.4. Common H_2S-impacted genes and pathways

Congruence analysis was performed to compare the two sulphide donors for equivalent concentration contrasts against control with the aim of identifying the genes and pathways impacted by both sources of H_2S (Fig. 6). Highly significant overlaps were found between DEGs at each of the concentrations (Fig. 6A). For example, at 100 µM, there were 34 upregulated and 26 downregulated genes shared by the two sulphide donors (Fig. 6B). When assessing the overlap of significantly enriched GO terms and reactome pathways, particularly strong similarity was found between NaHS and GYY4137 response GO terms at 100 µM. There were 16 GO terms significantly overlapping (Fig. 6C). Of these, 12 were upregulated by both sulphide donors including several involving iron ion transport, cellular iron ion homeostasis, and ferric iron binding. Two of the three shared downregulated GO terms comprised of an extracellular matrix structural constituent and collagen-containing extracellular matrix. The GO term "glutathione transferase activity" was significantly enriched for DEGs in the NaHS 1 µM vs control contrast and all three of the GYY4137 vs control contrasts as well as in the genes upregulated in the NaHS 100 µM vs 1 µM contrast. No significant overlap was detected in the reactome pathway at any concentration.

4. Discussion

The first line of defence of the human body to ambient H_2S intoxication is the olfactory organ [7]. H_2S is soluble in water and thus is expected to influence the nasal immune system of fish, but this interaction is unclear. To our knowledge, this is the first report discussing the molecular responses to H_2S of leukocytes from the olfactory organ of a teleost fish. Here we showed that though the tested pharmacological concentrations of H_2S had little effect on leukocyte viability, the transcriptomic landscape was altered dramatically in a dose-dependent manner and the form of the H_2S donor influenced the magnitude of these changes.

H_2S has either a promoting or inhibiting function on cellular viability [6,8,21]. In normal human hepatocytes, NaHS (10–1000 µM) treatment resulted in negligible effects on viability and proliferation. In hepatocellular carcinoma (HCC), however, exposure to 10–100 µM NaHS could significantly promote the growth and migration of HCC cells whereas 600–1000 µM NaHS inhibited a dose-dependent manner [22]. A 24-h exposure of porcine vascular wall–mesenchymal stem cells to NaHS resulted in insignificant effects on cell viability, but this same process interfered with the cell cycle by blocking the S phase [23]. On the other hand, 24-h exposure to NaHS promoted cellular proliferation in human mesenchymal stem cells [24]. Here, we found that H_2S concentrations of 1–100 µM had little effect on leukocyte viability suggesting minimal toxicity risk of the tested levels. Interestingly, cellular viability in GYY4137 was significantly lower than NaHS at 100 µM exposure dose. The kinetics of H_2S release of these sulphide donors—NaHS being an instantaneous donor while GYY4137 being a slow-releasing H_2S donor [19]—likely played a part in this apparent difference. The duality of functions of H_2S towards cellular proliferation can also be attributed to this striking contrasts with earlier studies in mammalian models [22].

The variation in the known chemical behaviour of these two sulphide donors in an aqueous solution was evidently manifested in the transcriptomic modifications following exposure. The number of DEGs and the magnitude of response, regardless of association tests and concentration exposures, were identified to be higher in nasal leukocytes exposed to NaHS than to GYY4137. This difference indicates that the magnitude of H_2S-induced changes in the nasal leukocyte are dependent on concentration. The changes are also influenced by the kind of sulphide donors that innately exhibit varying H_2S release kinetics.

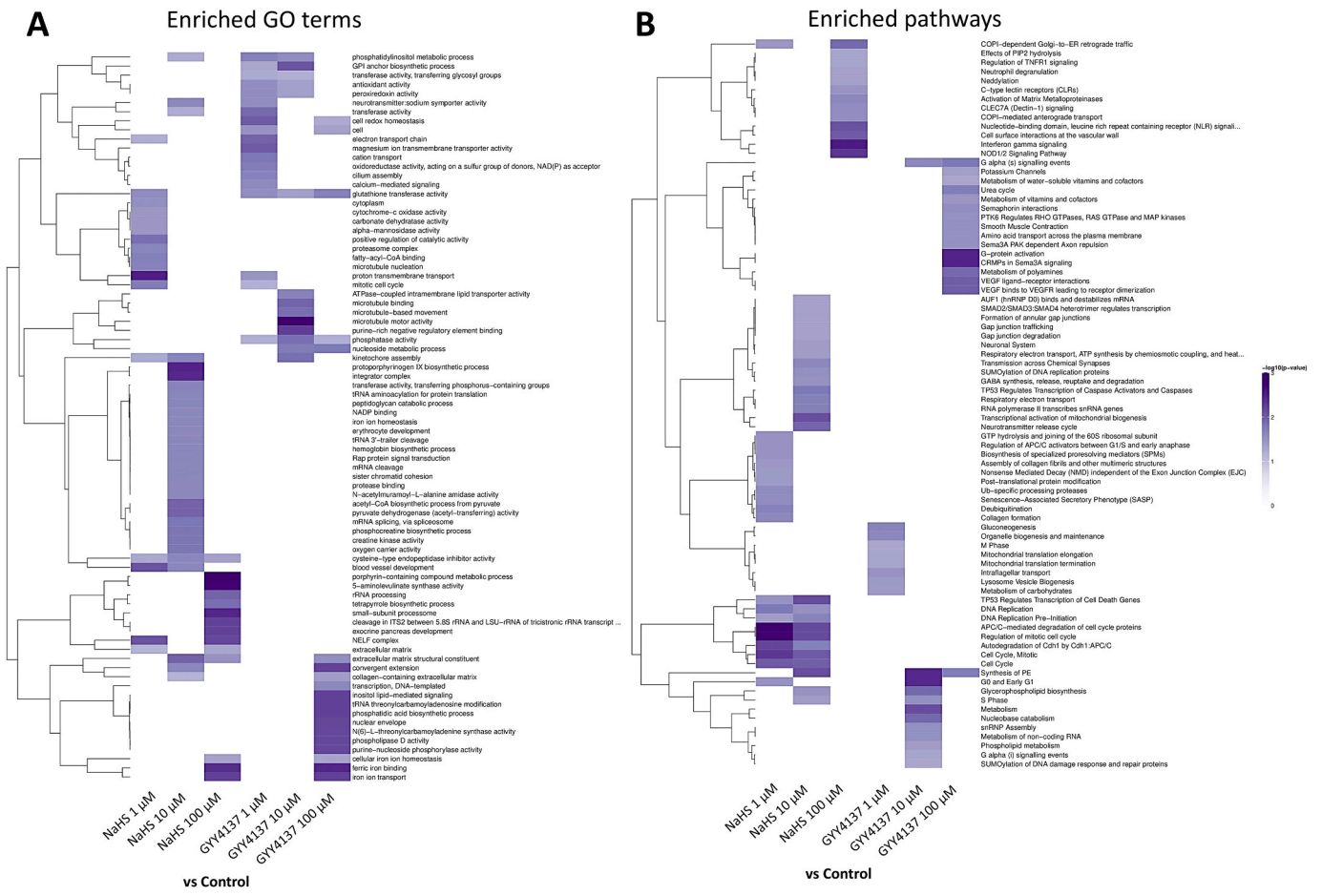

Fig. 5. Heatmaps of significantly enriched (A) GO terms and (B) Reactome pathways for control contrasts. For additional information, refer to Fig. 3.

However, the underlying mechanisms appear to share a high similarity between the two sulphide donors as discussed in the following section. When the NaHS encounters water, the salts quickly generate H_2S; therefore, we assumed that cells are exposed to high H_2S levels at the start and may not be sustained throughout the exposure period [20]. On the other hand, GYY4137 releases H_2S in hours to days potentially allowing the cells to respond to H_2S gradually [20,25]. It is very likely that nasal leukocytes were acutely shocked by H_2S in NaHS-exposed group; this in turn triggers the cells to mobilise robust and stronger responses to the chemical pressure as evidently shown by the number of affected genes. Murine macrophages were shown to respond differently and distinctively to these two sulphide donors [26], but the present study is the first to report a transcriptome-wide comparative survey on how these H_2S sources modify the molecular repertoire of immune cells.

Molecular processes governing immunological functions in salmon nasal leukocytes were significantly affected by the H_2S donors supporting their previously identified role in mammalian systems as a potent regulatory mediator of immunity [6]. Interferons (IFNs) are antiviral defence cytokines in jawed vertebrates that participate in various ways to inhibit virus replication and modulate immune responses [27]. We have found that both sulphide donors, especially when administered at the highest dose, interfered with interferon signalling such as the modulation of genes coding for *interferon regulatory factor 4*, *interferon regulatory factor 1*, and *interferon alpha 2*. Endogenous and pharmacological H_2S has both anti-inflammatory and proinflammatory effects [8]. Several interferons, such as IFN-α, are multifunctional immunomodulatory cytokine with a profound impact on the cytokine cascade including several anti-inflammatory properties [28]. Though the interplay of interferons and H_2S has not been functionally elucidated in fish, the present transcriptomic data suggest that they play crucial molecular signals in orchestrating a robust immune response to resolve H_2S-induced inflammation. The immune-modulatory functions of H_2S via interferon signalling is likely carried over to downstream immune processes such as in neutrophils. At the highest dose, GO analysis revealed that the neutrophil degranulation process was affected by the H_2S donors indicating their potential role in innate immunity. When stimulated, neutrophils, the most abundant leukocytes, migrate towards the inflammatory site and release granule-derived mediators by degranulation or exocytosis of membrane-bound secretory granules. These granules contain a diverse array of antimicrobial proteins and proteolytic enzymes, which participate in the defence mechanism [29]. H_2S is a mediator of neutrophil functions, and it can either promote or inhibit neutrophil migration [6,30]. Physiological H_2S levels have been demonstrated to support neutrophil viability; thus, stimulating cellular functions including their antimicrobial properties [6]. This mechanism may be at play in the observed effects of H_2S on the genes involved in neutrophil degranulation.

Vascular endothelial growth factors (VEGF) are produced by endothelial cells, macrophages, activated T cells, and a variety of other cell types. They mediate the recruitment and adhesion of leukocytes by binding to its receptors VEGFR-1 and VEGFR-2 [31]. We found that the VEGFR ligand-receptor interaction pathway was significantly affected in nasal leukocytes exposed to the H_2S donors at the highest dose. H_2S could trigger inflammatory responses [8], and the present study supports this based on the array of H_2S-affected genes detected with known functions in orchestrating inflammation. The enrichment of the VEGFR ligand-receptor interaction pathway following H_2S stimulation is likely a mechanism to resolve the effects of inflammatory triggers given that

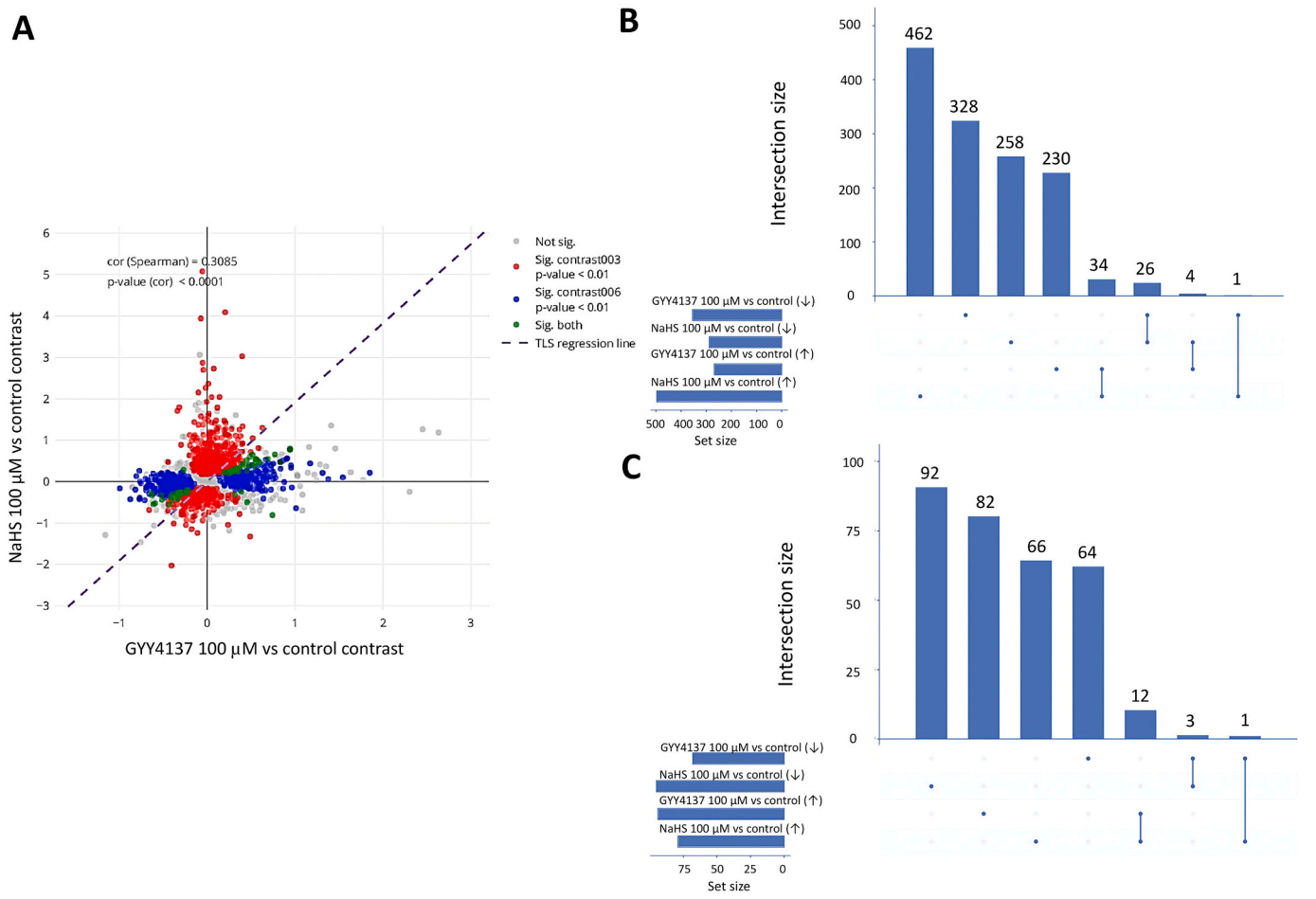

Fig. 6. Common molecular responses to H_2S of the two sulphide donors. A) A representative scatter plot comparing significant features in NaHS 100 µM vs control against GYY4137 100 µM vs control. Features are represented by points. The colour of the point indicates to which set the feature is assigned. For each feature the log2 (fold change) in the NaHS 100 µM vs control contrast (y-axis) and the log2 (fold change) in the GYY4137 100 µM vs control contrast (x-axis) is shown. **B)** Upset plot of significant DEGs. A plot showing the interaction between sets of up- and down-regulated features. The leftmost bar chart shows the size of each set used as an input. The top barchart shows the exclusive size of each set (i.e., each feature is only counted once in this bar chart). The dot-plot in the centre shows the sets interacting in each case. **C)** A plot showing the interaction between sets of up- and down-regulated GO terms. The leftmost bar chart shows the size of each set used as input. The top bar chart shows the exclusive size of each set (i.e., each GO term is only counted once in this bar chart). The dot-plot in the centre shows the sets interacting in each case. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the VEGF-VEGFR system mediates this process by suppressing inflammation [32,33]. It is also plausible that such a significant impact on this pathway may be related to the implied relationship of H_2S and VEGFR2 where the latter is believed to be a potential "H_2S receptor" [34,35]. VEGFR2 mediates most of the biological effects of VEGF. It was identified in vascular endothelial cells that the Cys1045–Cys1024 disulfide bond in VEGFR2 is a specific molecular switch targeted by H_2S providing the first direct evidence of the role of VEGFR2 as a "H_2S receptor" [34]. Thus, this study offered new insights into the presence of potentially similar interplays between H_2S and VEGFR2 in piscine immune cells. Future functional studies are expected to elucidate the conservation of such H_2S-VEGFR2 mechanism in vertebrates.

Sulphide can cause oxidative stress [11]. In terrestrial vertebrates such as chicken, airborne H_2S could weaken the antioxidant capacity and eventually lead to apoptosis and myocardial damage [21]. In rodents, ventilation with moderate tidal volumes of H_2S led to an excessive formation of reactive oxygen species (ROS) and activation of the antioxidant glutathione. This promoted the development of lung injury [36]. Mucosal surfaces of fish are prone to oxidative stress triggered by environmental pollutants and other chemical signals [3,37]. It remains unclear whether H_2S is an oxidant stressor as previously identified in different animal models. The transcriptomic changes provided insights into the potential interference of H_2S on the oxidative stress status of the nasal leukocytes. Both sulphide donors enriched the GO term glutathione transferase activity regardless of the dose suggesting that this arm of the antioxidant defence system of the nasal leukocytes is a key protective repertoire towards potential oxidant-induced changes from H_2S. Glutathione (GSH) is a tripeptide consisting of glycine, cysteine, and glutamic acid; it plays a critical role in protecting cells from oxidative damage and the toxicity of xenobiotics. It maintains redox homeostasis when it changes its formation from reduced GSH to oxidised GSH (GSSG, glutathione disulfide) [38]. Given the known function of glutathione in suppressing H_2S-induced oxidative stress [39], it is very probable that such a protective mechanism had been triggered in the nasal leukocytes.

We found that cellular processes such as iron transport, iron homeostasis, and iron binding (crucial for heme proteins and haemoglobin biosynthesis) were substantially affected by the two sulphide donors particularly at the higher dose. The capability of H_2S to influence these processes may be explained by the role of iron in altering the chemical reactivities of haemoproteins in mammalian systems [40,41]. The interaction of sulphide and iron can lead to the formation of polysulfides or thiosulfates or promote a covalent modification of the heme yielding a sulfheme derivative [41]. This sulphide binding reduces heme iron affecting the redox state of the protein and thereby affecting cellular functions [40]. The cost of these interactions in fish nasal leukocytes requires further investigations. In particular, it is compelling to target

how chemical changes in iron following reaction with H_2S dysregulates the biological function of heme proteins and the associated consequences in leukocytes' defence mechanisms. This interplay is relevant because there is a known tight relationship between heme, iron, and immunity in this immune cell [42]. The substantial changes in the processes related to these mechanisms as revealed by the transcriptomic data point to a possibility that such interplay may be present in fish nasal leukocytes.

This study provided the first transcriptome-wide analysis of the molecular processes in the nasal leukocytes induced by exogenous H_2S. There were differences in the responses between the two sulphide donors especially in the magnitude of response. However, the global transcriptomic profiles revealed that they affected numerous common biological processes including immune signalling, VEGFR ligand-receptor interaction, redox homeostasis, and iron binding and transport. Pharmacological H_2S levels did not significantly impact cellular viability; however, the transcriptomic changes point to a potent modulatory function of exogenous H_2S on nasal leukocyte physiology offering insights into the role of fish nasal mucosa on H_2S sensing and defence. H_2S is an environmental parameter whose functions in fish, especially in immunity, are not fully understood. The H_2S-responsive molecules and processes identified here addressed this gap and offered foundational information to understand this interaction further. This is particularly relevant in land-based Atlantic salmon production since the threats of H_2S toxicity is increasingly prevalent. Understanding this interaction may offer opportunities for mitigation and preventive measures such as the use of H_2S-responsive marker genes as early warning signs.

Author contributions

CCL conceived the research idea and secured funding. NAR and CCL performed the trial, analysed the samples and handled the data. NAR and CCL wrote the first draft of the manuscript.

Declaration of competing interest

The authors declare no competing interests.

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