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Short Communication

Comparative basal transcriptome profiles of the olfactory rosette and gills of Atlantic salmon (*Salmo salar*) unveil shared and distinct immunological features

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ABSTRACT

The molecular repertoire of the mucosa-associated lymphoid tissue (MALT) in the olfactory rosette in most teleost fish is unknown. Here we present the basal transcriptome of the olfactory rosette of Atlantic salmon (*Salmo salar*). To investigate its mucosal immune features, we performed a comparative transcriptomic analysis with the gills, one of the most studied organs possessing MALT. Pathway enrichment revealed that cytokine-cytokine interaction and the neuroactive ligand-receptor interaction pathways were at the core of the shared similarity between the two organs. The immunological features of the two organs were further characterised by the overrepresentation of several immune-related pathways, particularly important for pathogen recognition. The immunological differences between the two organs were underlined with the differential regulation of markers for interleukins, extracellular matrix, antimicrobial peptides, and complement. The basal transcriptome of Atlantic salmon olfactory rosette is a valuable molecular toolbox that will advance our understanding of nasal immunity in teleost fish.

1. Introduction

Teleost fish are ancient vertebrates with a dedicated and complex immune system that has protected them from various pathogens, and malicious stimuli over millions of years [1]. The teleost lineage shares three whole-genome duplication (WGD) events, two that occurred prior to their divergence (1R, 2R), and one teleost specific WGD (Ts3R) in their common ancestor [2,3]. In addition, salmonid-specific fourth vertebrate whole-genome duplication happened 80 million years ago [4]. This led to evolutionary innovation and diversification such as the expansion of several immune molecular groups, providing the opportunity to acquire genes with new or modified immune functions [5].

Recent discoveries in teleost fish confirm that innate and adaptive immunity are not isolated compartments and they share cells and molecules [6,7]. The primary (lymphocyte-generating) lymphoid organs are the thymus and head kidney, while secondary (immune response-generating) lymphoid organs include the spleen and mucosal-associated lymphoid tissues (MALT). Leukocytes are present in the majority of fish tissues, but distinct organisations are found in mucosal tissues including the three well-studied MALTs - gut-associated lymphoid tissue (GALT), gill-associated lymphoid tissue (GIALT), and skin-associated lymphoid tissue (SALT) [7]. Teleost MALT includes specialised diffuse B cells and T cells that have co-evolved to defend the microbe-rich mucosal environment [7,8]. Hence, the MALT can be considered the first line of defence in teleost fish [6].

The olfactory organ is predominantly attributed to the sense of olfaction in many vertebrates. Due to its anatomical features, the olfactory organ also participates in the defence against airborne antigens. The nasopharynx-associated lymphoid tissue (NALT) is a MALT that was initially considered to be only present in the olfactory organ of terrestrial vertebrates. The discovery of NALT in rainbow trout (*Oncorynchus mykiss*) highlighted that immune function associated with the olfactory organ (from hereon will be referred to as olfactory rosette) is a conserved feature of all vertebrates [9]. Teleost fish have two olfactory rosettes that sit inside the nasal cavity and are connected to the central nervous system (CNS) via the olfactory bulb [10]. An olfactory rosette contains a variable number of lamellae with two types of epithelia - a neuro-epithelium that exhibits sensory functions and a mucosal epithelium

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that secretes the mucus that covers the nasal cavity. One of the main implications of the discovery of NALT in fish is the potential for nasal vaccination, a minimally invasive strategy that has been shown to require only a small amount of antigen to trigger localised and systemic immunological stimulation [11].

Elucidating the molecular landscape of NALT in teleost fish is not only important to the further advancement of our understanding of nasal immunity but will also lead us a step closer to realising the potential of nasal vaccination as a disease prevention strategy. Here we describe the basal transcriptome of the olfactory rosette of Atlantic salmon (*Salmo salar*) smolts, in relation to the gill transcriptome. Atlantic salmon is a relevant model being one of the prominent farmed species in the world and improving its health remains a top priority. Earlier we have shown through microarray and targeted qPCR analysis that Atlantic salmon NALT could mount strong responses to immunotoxicants, providing evidence of their immune functions [12–15]. To our knowledge, there are no studies yet applying high-throughput sequencing to elucidate the molecular landscape of the MALT associated to olfactory rosette in Atlantic salmon. The presented basal transcriptome data highlight the similarities and distinctiveness between the olfactory rosette and the gills, an organ with a well-studied MALT. Moreover, the sequenced transcriptome can be used as a reference to further explore the immune features of Atlantic salmon NALT.

2. Materials and methods

2.1. Animal ethics

All animal handling procedures were in accordance with the Guidelines of the European Union (Directive 2010/63/EU) and the Norwegian Animal Welfare Act of 2009. The fish specimens used in the study were collected from a trial approved by the Norwegian Food Safety Authority under FOTS ID 20/37233. Key personnel of the trial hold a FELASA C Certificate.

2.2. Description of the specimens

The samples were collected during a fish trial, where amoebic gill disease was induced in Atlantic salmon smolts followed by a treatment with peracetic acid [16]. The samples were obtained from the control group that was neither infected nor treated. Briefly, the fish were reared in a 800 L tank operating in a flow through system under the following conditions: water flow rate of 6–7 L/min, water temperature at 14.5 °C, dissolved oxygen at >85% saturation, salinity at 35 ppt, photoperiod set at 24 light: 0 darkness, and continuous feeding regime with a commercial diet (Skretting Nutra Olympic 3 mm, Averøy, Norway) administered through a belt feeder. Six fish (weight: 82 ± 5.5 g; length: 20.3 ± 0.7 cm) were dip netted from the tank and humanely euthanised with a bath overdose of Benzoak Vet (ACD Pharmaceuticals AS, Leknes, Norway). From the left side, gills from the second gill arch and olfactory rosette were dissected and suspended in RNAlater (Ambion©, Connecticut, USA), kept at room temperature for 12 h for penetration and afterwards stored at −80 °C until RNA isolation.

2.3. RNA isolation and quality evaluation

Total RNA was isolated from the gills and olfactory rosette using the Agencourt RNAdvance™ Tissue Total RNA Purification Kit (Beckman Coulter Inc., CA, USA) with the aid of Biomek 4000 automated workbench station (Beckman Coulter, Inc., CA, USA). The purified RNA was quantified using NanoDrop 8000 Spectrophotometer (ThermoFisher Scientific, USA) and integrity was further evaluated using the Agilent® 2100 Bioanalyzer™ RNA 6000 Nano Kit (Agilent Technology Inc., CA, USA). All samples had an RNA Integrity Number (RIN) above 8.0.

2.4. Library preparation and RNA-sequencing

Total RNA samples were sent to the Norwegian Sequencing Centre (OUS, Norway) for RNA-seq. Briefly, RNA-seq libraries were prepared using strand specific TruSeq RNA Library Prep Kit (Illumina, CA, USA) following manufacturer's protocol. Thereafter, the 12 libraries were pooled together and sequenced in half a lane of NovaSeq S4 flowcell, 150 bp paired end reads. The sequencing raw data is available in ArrayExpress under accession number E-MTAB-12723.

2.5. Data analysis

Raw sequence data were pre-processed to remove/trim adapter sequences and low-quality reads using BBDuk (part of BBMap v.38.18; parameters: ktrim = r k = 23 mink = 11 hdist = 1 tbo tpe qtrim = r trimq = 15 maq = 15 minlen = 36 forcetrimright = 149) [17]. Cleaned data was aligned against the *Salmo salar* ICSASG_V2 genome (ENSEMBL) using HISAT2 v.2.2.1 (parameters: –rna-strandness RF) [18]. Stringtie v.2.1.5 [19] was used to estimate the number of reads (raw count data) aligning against the reference genes in ICSASG_V2 ENSEMBL r104 GTF annotation.

Initial data exploration and analysis of raw count data was performed using SARTools (v.1.7.4) R (v.4.1.1) package [20]. Normalization and differential expression analysis were done according to the DESeq2 (v.1.34.0) R library [21]. The resulting data was filtered for features with very low counts and high dispersion using the independent filtering method of DESeq2. Log2 fold-change (logFC) was calculated for each gene along with the adjusted *p*-values (p-adj) applying the Benjamini-Hochberg (BH method). Genes expressed distinctly in each organ were detected by evaluating the normalized count value within each replicate (≥ 10 in the specific organ and < 10 in the other organ, in each replicate). A full description of the analysis, including R session information and parameters can be found in Supplementary Material S1 'Statistical report: Pairwise comparison of conditions with DESeq2'. The DESeq2 output was surveyed for previously assessed genes of interest in Atlantic salmon related to immune competence [22].

Analysis of gene ontology was performed in R (v.4.2.1), with the package topGO v.2.48.0 [23]. Information on gene ontology for Atlantic salmon (Ssal_v.3.1) was downloaded from the ENSEMBL Biomart (01 September 2022) and used to create the gene universe mapping required by topGO. Input gene lists contained genes i) distinctly expressed in gill, ii) distinctly expressed in the olfactory rosette, and iii) significant DEG's (p-adj < 0.05), divided into up- and downregulated genes. Individual analysis for biological process, cellular component and molecular function was conducted. Fisher statistics and the "elim"-algorithm were applied [23], with a significance threshold of p-adj value < 0.05 for enrichment. Visualisation of GO term enrichment was prepared using GOplot (v.1.0.2) [24] and ggplot2 (v.3.4.0) [25]. For each GO term, a z-score was calculated (1): a positive value indicates a tendency for genes within the term to be upregulated and a negative value a tendency for down-regulation [24].

$$\text{z} - \text{score} = \frac{(\#\text{upregulated genes} - \#\text{downregulated genes})}{\sqrt{\text{Total number of genes}}} \quad (1)$$

Ensembl gene ids were mapped against Uniprot ids applicable for KEGG enrichment analysis, using AnnotationHub (v3.4.2) and AnnotationDbi (v.1.58.0) (mapping provided by AH104987, 06 January 2023). As the mapping is partial, input gene lists were more than halved in size. Inputs lists consisted of genes expressed in i) the olfactory rosette and ii) the gill (normalized count in each sample from the organ had to be 10 or higher). Gene set enrichment analysis was performed using clusterProfiler (v.4.7.1) [26,27], and the *gseKEGG*-function (permutations 10,000, minimum gene set size 15, maximum gene set size 800, *p*-value cut-off 0.05 and p-value adjustment method Benjamin-Hochberg). To explore the olfactory rosette DEGs and non-DEGs further an overrepresentation

analysis applying the *enrichKEGG* function was used on i) a set DEGs (p-adj <0.05, log2 foldchange>|2|), and ii) a set of genes, where also non-DEGs were included. The latter set was limited to genes showing a log2 fold change between −2 and 2. The *enrich*KEGG function was applied as follows; *p*-value cut-off 0.05, p-adjustment method Benjamin-Hochberg, q-value cut-off = 0.2, minimum gene set size 100.

Two enriched pathways, from KEGG enrichment analysis, found in both organs were selected for further investigation. Core enrichment gene sets were compared and checked for overlap. In addition, an analysis of GO term enrichment was performed, as described above, except that there was no filtering for the normalized counts. The resulting output (GO terms and p-adjusted value) was summarised using Revigo (small 0.5, default settings) [28]. A flow-diagram of the analyses are presented in Fig. 1.

3. Results and discussion

The immunological role of MALT associated to the olfactory rosette was recently elucidated in teleost fish using rainbow trout as a model [9]. However, further understanding of its immunological function is restricted by limited genomic resources since the olfactory organ is not commonly a target in immunological and physiological studies in fish, especially in Atlantic salmon. Here we present the basal transcriptome profile of the olfactory rosette of Atlantic salmon smolts. Comparative transcriptomic analyses revealed shared and distinct features with a well-studied mucosal organ in teleost fish – the gills. The gills are multifunctional organs, besides their immune function, they are important for maintaining homeostasis, including respiration, acid-base regulation, excretion of urea, and osmoregulation [29].

3.1. General features of the olfactory rosette and gills transcriptomes

Initial exploration by principal component analysis (PCA) of the transcriptome profiles of the two mucosal organs shows a clear separation (Fig. 2), indicating that the two organs have different basal molecular architectures. This is not surprising since they have distinct physiological functions – the olfactory rosette being a chemosensory organ, while the gills are for gas- and ion exchange. Both organs contained two of the fundamental MALTs in fish [6], and as both are constantly in contact with the external environment, shared common functions can be expected too as highlighted in the next sections.

The DESeq2-analysis revealed 24,880 differentially expressed genes (DEGs) between the two mucosal organs. Also, through filtering the normalized counts, 175 genes were determined to be distinctly expressed in the gills, while the olfactory rosette exhibited 1180 distinctly expressed genes. These groups of genes were further explored in the succeeding sections.

3.2. Gene ontology of the olfactory rosette and gills transcriptomes

The functional characteristics of the distinct and shared genes was further investigated by analysing Gene Ontology (GO) enrichment among the DEGs. There were 129 and 80 enriched GO terms (p-adj < 0.05) from the group of distinctly expressed genes in the olfactory rosette and the gills, respectively. The DEGs resulted in 398 enriched GO terms for upregulated genes, and 344 enriched GO terms for genes that were downregulated in the olfactory rosette (in relation to the gills). The plots in Fig. 3 together with Table 1 show the remaining terms after additional filtering: a gene count for each enriched term of >200, and p-adjusted value <0.001 for the lists of DEGs, while a count of 10, and p-adj <0.01 was required for the distinct genes (complete list of the significantly enriched terms can be found in Supplementary Material, S2). GO terms related to chemosensory (e.g., *detection of chemical stimulus involved in sensory perception of smell*, *sensory perception of smell*), cellular transport (e.g., *transmembrane transport, potassium ion transmembrane transport*) and the cell membrane (e.g. *postsynaptic membrane*, *plasma membrane*) were enriched in the genes identified as distinct to the olfactory rosette gene set, while GO terms associated with regulation of transcription and cellular processes (e.g., *regulation of cellular processes, regulation of transcription*) were enriched in the gene set distinct in the gills (Table 1). The enrichment of GO terms related to chemosensory in the olfactory rosette supports the vital role of this organ in detecting and processing of chemical stimuli in the aquatic environment, lending additional insights into our previous observations from several immunotoxicological studies in Atlantic salmon [13–15].

We focused the GO enrichment analysis by separating the DEGs into upregulated or downregulated genes (Table 1). The gene expression levels in the olfactory rosette are all in relation to the gill transcriptome (reference organ). The most significant GO terms for upregulated DEGs included the terms *microtubule-based processes*, *nervous system development* and the *endoplasmic reticulum membrane* for the cellular component (CC) and biological process (BP) terms. Among the molecular function (MF) terms, *RNA binding*, *calcium ion binding* and *transmembrane transporter activity* were prominent. These fundamental processes are critical

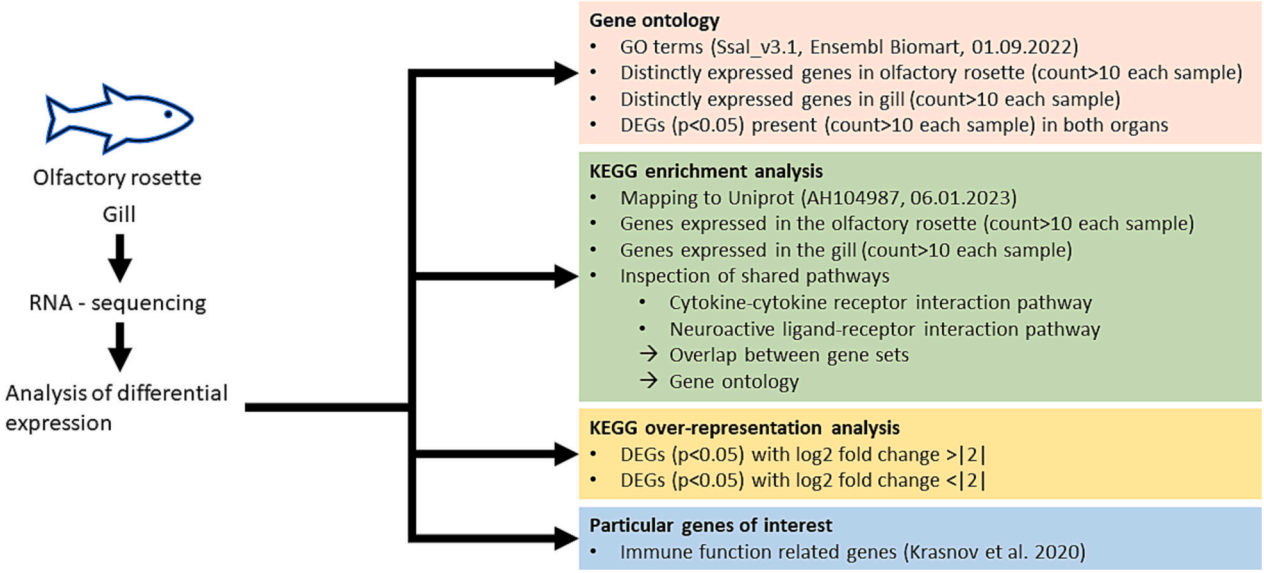

Fig. 1. Flow diagram providing an overview of the analyses described in Section 2.5.

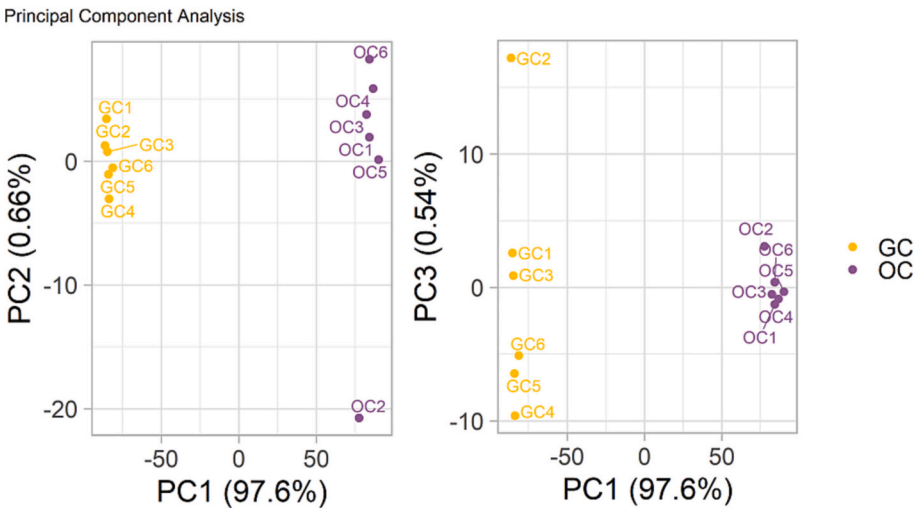

Fig. 2. Principal Component Analysis (PCA), depicting the separation of the transcriptome profiles of the olfactory rosette (OC) and gills (GC).

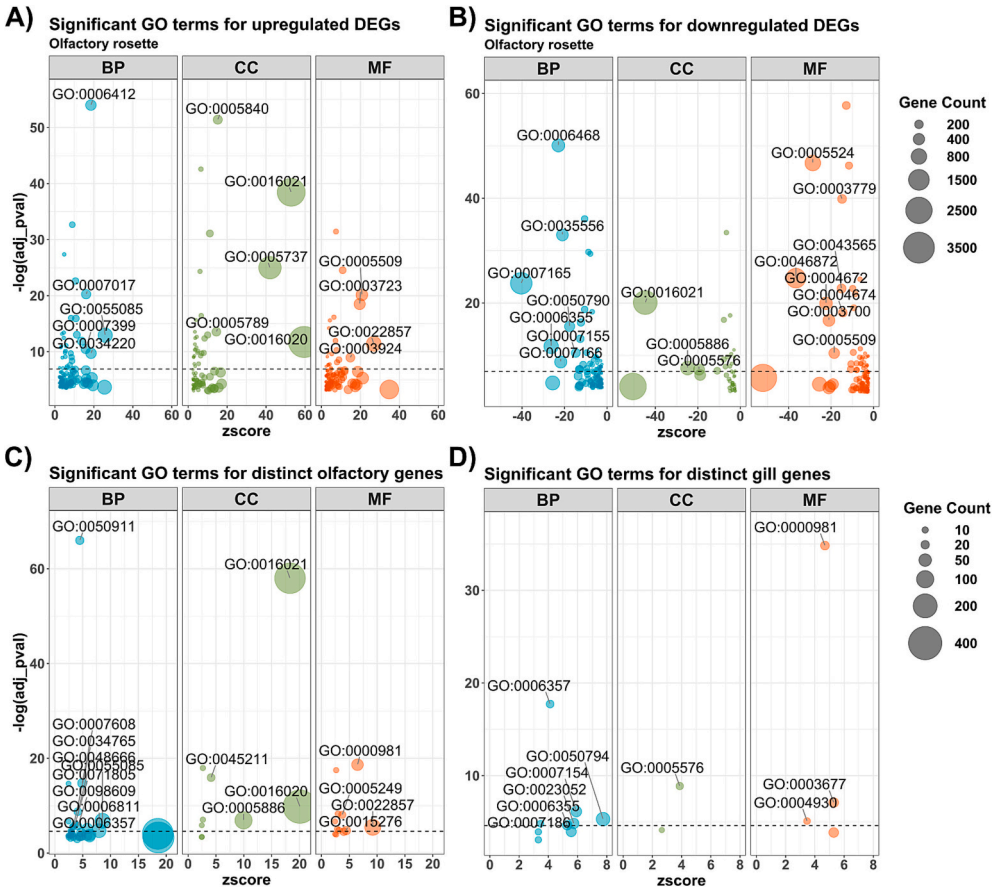

Fig. 3. Gene Ontology (GO) plots for the DEGs identified in the olfactory rosette and the gills. A) Shared genes that are upregulated in the olfactory rosette (OR), B) Shared genes that are downregulated in the OR. C) Distinct olfactory genes (log2FC from DESeq with gill as reference). D) Distinct gill genes (log2FC from DESeq with OR as ref). Dotted line represents a p-adjusted value of 0.001. BP = Biological process; CC = Cellular component; MF = Molecular function.

for the physiological homeostasis of these organs, as well as in their ability to respond to stimuli. The genes connected to these terms showed lower counts in the gills in relation to the olfactory rosette (e.g., *elavl1, ptbp2, cdh6, call4, ndufv1, aloxe3*). For the shared downregulated genes, we identified BP-terms associated with cellular signalling, including *intracellular signal transduction*, *signal transduction* and *cell surface receptor signalling pathway*. Other noticeable BP terms were *protein phosphorylation*, *regulation of catalytic activity* and *cell adhesion*. For CC the most notable GO term was *membrane*. MF terms were mostly related to binding (*ATP binding*, *actin binding*, *metal ion binding* and *calcium ion binding*) and kinase activity (*protein serine/threonine kinase activity*, *protein kinase activity*.

Table 1
Summary of annotated GO terms from plots in Fig. 2. Sub-ontologies of GO terms: BP = biological process; CC = cellular component; MF = molecular function)

Most important GO terms for distinct genes in the olfactory rosette		
BP	GO:0050911	Detection of chemical stimulus involved in sensory perception of smell
BP	GO:0007608	Sensory perception of smell
BP	GO:0034765	Regulation of monoatomic ion transmembrane transport
BP	GO:0048666	Neuron development
BP	GO:0055085	Transmembrane transport
BP	GO:0071805	Potassium ion transmembrane transport
BP	GO:0098609	Cell-cell adhesion
BP	GO:0006811	Monoatomic ion transport
BP	GO:0006357	Regulation of transcription by RNA polymerase II
CC	GO:0016021	Membrane
CC	GO:0045211	Postsynaptic membrane
CC	GO:0016020	Membrane
CC	GO:0005886	Plasma membrane
MF	GO:0000981	DNA-binding transcription factor activity, RNA polymerase II-specific
MF	GO:0005249	Voltage-gated potassium channel activity
MF	GO:0022857	Transmembrane transporter activity
MF	GO:0015276	Ligand-gated monoatomic ion channel activity
Most important GO terms for distinct genes in the gills		
BP	GO:0006357	Regulation of transcription by RNA polymerase II
BP	GO:0050794	Regulation of cellular process
BP	GO:0007154	Cell communication
BP	GO:0023052	Signaling
BP	GO:0006355	Regulation of DNA-templated transcription
BP	GO:0007186	G protein-coupled receptor signaling pathway
CC	GO:0005576	Extracellular region
MF	GO:0000981	DNA-binding transcription factor activity, RNA polymerase II-specific
MF	GO:0003677	DNA binding
MF	GO:0004930	G protein-coupled receptor activity
Most important GO terms for upregulated DEGs		
BP	GO:0006412	Translation
BP	GO:0007017	Microtubule-based process
BP	GO:0055085	Transmembrane transport
BP	GO:0007399	Nervous system development
BP	GO:0034220	Monoatomic ion transmembrane transport
CC	GO:0005840	Ribosome
CC	GO:0016021	Membrane
CC	GO:0005737	Cytoplasm
CC	GO:0005789	Endoplasmic reticulum membrane
CC	GO:0016020	Membrane
MF	GO:0005509	Calcium ion binding
MF	GO:0003723	RNA binding
MF	GO:0022857	Transmembrane transporter activity
MF	GO:0003924	GTPase activity
Most important GO terms for downregulated DEGs		
BP	GO:0006468	Protein phosphorylation
BP	GO:0035556	Intracellular signal transduction
BP	GO:0007165	Signal transduction
BP	GO:0050790	Regulation of catalytic activity
BP	GO:0006355	Regulation of DNA-templated transcription
BP	GO:0007155	Cell adhesion
BP	GO:0007166	Cell surface receptor signaling pathway
CC	GO:0016021	Membrane
CC	GO:0005886	Plasma membrane
CC	GO:0005576	Extracellular region
MF	GO:0005524	ATP binding
MF	GO:0003779	Actin binding
MF	GO:0043565	Sequence-specific DNA binding
MF	GO:0046872	Metal ion binding
MF	GO:0004672	Protein kinase activity
MF	GO:0004674	Protein serine/threonine kinase activity
MF	GO:0003700	DNA-binding transcription factor activity
MF	GO:0005509	Calcium ion binding

3.3. Pathway enrichment analysis of the olfactory rosette and the gill transcriptomes

To further explore the functional features of the two mucosal transcriptomes, we performed pathway enrichment analysis (Fig. 4). The KEGG pathway gene set enrichment analysis considered all genes found in each of the organs (each gene required to have a count of 10 or higher in each sample) and also considered the log2FC in relation to the other organ. Five pathways were significantly enriched (padj<0.05) in the olfactory rosette (Fig. 4A). The *neuroactive ligand-receptor interaction pathway* showed increased activity, while the four other pathways showed decreased activity relative to the gills. The *neuroactive ligand–receptor interaction signalling pathway* is directly related to neuro function, where neuroactive ligands influence neuronal function by binding to intracellular receptors and are capable of binding transcription factors and regulating gene expressions [30]. The olfactory rosette of fish contains olfactory sensory neurons (OSNs) responsible for the detection of chemical and biological stimuli in the environment [31],

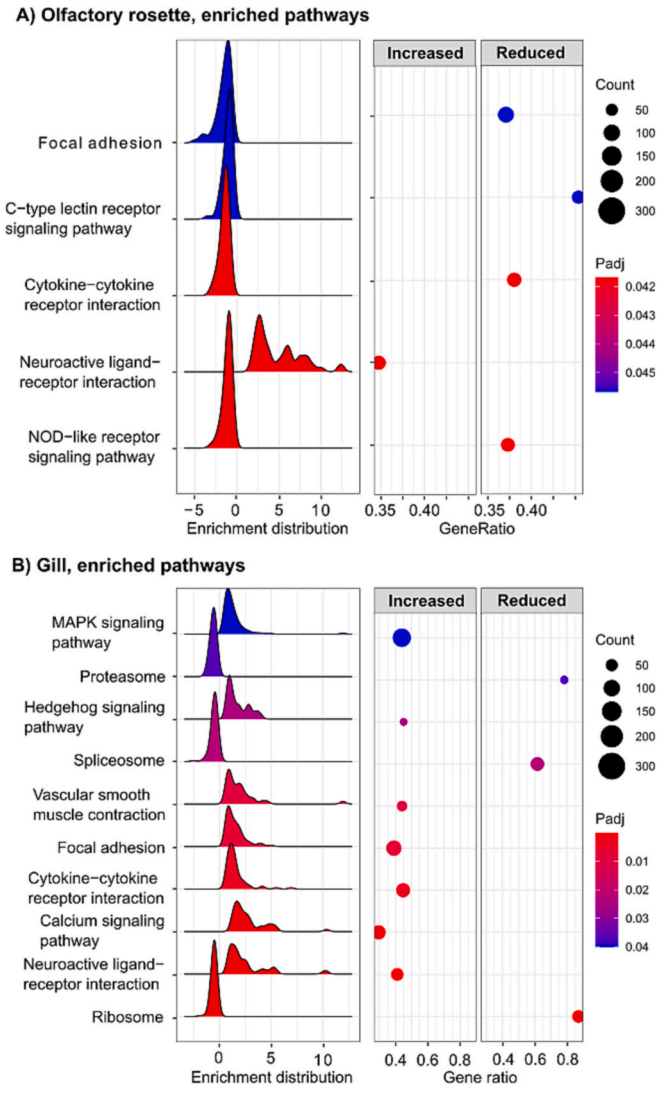

Fig. 4. KEGG pathway enrichment plots, to the left are density plots indicating the frequency of fold change values per gene within each gene set, and to the right is the gene ratio (count of core enrichment genes) / (count of pathway genes) for the A) olfactory rosette and B) gills. The enriched pathways were denoted either increased or decreased, based on the other organ. The size of the dot corresponds to the count number and the p-adjusted values are indicated by a colour gradient.

and the enrichment of the *neuroactive ligand–receptor interaction signalling* pathway may perhaps be related to this key neuroimmune feature. Cytokines are foremostly known as potent intercellular regulators of innate and adaptive immunity, though they serve other important functions as well in cell growth, differentiation, cell death, development and repair processes [32]. Enrichment of *cytokine-cytokine receptor interaction pathway* in the olfactory rosette, though gene expression levels appeared lower than the gills, indicates that under basal condition, cytokines are active but to a lesser extent than in the gills. If this could be related to the larger surface area of the gills interacting directly with the aquatic environment that contains several stimuli remains to be functionally elucidated. It is known that gills contain a significant amount of peripheral blood leukocytes [33]. Since the gill specimens were not perfused to remove blood, the enrichment of *cytokine-cytokine receptor interaction pathway* could be due to higher number of blood leukocytes in gills compared to the olfactory rosette.

Teleost fish exhibit a highly diverse pathogen recognition receptors (PRRs) for detecting and responding to pathogen-associated molecular patterns (PAMPs), and these are crucial immune arsenals at mucosal barriers [34]. Two of the signalling pathways (i.e., C-type lectin receptor signalling and NOD-like receptor signalling) associated with this process were enriched in the olfactory rosette. C-type lectin receptors (CLRs) comprise a subfamily of PRRs that senses glycans present in microbial pathogens, binds to their mannose moieties and opsonises them, thereby activating phagocytosis and/or the complement pathway [35]. In mammalian models, c-type lectin receptors have been identified to play an important function in respiratory disorders such as asthma by recognising fungal-derived allergens and other structurally similar allergens [36]. Nucleotide-binding oligomerization domain-like receptors (NOD-like receptors or NLRs) are a family of intracellular receptors possessing a C-terminal LRR (leucine-rich repeats) domain and a central NACHT domain (named after NAIP, CIITA, HET-E, and TP1) which can orchestrate a host immune response against microbial invasions and endogenous danger signals [37]. In humans, several inflammatory airway diseases (e.g., polyps, allergic rhinitis) have been linked with differential regulation of NLRs in the nose [38,39]. We have limited information on PAMP-PRR interactions in the nasal mucosa of fish. Nonetheless, we believe that CLRs and NLRs are likely crucial for the nasal mucosa of salmon to respond to constant pathogenic pressure in their immediate environment, thereby offering further implications to their role in host immunity.

Three of the enriched pathways in the olfactory rosettes were also found to be enriched pathways in the gills, however the *cytokine-cytokine receptor interaction pathway* and *focal adhesion* core gene enrichment sets were expressed at a higher level in the gills relative to the olfactory rosette (Fig. 4B). Recent evidence suggest that gills may be involved in the sensing, and even mounting a robust response to stressors [40,41]. Focal adhesion serves as dynamic signalling hubs within the cell and connect intracellular actin to the extracellular matrix (ECM) and is responsive to environmental cues [42]. Genes in this pathway are crucial to cell adhesion and signal transduction and play regulatory roles in the ability of gills to respond to osmotic stress and hypoxia [43,44]. Important signalling pathways including *calcium signalling pathway, MAPK signalling pathway* and *Hedgehog signalling pathway* were among the enriched pathways in the gills. The mitogen-activated protein kinase (MAPK) cascade is an ancient and evolutionarily conserved signalling pathway involved in several physiological processes [45], including as key transducers of osmosensory signals in fish gill cells [46]. On the other hand, Hedgehog signalling pathway has been implicated in the environmental plasticity of cichlid gill arch anatomy [47]. Enrichment of these pathways in the current study supports their key roles in salmon gill physiology. The *ribosome, spliceosome* and *proteasome* pathways showed lower gene expression in the gills in relation to the olfactory rosette. This difference offers insight into the distinctive control under normal conditions of fundamental physiological processes such as mRNA processing and protein degradation between the two mucosal organs. It is important to emphasise that the increased and decreased reference to the enriched pathways only indicates higher or lower counts in relation to the organ of comparison, and not that the pathway is substantially highly active or in quiescence. Nonetheless, the comparison of the trends provided an interesting reference on the physiological pathways that are likely at play under unstimulated conditions.

3.4. Focus on cytokine-cytokine interaction and neuroactive ligand-receptor interaction pathways in the olfactory rosette and the gills

Further investigation of the core enrichment gene sets of the *cytokine-cytokine interaction* and the *neuroactive ligand-receptor interaction pathways* for both organs revealed significant overlap in core gene enrichment sets for the *cytokine-cytokine interaction* pathway (Fig. 5A), while the core gene enrichment sets of the *neuroactive ligand-receptor interaction pathway* consisted of two distinct gene sets (Fig. 5B**,** Supplementary Material S3**)**. The large gene overlap in the *cytokine-cytokine interaction* pathway indicates that the cytokine repertoire and likely its downstream regulatory processes are perhaps similar between the two organs, which further supports their commonality as key mucosal organs of Atlantic salmon. The lower expression values found in the olfactory rosette compared to those in the gills suggests that under basal conditions the pathway operates at a relatively lower activity in the olfactory rosette than the gills, mirroring the tissue-specific enrichment revealed in Fig. 4. The tissue specific core enrichment gene sets of the *neuroactive ligand-receptor interaction* pathway indicate tissue specific pathway behaviour, which, to our knowledge have not been comparatively described earlier. We investigated the distinctiveness of the *neuroactive ligand-receptor* interaction pathway by focusing on a gene group level for GO analysis of the two distinct core gene enrichment sets and found 28 overlapping GO terms, and 49 and 67 unique GO terms for the olfactory rosette and gills core enrichment genes sets, respectively (Fig. 5C). Among the GO terms to the olfactory rosette were GO terms related to immune functions including *immunoglobin production,* and *regulation of immune effector process* (Fig. 5D). GO terms related to immunity were also exclusively identified in the gills including *production of molecular mediator of immune and response*, *immune response-activating cell surface receptor signalling pathway* and *hematopoietic stem cell differentiation* (Fig. 5E**,** Supplementary Material S3). These distinctions point to a potential difference in the regulatory mechanism concerning neuroimmune interaction in these two mucosal organs. It is also interesting to highlight that GO terms related to oxidative stress regulation, including, *superoxide metabolic process*, and *regulation of reactive oxygen species metabolic process*, were clearly represented in the olfactory rosette. We have shown earlier that the olfactory rosette of Atlantic salmon could mobilise a strong antioxidant response to oxidative stressors [13,14], thereby substantiating the overrepresentation of these GO terms within *neuroactive ligand-receptor interaction* pathway in the olfactory rosette. The core enrichment gene sets and their respective GO terms can be found in Supplementary Material S3**.**

3.5. Overrepresentation analysis of the olfactory rosette and gills transcriptomes

An overrepresentation analysis was performed by defining the gene list of interest as significant DEGs between the olfactory rosette and gills, with the latter as the reference organ, and with a log2 fold change larger than |2| (i.e., genes that showed up- or downregulation in the olfactory rosette in relation the gills) (Fig. 6). The resulting output included the *neuroactive ligand-receptor pathway* which was also found common for both organs in the enrichment analysis. Four additional pathways were identified to be significantly overrepresented, including *cell adhesion molecules*, *calcium signalling*, *adrenergic signalling in cardiomyocytes* and *purine metabolism*. Density plots of the log2 fold-change of the genes found for each respective pathways show a tendency for upregulation of genes in the *neuroactive ligand-receptor interaction* and *cell adhesion*

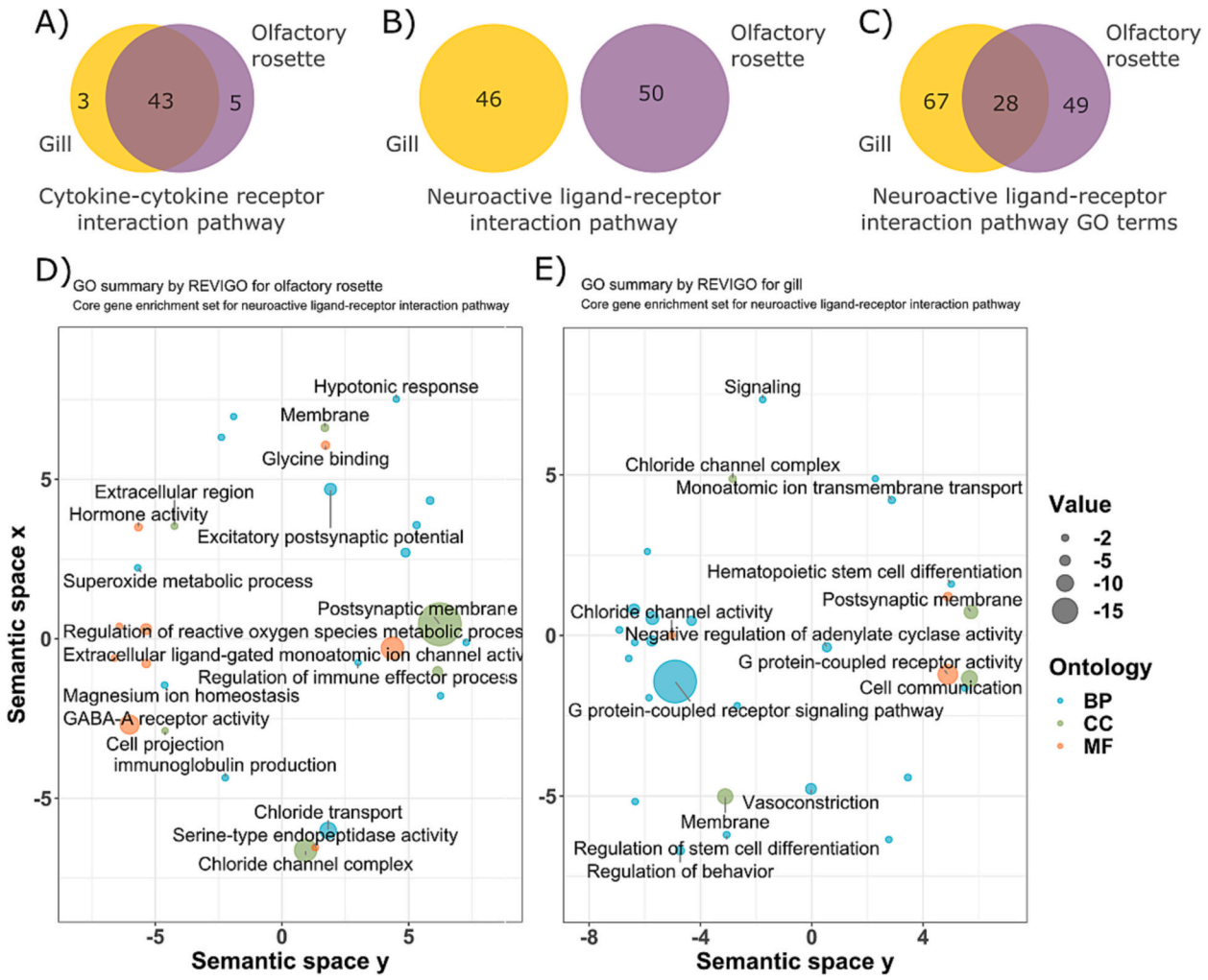

Fig. 5. Core enrichment gene sets of the cytokine-cytokine receptor interaction and the neuroactive ligand-receptor interaction pathways. A) Venn diagram for the core enrichment gene sets of the cytokine-cytokine interaction pathway. B) Venn diagram for the core gene sets of the neuroactive ligand-receptor interaction pathway. C) Venn diagram for the GO terms of the core gene sets within the neuroactive ligand-receptor interaction pathway. D-E) Scatter plot of GO term summary (REVIGO) for the core enrichment gene set for the neuroactive ligand-receptor interaction pathway for the D) olfactory rosette and E) gill. Revigo uses Multidimensional Scaling (MDS) to reduce the dimensionality of a matrix of the GO terms pairwise semantic similarities, which generates the x- and y-axis. I.e., similar GO terms are positioned closer together in the graphs. Value is $\log_{10}$ of the p-adjusted value for the GO term.

molecules pathways, while the *purine metabolism* pathway shows a tendency for genes to be downregulated (Fig. 6).

To explore potential similarities between the two organs, we further filtered for log2 fold change values ranging from −2 to 2, targeting the less quantitatively different genes (Fig. 7). As expected, several basal physiological pathways (e.g., *protein processing in endoplasmic reticulum*, *ribosome*) were included as significantly overrepresented, emphasising the physiological homeostasis crucially maintained in these organs. The immunological feature of the two organs was highlighted by the overrepresentation of several immune-related pathways, including the *NOD-like signalling pathway, mTOR signalling pathway*, *C-type lectin receptor signalling pathway*, and *FoxO signalling pathway*. This indicates that the MALT in the olfactory rosette, like in the gills, exhibits the molecular repertoire of an immune tissue capable of recognising, processing, and mounting a response to foreign antigens, such as pathogens. As discussed in Section 3.3, c-type lectin receptors and NOD-like receptors are a large family of PAMP that detect PRR, process the danger and transduce signals to activate the appropriate immune cascade. These fundamental mechanisms are early mediators of innate mucosal responses. Cellular metabolism is an important regulator of immune cell function, where the regulation mTOR pathway plays a crucial role in integrating environmental cues to meet the metabolic demands in the immune microenvironment [48]. In fish, the mTOR pathway has been shown to have a role in intestinal immunity [49] and in the phagocytic capacity, proliferation, and Ig production of B cells [50]. The transcription factors in the Forkhead box-O (FOXO) signalling pathway have a fundamental function in the development and differentiation of immune cells [51], in particular, has been linked to mucosal immunity [52]. These immune signalling pathways highlight the role of MALT associated to the gills and olfactory organ play as the first line of defence of fish equipped with the molecular machineries for pathogen recognition and initiation of immune response. Moreover, this further supports earlier evidence on the immunological function of MALT present in the olfactory organ of fish [9].

3.6. *Highlights of the mucosal immunological features of the olfactory rosette*

A multigene expression assay developed earlier for Atlantic salmon [22] was used as a basis to further explore the immunological distinctive features of the MALT found in the olfactory rosette and the gills. The genes included in the assay were identified as core immune competence biomarkers for post-smolt Atlantic salmon. Sixty-four of the genes from the assay were found to be present in the DESeq-data, and forty of these

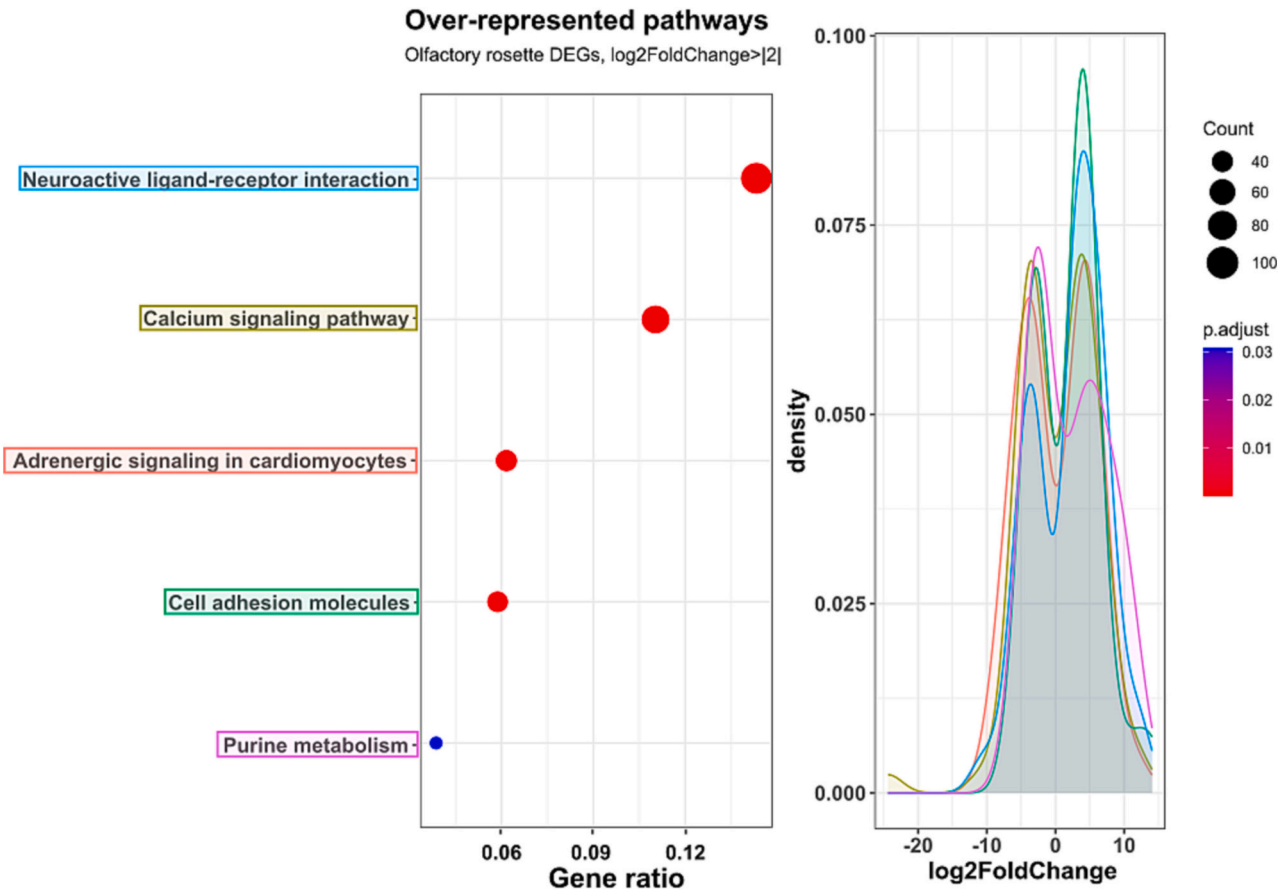

Fig. 6. Overrepresented pathways for olfactory rosette DEGs with a log2 fold change≥ |2|. A) Pathways with adjusted *p*-value and gene ratio, B) A smoothed density distribution of log2 fold change of the genes found in each pathway. Gene ratio = number of genes found for pathway/total number of genes found for all detected pathways. The size of the dot indicates the count of the genes under different pathways while the colour gradient represents the adjusted *p* values.

were found to be differentially expressed (Supplementary Material S4). Fifteen of these genes were significantly upregulated in the olfactory rosette (i.e., relative to the gills), while 25 were downregulated. Among the upregulated immune-related genes were cytokines including *interleukin 10 receptor b* (*il10rb*), *interleukin 18* (*il18*), *interleukin 20 receptor* (*il20ra*), and *chemokine ligand 19* (*ccl19*). It was shown earlier that CCL19 is a chemokine that controls the organization and function of NALT in a mammalian model [53]. In the seminal paper on nasal immunity in rainbow trout fish, *ccl19* was identified to be highly upregulated in the olfactory rosette following nasal vaccine delivery [9]; it was followed up by the discovery of its role in orchestrating both nasal and systemic antiviral immune responses in the same fish species [54]. Therefore, the present study supports the earlier proposition that *ccl19* plays a central regulatory role in the function of NALT in vertebrates. On the other hand, several cytokines were more expressed in the gills than the olfactory rosette including *interleukin 13 receptor a2* (*il12ra2*), *interleukin 1 r2* (*il1r2*), *colony stimulating factor 3 receptor* (*csf3r*) and *tumor necrosis factor* (*tnf*). Cytokines have pleiotropic functions, but their roles in immunity are outlined by their regulatory control of the growth and activity of immune cells, including during inflammation and pathogen clearance. For instance, *il18* belongs to the IL1 family and participates as a promoter of inflammation [32]. We found that *matrix metalloproteinases 9* (*mmp9*) and *13* (*mmp13*), which degrade extracellular proteins were upregulated in the olfactory rosette. It was implicated earlier that these *mmps* participate in the remodelling of extracellular matrix as an essential strategy of salmon to pathogens and stressors [55]. The panel of analysed genes revealed that the olfactory rosette possesses antibacterial activity as with the gills. Two known antimicrobial peptides (amp), *cathelicidin* (*camp*) and *hepcidin* (*hamp*) were upregulated in the olfactory rosette, while the expression of genes with known antibacterial functions *myeloperoxidase* (*mpo*) and *lysozyme c II* (*lysc2*) were higher in the gills. The amp-mediated response to bacterial stimuli in the olfactory rosette is not yet documented, however, previous knowledge about the antibacterial functions of *camp* [56] and *hamp* [57], underscores the innate arsenal of the NALT to respond to bacterial stimuli. The complement system plays a role in the generation of activated protein fragments for microbial killing, phagocytosis, inflammatory reactions, immune complex clearance, and antibody production [58]. During *Ichthyophthirius multifiliis*-infection in trout, expression of complement-related genes in the olfactory rosette changed significantly when the parasite level was highest [59]. *Complement c1q c chain* (*c1qc*) and *complement c1q-like protein* (*c1ql2*) were significantly downregulated while *complement factor 4* (*c4*) was found to be upregulated in the olfactory rosette. Though the nasal immune function of *c4* has not yet functionally elucidated in fish, earlier evidence in mammalian indicates that C4 was crucial for immune defence and inflammation control during rhinosinusitis [60], thus, we conjecture a similar role in the nasal defence of salmon against waterborne stimuli. This targeted identification of immunocompetence markers in the olfactory rosette and their comparative expression in the gills provide evidence that the olfactory rosette has the key immune molecules previously identified and characterised in other mucosal organs of salmon crucial for immune responses to pathogenic and stress insults [22,55].

3.7. Conclusions

Genomic resources are important toolboxes to elucidate the molecular functionality of MALT present in mucosal organs. In this paper, we

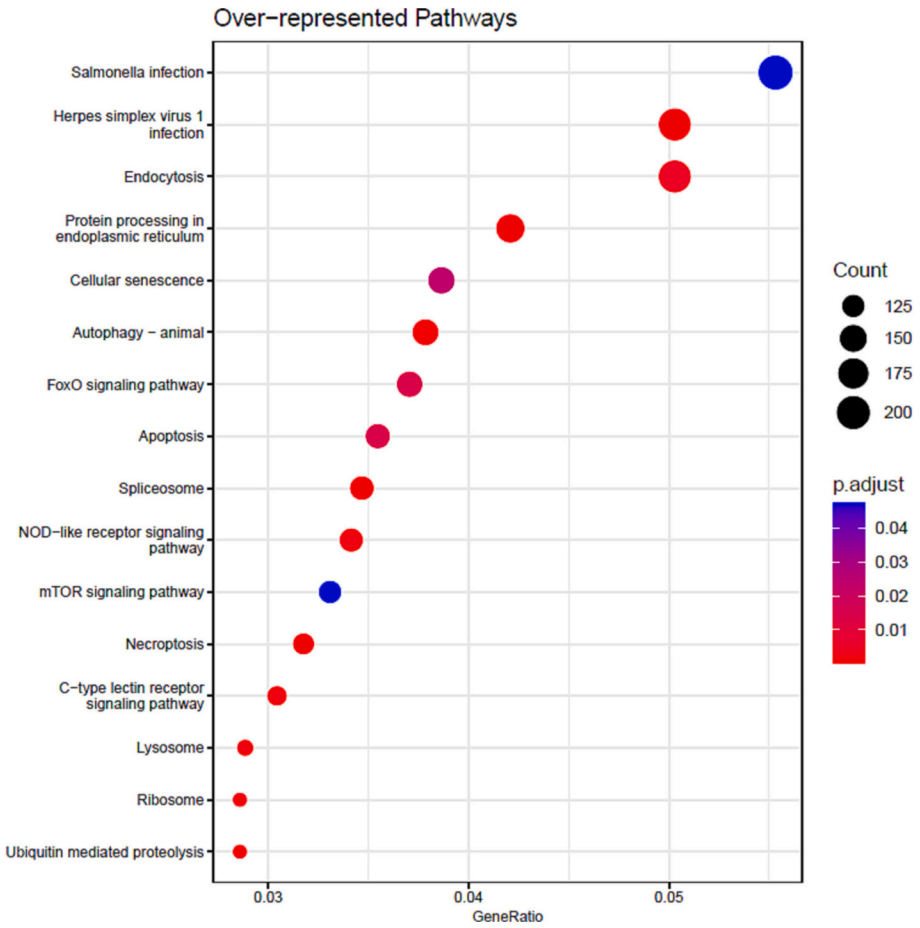

Fig. 7. Overrepresentation analysis focused on the shared genes expressed ($-2 < \log2FC < 2$) in the olfactory rosette and the gills. Enriched functional pathways are shown along the Y-axis and the gene ratio along the X-axis (for explanation of axis and legends see Fig. 6).

presented the molecular landscape of Atlantic salmon olfactory rosette. It is a main organ of the olfactory system, and the presence of MALT associated to the mucosal epithelium highlight its role in immunity [9,10]. The nasal microenvironment underlines the crossroads between olfaction, one of the most ancient sensory systems, and immunity, the system and mechanism that has protected fish for millions of years. Basal transcriptomic analysis revealed that olfactory rosette and the gills, a well-studied mucosal organ, shared functional similarities, especially on key pathways necessary to maintain physiological homeostasis including cellular regulation, transcription and membrane transport. Pathway enrichment revealed that cytokine-cytokine interaction and the neuroactive ligand-receptor interaction pathways were at the core of the shared similarity between the two organs, though the regulation at basal condition differed. The immune features of the MALT present in the two organs were further characterised by the overrepresentation of several immune-related pathways, particularly important for pathogen recognition and processing. Further targeted profiling of immunological feature using the gene panel from an earlier developed multigene assay for immunocompetence in Atlantic salmon supports that the MALT associated to the mucosal epithelium of the olfactory rosette possesses molecules crucial for immune defences against pathogens and stressors. The present study provides a fundamental molecular toolbox for comparative mucosal immunology and will be valuable for further exploration of the role of nasal immunity in Atlantic salmon.

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Author contributions

- C.C.L. conceived the research idea.
- C.C.L. collected the samples.
- M.I. and A.Y.M.S. performed the bioinformatic analyses.
- M.I. and A.Y.M.S. visualized the data.
- C.C.L. and M.I. interpreted the data.
- C.C.L. and M.I. wrote the first draft of the manuscript.
- All authors contributed to the writing and review of the final version of the manuscript.

Author statement

Carlo C. Lazado: acquired the funding, conceptualized the project and the analysis, interpreted the data, wrote, edited and reviewed the manuscript. **Marianne Iversen**: analysed, visualized, and interpreted the data, co-wrote the first draft of the manuscript. **Arvind Y.M. Sundaram**: analysed and interpreted the data, reviewed 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 the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest. Mention of trade names and service providers is solely for reporting and does not imply recommendations.

Data availability

The sequencing file has been deposited in ArrayExpress. Accession number is provided in the manuscript.

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