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Differential tissue immune stimulation through immersion in bacterial and viral agonists in the Antarctic *Notothenia rossii*

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ABSTRACT

The genome evolution of Antarctic notothenioids has been modulated by their extreme environment over millennia and more recently by human-caused constraints such as overfishing and climate change. Here we investigated the characteristics of the immune system in *Notothenia rossii* and how it responds to 8 h immersion in viral (Poly I:C, polyinosinic: polycytidylic acid) and bacterial (LPS, lipopolysaccharide) proxies. Blood plasma antiprotease activity and haematocrit were reduced in Poly I:C-treated fish only, while plasma protein, lysozyme activity and cortisol were unchanged with both treatments. The skin and duodenum transcriptomes responded strongly to the treatments, unlike the liver and spleen which had a mild response. Furthermore, the skin transcriptome responded most to the bacterial proxy (cell adhesion, metabolism and immune response processes) and the duodenum (metabolism, response to stress, regulation of intracellular signal transduction, and immune system responses) to the viral proxy. The differential tissue response to the two proxy challenges is indicative of immune specialisation of the duodenum and the skin towards pathogens. NOD-like and C-type lectin receptors may be central in recognising LPS and Poly I:C. Other antimicrobial compounds such as iron and selenium-related genes are essential defence mechanisms to protect the host from sepsis. In conclusion, our study revealed a specific response of two immune barrier tissue, the skin and duodenum, in *Notothenia rossii* when exposed to pathogen proxies by immersion, and this may represent an adaptation to pathogen infective strategies.

1. Introduction

Antarctica's isolation and cold-stable waters led to the evolution of organisms highly adapted to these extreme conditions. Among the teleost fishes, the notothenioid acquired antifreeze glycoproteins, a higher cellular mitochondrial density, constitutive levels of antioxidant enzymes and increased lipid contents of biological membranes [1]. Their haemoglobin lost oxygen affinity or was lost altogether in some species and they lost a heat-shock protein response to temperature [2]. Less attention, however, has been paid to the effect of isolation on the evolution of the immune system in Antarctic organisms, including the notothenioid.

Compared with other vertebrates, teleost fishes are protected from pathogens by innate and acquired immune responses. Teleost fishes have a well-developed innate immune response, possibly due to their constant contact with aquatic microbiota, including potential pathogens [3]. When fish from temperate waters, many from aquaculture, are in contact with pathogen-related molecules, such as lipopolysaccharide (LPS), significant changes in gene expression include pathogen-recognition receptors (PRRs), antimicrobial peptides and complement protein genes [4,5]. Significant endocrine and physiological changes also occur in plasma cortisol and luteinising hormone [6], mitochondrial metabolism, respiratory burst and reactive oxygen species [7].

Studies of Antarctic fish responses to pathogens and pathogen agonists remain sparse. Exposure to LPS caused a modification in iron metabolism genes in the notothenioids *N. rossii* and *N. coriiceps* [8]. *N. coriiceps* exposure to heat-killed *Escherichia coli O11:B4* (*E. coli*) and

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polyinosinic: polycytidylic acid (Poly I:C) in the water for 6 and 12 h modified toll-like receptors (TLR) and major histocompatibility complex I and II genes expression [9]. In contrast, LPS and Poly I:C injections failed to modify the expression of TLR and interleukin-related genes in both *Harpagifer antarticus* and *H. bispinis* at 2 °C, but immune-related genes were upregulated at 5 and 8 °C both in control and immune-challenged *H. antarticus* but not *H. bispinis* [10]. Transcriptome studies to characterise the global response to an immune challenge have generally been focused on a single tissue which means the analysis of tissue sensitivity to a common challenge in the same animal has not been established [11,12].

N. rossii is a notothenioid widely distributed in Antarctica, with more abundance at lower latitudes, and inhabiting waters with a temperature of −2 to 2 °C across an annual cycle. Overfishing in the 1970s led to the near extinction of *N. rossii* due to the associated population collapse and caused modifications in the population structure and physiology, such as slower growth and resilience [13]. Although *N. rossii* populations have been slowly recovering [14], it is still unclear whether overfishing affected the genetic structure and immune system-related alleles.

The present study was designed to evaluate the tissue-specific responses to bacterial and viral agonists in *N. rossii*. We were interested in examining if the *N. rossii* immune response to LPS and Poly I:C diverged from that of other notothenoids (*N. coriiceps*) and non-Antarctic teleost fish. For that purpose, *N. rossii* individuals were subjected to baths of LPS and Poly I:C and the blood plasma immune response and duodenum, skin, liver and spleen transcriptomes were analysed. We found significant differences in the responsiveness of the different tissues although surprisingly little change was observed in plasma immune parameters.

2. Materials and methods

2.1. Experimental conditions and sample collection

Fish capture, handling and experimentation were based on a permit issued by the Portuguese Environmental Agency under the regulations of the Madrid Protocol. *Notothenia rossii* (29.4 ± 0.71 cm length and 296 ± 19.4 g weight) were captured during the summer of 2019 (January and February) using a hook-and-line between 5 and 20 m deep in Maxwell Bay near the Chinese Great Wall Station located at King George Island, northwest of the Antarctic Peninsula (GPS coordinates: 62°13‴S, 58°58′W). Before experimentation, the fish were maintained in a 200 L flow-through seawater circuit of plastic tanks with aeration for at least five days (n = 6 per tank, density 8.9 ± 0.32 kg/m^3). Fish were fed daily with limpets, salps, amphipods and fish muscle. The water temperature (2.1 ± 0.5 °C), salinity (29 ± 0.5 ppt) and oxygen levels (10.7 ± 0.4 ppm) were monitored three times a day (7 a.m., 2 p.m. and 9 p.m.). No mortality was observed during acclimation or experiments.

The fish were lightly anaesthetised in 2-phenoxyethanol (0.1 mL/L, Sigma-Aldrich, Spain) and divided into three treatment groups (n = 6 per group; Supplementary Fig. S1): 1) the control group received only seawater; 2) the LPS group received LPS (*Escherichia coli* O111:B4 dissolved to 2.5 μg/mL in 1.1% NaCl, L2630, Sigma-Aldrich) mixed in the tank to a final concentration of 1.25 mg/L; 3) the Poly I:C group received synthetic Poly I:C (dissolved to 2.5 μg/mL water in 1.1% NaCl, P1530, Sigma-Aldrich) mixed in the tank to a final concentration of 1.25 mg/L poly I:C. The doses of LPS and Poly I:C were in the range of those used in immune challenge studies in fish [9,15–18]. The water flow in the tanks was stopped during the 1-h exposure, and temperature and oxygen levels were monitored.

At the end of the experiments, 8 h after the immune challenge, the fish were sacrificed with excess 2-phenoxyethanol (1 mL/L, Sigma-Aldrich). Fish were weighed (nearest g), and blood was collected from the caudal vasculature using a heparinised 1 mL syringe fitted with a 21-gauge needle. The liver and spleen were weighed (nearest mg) to determine their relative weight (liver or spleen weight/body weight x100). The anterior intestine (duodenum region), skin, liver, and spleen were dissected and fixed in RNAlater (Sigma-Aldrich) at four °C for 24 h before storage at −80 °C. An aliquot of blood was used to determine the haematocrit, and another was centrifuged at 10,000 g (4 °C) for 4 min to collect the plasma. For the hematocrit, blood was collected into heparinised micro hematocrit capillary tubes (VWR, Portugal) and centrifuged at 10,000 g for 5 min at 4 °C in a hematocrit centrifuge (VWR). The packed cell volume was measured as a percentage of total volume. The plasma was frozen at −80 °C until total protein, cortisol levels, lysozyme and antiprotease activities were analysed.

2.2. Blood plasma biochemistry

Total plasma proteins were measured using a Quick Start™ Bradford Protein Assay kit (Bio-Rad, Portugal) adapted to 96-well plates as previously described [19]. Measurements were performed at 590 nm in a spectrophotometer at 25 °C (Agilent Technologies, USA). Lysozyme activity and total antiprotease activity were measured as described earlier [19]. In the optimised protocol, 20 μl of porcine pancreas trypsin (5 mg/mL, Sigma-Aldrich) was mixed with 10 μL of *N. rossii* plasma for 10 min. Then 200 μL of 0.1 M phosphate buffer pH 7.0 and 250 μl of 2 % Azocasein (A-2765, Sigma-Aldrich) was added. The reaction was incubated for 1 h at 4 °C (similar to the temperature *N. rossii* experience in nature), 500 μL of 10 % trichloroacetic acid (Sigma-Aldrich) was added, and the reaction was incubated for 30 min at room temperature (21 °C) before centrifugation at 10,000 *g* for 10 min. The supernatant (100 μl) was collected and added in duplicate to a 96-well plate, followed by 100 μl sodium hydroxide (1 N, NaOH, VWR, Spain). A blank reaction was prepared with NaOH (100 μl) and phosphate buffer (100 μl). Reaction products were measured at 450 nm in a spectrophotometer (Agilent Technologies). Plasma cortisol was measured using an in-house radioimmunoassay (RIA) as previously described [19].

2.3. RNA extraction, sequencing library preparation and sequencing

Total RNA extraction of duodenum, skin, spleen and liver (~25 mg, n = 3/tissue) from control, Poly I:C and LPS-challenged fish and sequencing library preparation was carried out exactly as previously described [19]. The 36 paired-end complementary DNA (cDNA) libraries with an insert size of 250 base pairs (bp) were sequenced using an Illumina HiSeq X Next-Generation Sequencing system (Shanghai Ocean University, China).

2.4. Transcriptome assembly and gene expression analysis

A total of 7,865,495,667 million paired-end (PE) raw transcriptome reads were generated from sequenced libraries of the liver (Lv_1 to Lv_3), duodenum (Du_1 to Du_3), spleen (Sp_1 to Sp_3), and skin (Sk_1 to Sk_3) from the control (C1–C3, n = 3 libraries/tissue), Poly I:C (P1–P3, n = 3 libraries/tissues) and LPS-treatment groups (L1-L3, n = 3 libraries/tissue). The Galaxy v21.01 platform was used for RNA-Seq quality control using FastQC and FastP as described earlier [19]. Index and adapter sequences were trimmed using Trimmomatic v4.4.10, and low-quality trimmed sequences were eliminated using cutadapt when the FPKM was <1 and/or the length was <300 base pairs (bp). *De novo* assembly of all reads was performed in Trinity v2.11.0 with the default conditions for the "-normalise reads" parameter as previously described [19]. TransRate software (version 1.0.3) was used for quality filtering and optimisation of the assembly since no reference genome was available [19]. The reference transcriptome obtained was used to map the reads from each tissue sequencing library and run the differential gene expression analysis. Clean reads were mapped using the RSEM package v1.3.3 as described earlier [19]. The raw sequence reads were deposited to the NCBI Sequence Reads Archive with BioProject accession ID PRJNA1051132. Differentially expressed genes (DEGs) were identified using the DESEq2 package v1.30.0 in RStudio v1.4.1103 (FDR <0.05) through pairwise comparisons between each group (control,

Poly I:C and LPS) for each tissue. Heatmaps were generated for DEGs using the heatmap package v1.14.0 from RStudio v1.4.1103. Multivariate Principal Components Analysis (PCA) was performed using DEGs in ggplot2 v3.3.0 in RStudio with confidence ellipses set at > 95%. The significance threshold was set at FDR <0.05.

2.5. Gene functional annotations

Gene annotation of assembled contigs was performed with DIAMOND v0.9.14.115 against the NCBI and UniProtKB/Swiss-Prot databases using BlastX v2.9.0 (e-value cutoff of 1e−5) [20]. In addition, functional annotation was obtained using the best deduced open reading frame (ORF) given by Transdecoder v5.5.0 as previously mentioned [19]. GO annotation was obtained from the UniProt/Swiss-Prot database and KEGG from KAAS software and GO-enriched terms were summarised by REVIGO as previously described [19], which removed redundant GO terms based on semantic similarity and gave a summarised output in Venn diagrams. In parallel, KEGG enrichment analysis of DEGs (FDR <0.05) was conducted using clusterProfiler in RStudio with the default parameters, and the top 10 enriched terms in duodenum and skin under LPS and Poly I:C were plotted by ggplot2 software v3.3.0 in RStudio.

2.6. Quantitative PCR

A group of genes that were differentially expressed in the transcriptomes generated (*cl7*, *cl4*, *slc12a1*, *slc12a2*, *pparγ* and *tnf-α*) were chosen to confirm their expression by real-time quantitative PCR (RT-qPCR) using specific primers designed in house or available in the literature (TRY) [21]. The reference genes selected for normalisation, β-actin (TR9194|c3_g4_i8) and *18s rRNA* did not vary in expression between experimental groups. The qPCR reactions were performed in 384-well plates (Axygen, Germany) using a Cybio Felix Pipetting Robot Real-Time PCR Detection System (Analytik Jena, Germany) in a final reaction volume of 10 μl containing 2 μl of cDNA, 5 μl of FORGET-ME-NOT™ (Biotium, USA) and 0.3 mM of forward and reverse primers (Supplementary Table S1). The thermocycle used was: 95 °C for 30 s, followed by 40 cycles of 95 °C for 5 s and 10 s at the annealing temperature. Melting curves 60 °C–95 °C with an increment of 0.5 °C for each 10 s were performed to confirm reaction specificity. To quantify transcript expression levels, standard curves for each gene were included in RT-qPCR reactions using serial dilutions (1:10) from 50 ft/μl to 50 ag/μl of the quantified amplicon. All RT-qPCR analysis included a no template control and a -RT control. PCR efficiencies were >90 % for each target gene transcript analysed. Gene expression levels were normalized using the geometric mean of the two reference genes (18s and β-actin) based on the standard curve method [22].

2.7. Statistical analysis

The effect of treatments on the biochemical parameters and hepatosomatic and spleen relative weights was analysed by One-way analysis of variance (ANOVA) followed by the Holm-Sidak method (blood plasma biochemical analyses) with a significance level of 5%. The graphs were generated using GraphPrism v6.01. The correlation between the transcriptome results (FPKM, Log 2 fold change) and RT-qPCR (Log 2 fold change) were determined using a Pearson correlation, generated by ggplot2 v3.3.0 and corrplot v0.92 software packages in RStudio.

3. Results

3.1. Morphological and biochemical parameters

No significant differences were detected in the relative hepatosomatic and spleen weights (data not shown) or in plasma protein (Supplementary Fig. S2A), lysozyme activity (Supplementary Fig. S2B) and cortisol (Supplementary Fig. S2C) between the experimental groups. In contrast, Poly I:C, but not LPS, caused an approximately 14% antiprotease inhibition (p = 0.012), indicating higher plasma protease activity in this group (Supplementary Fig. S2D). Similarly, Poly I:C also caused a slight but significant reduction in haematocrit from 39% to 35% (p = 0.0344; Supplementary Fig. S2E).

3.2. Transcriptome quality

A total of 283.16 million (M) raw reads were obtained: 162.27 from the liver libraries, 126.51 from the spleen, 137.65 from the duodenum and 139.90 from the skin (Supplementary Table S2A). After quality filtering, 161.46 M reads were obtained from the liver, 125.20 from the spleen, 124.06 from the duodenum and 129.24 from the skin. Upon assembly, there were 82,913 contigs from the liver, 130,331 from the spleen, 95,423 from the duodenum and 48,188 from the skin. The median contig length (N50) was 1836 bp, and the GC content across the dataset was 45 % (Supplementary Table S2A). The average alignment ratio for the sequenced reads against the *N. rossii* reference transcriptome was 90% for the liver, 83% for the spleen, 85% for the intestine and 86% for the skin (Supplementary Table S2B). Annotated contigs were 86% (71,686 contigs), 65% (84,083 contigs), 59% (56,063 contigs) and 54% (26,137 contigs) for liver, spleen, duodenum, and skin, respectively.

3.3. Differentially expressed genes

Pairwise comparisons between the immune challenges and control for each tissue revealed a high variation in DEGs with tissue-specific responses and little overlap (Table 1, Fig. 1). In the duodenum, the control compared to LPS challenge generated 220 DEGs, of which 199 were up- and 21 down-regulated, while Poly I:C challenge generated 1582 DEGs, of which 1531 were up- and 51-down regulated (Supplementary Table S3A-3B). In the skin, the control compared to LPS treatment generated 1827 DEGs (698 up- and 1129-down regulated), while only 195 (62 up- and 133-down regulated) were generated by control compared to Poly I:C challenge (Supplementary Table S3C-3D). A low number of DEGs were generated in the spleen by control compared to any of the challenges: 11 for LPS (9 up- and two down-regulated) and 23 for Poly I:C challenge (11 up- and 12 down-regulated) (Supplementary Table S3E-3F). In the liver, 49 DEG were generated by control compared to LPS (46 up- and three down-regulated) and 131 for Poly I:C (75 up- and 56 down-regulated) (Supplementary Table S3G-3H).

In the LPS challenge, the skin and duodenum shared 26 DEGs and skin and liver 10, with no share of DEGs by any other tissue combination (Fig. 1A). In the Poly I:C challenge, the skin and duodenum shared 40 DEGs, the duodenum and liver 17, skin, duodenum and liver 2, and less than 3 DEGs were shared by skin and liver (Fig. 1B). A PCA analysis of the DEGs for the four tissues and group treatments revealed that skin and duodenum were divergent to the spleen and liver (Fig. 2A). Separate PCA for each tissue revealed homogeneity of the control samples, while any treatment caused different levels of variation depending on the tissue, suggesting differences in sensitivity among individuals (Fig. 2B-

Table 1
Dataset annotation statistics.

Transcript annotation	Number	Percentage (%)
Annotated	237,969	67
BlastX and BlastP hits to NCBI	172,541	73
BlastX and BlastP hits to SwissProt	172,782	73
KEGG annotation	80,381	34
Gene Ontology annotation	172,921	73
Non-annotated	118,886	33

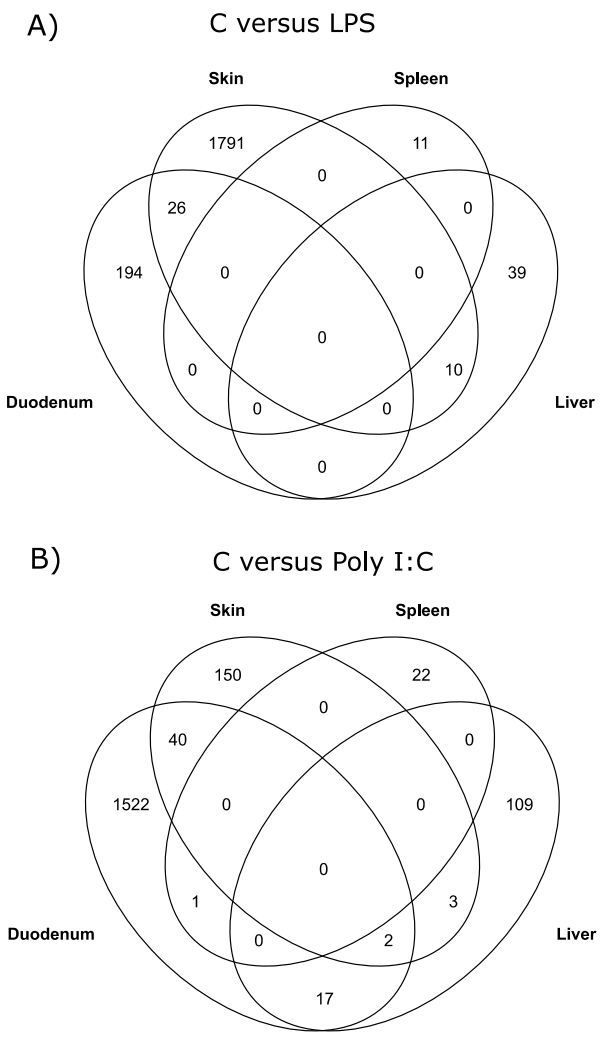

Fig. 1. Venn diagram showing the number of specific and shared DEGs in the four tissue transcriptomes of the A) Control (C) versus LPS and B) Control (C) versus Poly I:C (FDR<0.05).

E). At least 70% of the variation was accounted for in each tissue's first two dimensions of the PCA (Fig. 2). In the skin, the response to LPS was clear, although more divergent for the Poly I:C challenge (Fig. 2B). In contrast, in the duodenum, the LPS challenge had a more divergent response than poly I:C (Fig. 2C). Liver and spleen samples were more similar and least variable, although they also seemed to respond differently to the challenges (Fig. 2D and E). A clustering heatmap confirmed these observations (Fig. 3). Only the duodenum and spleen samples clustered entirely according to treatment. The skin and liver had overlapping samples from different treatment groups, especially Poly I:C-treated skin (Fig. 3B) and LPS-treated liver (Fig. 3D).

3.4. Functional analysis

In the LPS-treated fish, the skin had the most robust transcriptomic response and was represented by a large number of GO terms: 1668 Biological Process (BP), 242 Cellular Component (CC), 155 Molecular Function (MF). The most significant BP were linked to the regulation of biological and cellular processes, epithelial cell differentiation and tissue development, cell-cell adhesion (tight junction organisation/assembly), metabolic processes, in particular those linked to organonitrogen compounds and macromolecule, and immune response (Fig. 4, Supplementary Table S4). The duodenum was represented by about 10% of the skin GO terms (167 BP, 62 BP, 8 MF), of which approximately 69% BP overlapped (Fig. 4, Supplementary Table S4). In the liver, GO terms were less represented (15 BP, 55 CC, 36 MF) and were related to those in skin and duodenum and included primarily protein and amino acid-related biosynthetic processes. In the spleen, there were few significant GO terms (16 MF), and only transferase activity appeared to be related to immune response.

In the Poly I:C treated fish, the duodenum had the most robust response and was represented by a large number of GO terms (1325 BP, 221 CC, 196 MF), of which the most significant BP was related to the regulation of biological and metabolic processes, energy generation, response to stress and homeostatic processes, regulation of intracellular signal transduction and developmental processes, as well as immune system responses (Fig. 4, Supplementary Table S4). The skin was represented by 11% of GO terms (201 BP, 82 CC, 52 MF) compared to the duodenum, of which 52% overlapped with GO terms present in the duodenum. Those represented with the most significance were protein and amino acid biosynthetic processes and energy generation (Fig. 4, Supplementary Table S4). The liver was represented by a similar number of GO terms (228 BP, 17 CC, 4 MF), a large majority related to lipid (fatty acid), purine and carbohydrate metabolism. There was a high overlap between liver and duodenum GO term (72%) related processes in mitochondria, including carbohydrate and lipid metabolism and apoptosis. However, there was minimal overlap between GO terms in liver and skin (only 12% overlap). The spleen had no significant representation of GO terms.

Comparison of the response to LPS and Poly I:C within each tissue revealed common patterns. In the skin, 56% of the BP terms generated by the Poly I:C treatment overlapped with those from the LPS treatment. They included actin filament-based process, cellular macromolecule/protein/small molecule metabolic process and their regulation, and developmental processes, among others (Supplementary Table S4). Similarly, in the duodenum, 70% of the GO terms generated by LPS treatment overlapped with those from Poly I:C. These included metabolic processes, responses to chemicals, and stress and developmental processes, among others (Supplementary Table S4).

In the duodenum, the highly enriched KEGG pathways under LPS stimulation were related to metabolic pathways, tight junctions, cellular and humoral immune responses from both innate and acquired immunity, including cell adhesion, pathogen recognition receptors (e.g. C-type lectins), antigen processing and presentation, leukocyte migration, phagosome and mucosal network for immunoglobulin production (Fig. 5, Supplementary Table S5). In the same tissue, Poly I:C promoted pathways were related to metabolic pathways (carbon metabolism), bacterial metabolites (propanoate), humoral immune components such as phagosome, NOD-like receptor, interleukin-17), and hormones (e.g. insulin, cortisol and estrogen) as well (Fig. 5, Supplementary Table S5).

In the skin, the most enriched KEEG pathways were metabolic pathways, Wnt signaling pathway, adherens junctions, and cellular and humoral immune responses from both innate and acquired immunity, in response to LPS (Fig. 5, Supplementary Table S5). While, in response to Poly I:C, the enriched pathways related to ribosome and metabolic pathways were mostly present (Fig. 5, Supplementary Table S5).

The other tissues (spleen and liver) had significantly lower enriched KEGG pathways (Supplementary Table S5). In the spleen, metabolic pathways with lipid metabolism in evidence were the most enriched pathways in response to LPS. For Poly I:C, spliceosome pathway was highly enriched (Supplementary Table S5).

In the liver, the most enriched KEGG pathways were ribosome, biosynthesis of amino acids and metabolic pathways emphasizing carbon metabolism under LPS stimulation. In the same tissue, Poly I:C induced modifications in metabolic pathways (Supplementary Table S5).

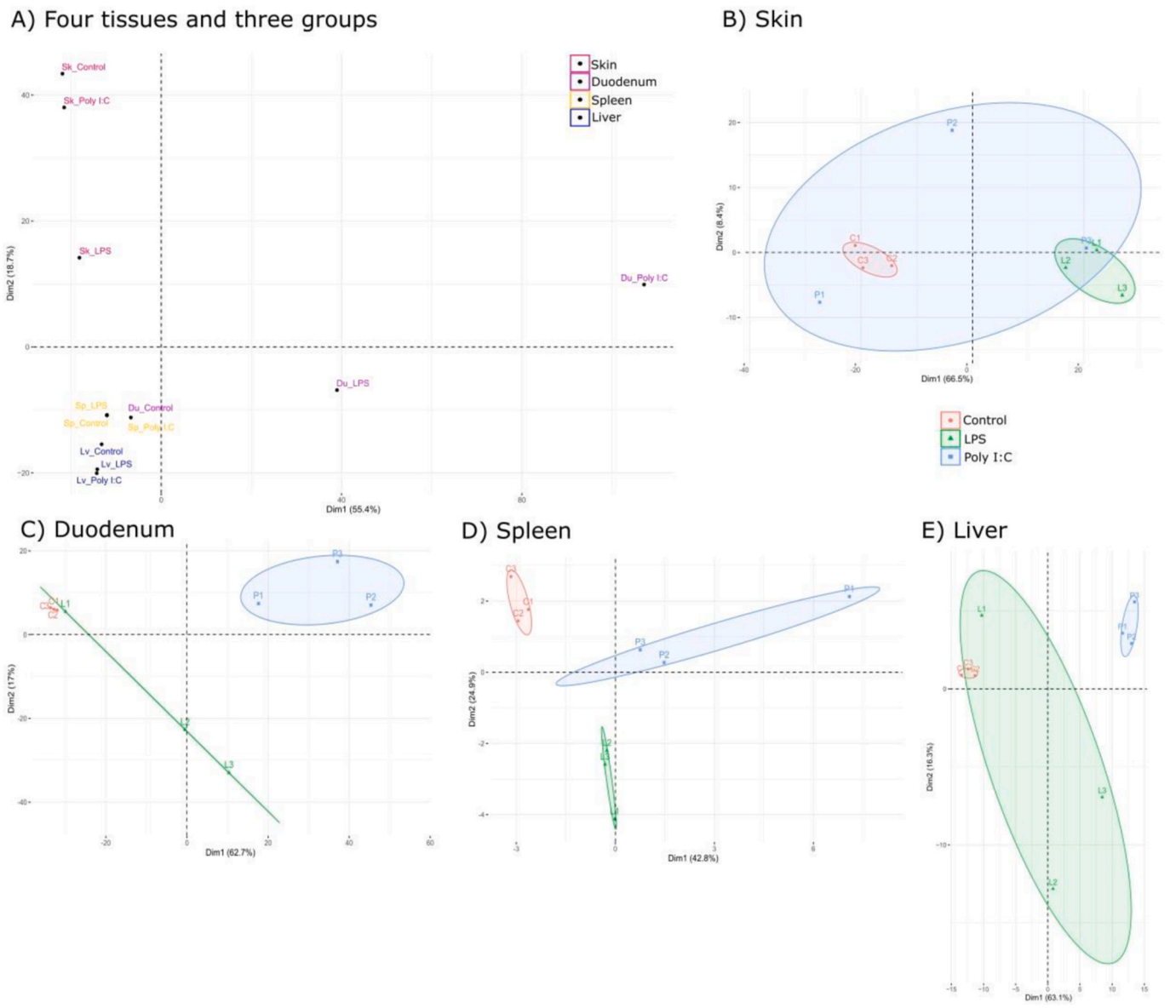

Fig. 2. Principal components analysis (PCA) of the DEGs identified in the A) duodenum, B) skin, C) spleen and D) liver transcriptomes of control, LPS and Poly I:C treated fish. PCA of DEGs (log2 expression) between the control (C1–C3), LPS (L1-L3) and Poly I:C (P1–P3)-challenged fish and in the four tissue transcriptomes (FDR <0.05). The control replicates are indicated with orange circles; the LPS replicates are indicated by green triangles, and the Poly I:C by blue squares.

3.5. Immune genes

As indicated by the GO and KEGG analysis, LPS and Poly I:C caused an enrichment in immune genes and immune pathways. In the Control compared to LPS-treated fish, iron metabolism pathways were highly modified in the skin but not in the duodenum, and several toxin gene transcripts, such as *stonustoxin* (*stxa*, *stxb*), *neoverrucotoxin* (*nvtx*) and *sagatoxin* (*srs1al*) were highly expressed in the skin but largely absent in the duodenum (Supplementary Table S3).

In contrast, pathogen recognition receptors (PRRs), namely, scavenger receptors (SR-As, *scarb1* and *ssc4d*), C-type lectin receptors (CLRs, *mrc1*, *mrc2* and *clec11a*) and NOD-like receptors (NLRs, *nlrc3*, *nlrp1* and *nlrp3*), were highly upregulated in the duodenum of Control compared to Poly I:C treated fish but absent from the skin, spleen, and liver (Supplementary Table S3). Similarly, several genes of the iron metabolism pathway were highly upregulated in the duodenum of the control group, but only a few were upregulated in the liver and none in the skin or spleen (Supplementary Table S3). Similarly, numerous genes of innate humoral immunity were highly upregulated in the duodenum but not at all in the spleen, and a small number were modified in the skin and liver (Supplementary Table S3).

3.6. Gene expression by RT-qPCR

Expression of *cldn7*, *cldn4*, *slc12a1*, *slc12a2*, *pparγ*, *tnf-α* and *prss1* was determined in the duodenum and skin of *N. rossii*. No expression of *pparγ* and *tnf-α* was detected by RT-qPCR in both tissues. A positive correlation was observed in duodenum (Pearson correlation coefficient = 0.515, p = 0.0003) and skin (Pearson correlation coefficient = 0.440, p = 0.0025) for the change in gene expression detected by RNA-seq (FPKM, Log 2 fold-change) and RT-qPCR (Log 2 fold-change).

4. Discussion

Exposure of *N. rossii* to bacterial (LPS) or viral (Poly I:C) challenges by immersion provoked a differential gene expression response in the duodenum, skin, liver, and spleen, with the barrier tissues skin and duodenum the most modified and the spleen the least responsive.

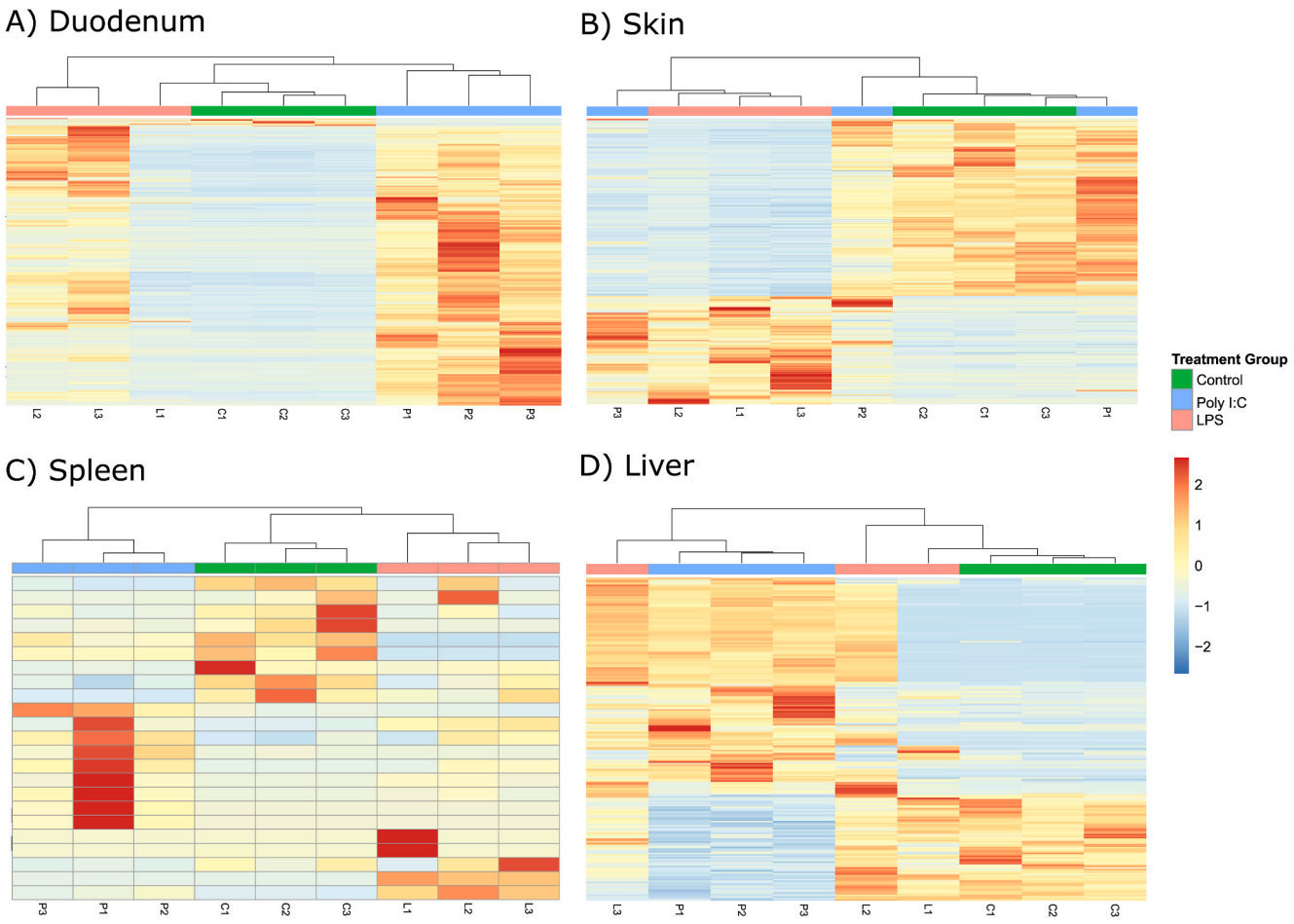

Fig. 3. Heatmap generated from DEGs identified in the A) duodenum, B) skin, C) spleen and D) liver transcriptomes of control, LPS and Poly I:C treated fish. A heatmap of clustered DEGs (log2 expression) in control (C1–C3), LPS (L1-L3) and Poly I:C (P1–P3)- challenged fish in the duodenum, skin (FDR<0.001), spleen and liver (FDR<0.05) tissue transcriptomes. Note the clustering of the expression pattern by treatment. The red colour gradient indicates increasing abundance (up-regulation), the blue colour gradient indicates decreasing abundance (down-regulation), and yellow indicates no significant up- or down-regulation.

Furthermore, there was a significant difference in tissue responsiveness depending on the pathogen used for exposure, with the duodenum more responsive to the viral proxy and the skin more responsive to the bacterial proxy. The most affected processes represented in the four tissue transcriptomes under both challenges were largely metabolic (i.e., protein, glucose, lipid, and energy metabolism) and immune. There was a conserved response by the more responsive tissues to both immune challenges, which included transcription factors, ribosomes and metabolic energy production pathways linked to carbohydrates, lipids, glucose, and ATP. Immune system defences are costly, especially for wild vertebrates and potentially compete with other physiological processes for limited nutrients [23]. For instance, during an infection, stimulated macrophage turn over ATP in the cell ten times per min (hypermetabolic state) and increase the glucose and glutamine utilisation [24], and when TNF-α or IL-1 is administered can undergo a 30% increase in their respiration rate [25]. The main transcriptional modifications associated with the immune response were PRRs, CLRs, NLRs and other humoral and cellular immunity-related genes. Transcriptional modifications related to iron and selenium metabolism were presumably linked to the involvement of these metals in innate immunity against microbial pathogens such as bacteria and viruses.

The lack of a response in plasma biochemical parameters and the sizeable transcriptional response to bacterial and viral stimuli underline the effectiveness of the physical barrier functions of the skin and duodenum. The same outcome for the biochemical parameters analysed was also observed in a previous study in *N. coriiceps* seven days after intraperitoneal LPS injection. The route of administration of LPS (immersion or i.p.) and time of sampling after exposure (8 h or 7 days) in the two studies do not seem to influence plasma parameters since similar results were observed in the two experiments. However, in contrast to our current findings, the study in which LPS was administered i.p. failed to encounter a robust transcriptional response in the duodenum, skin and head kidney [19]. Although no modification in plasma antiprotease (trypsin inhibition) was detected in the blood plasma, trypsin genes (*prss1*, *prss2*) were highly upregulated in the control duodenum, liver and skin. We speculate that the increased amount of trypsin released in the blood may be due to interference with antiprotease enzyme activity, which indicates low capacity for trypsin inhibition activity. Few studies have addressed changes in biochemical parameters in relation to Poly I:C administration. In chinook salmon Poly I:C i.p. injection caused an increase in the haematocrit [12], contrasting with our study where a decrease in haematocrit, and anti-trypsin inhibition was observed.

An unexpected outcome of our study was the differential tissue response to the two stimuli. Immersion in LPS elicited a solid transcriptional response in the skin and, while still significant, a much more subdued response in the duodenum and liver. The same was true of Poly I:C, but the duodenum and skin reversed responsiveness. The higher transcriptional responses could reflect the more common routes of infection of each type of pathogen, which led to higher sensitivity observed in the corresponding tissues [27]. This probably explains why the skin and duodenum, which were directly exposed to the bacterial and viral proxies, had a much stronger transcriptional response than

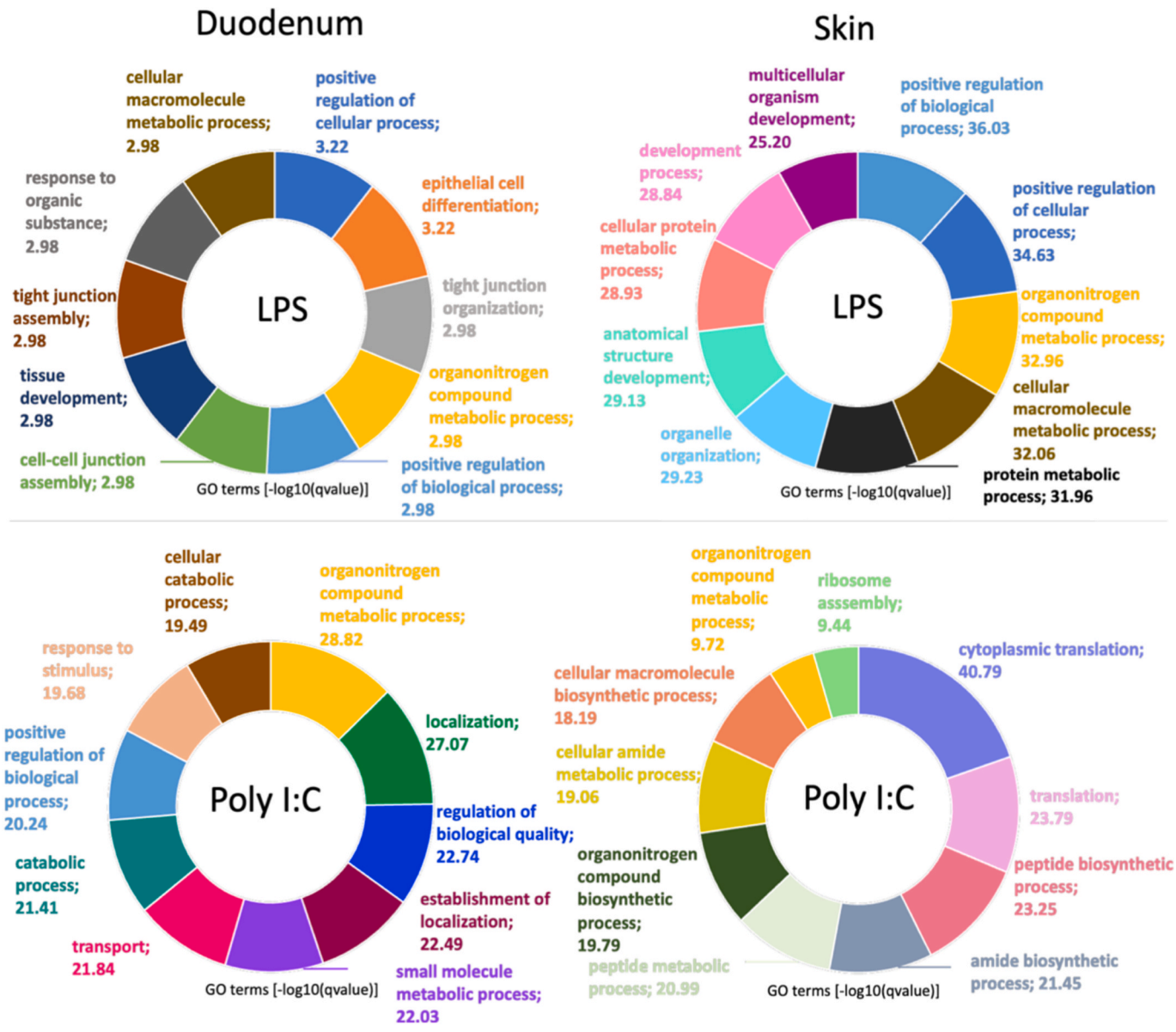

Fig. 4. The ten most GO-enriched terms related to biological processes in the duodenum and skin between Control versus LPS (LPS) and Control versus Poly I:C (Poly I:C) in *N. rossii*. The values are represented by adjusted p-value (qvalue) and negative log10 of adjusted p-value (enrichment).

tissues that were not directly exposed and had secondary immune functions, such as the spleen and liver.

LPS and Poly I:C caused common changes across tissues, mostly BP linked to metabolic pathways, although tissue-specific responses were also identified. The metabolic response included considerable changes in the expression of gene transcripts related to iron and selenium metabolism pathways in the four tissues studied. Such transcripts have a role in anti-bacterial defences [8,28] and have been previously identified in *N. coriiceps* and *N. rossii* [29]. Since iron is essential for bacterial replication, a reduction in its availability in the circulatory system of the host is considered a defence mechanism to provoke bacterial starvation [8]. There are few studies relating iron to viral infections in fish, unlike selenium, which has been proposed to have antiviral properties [30]. In teleost fish, selenium has primarily been studied from a dietary and nutritional perspective in relation to growth, as an antioxidant and immune stimulant [31]. The higher number of DEGs related to selenium in the present study (*methanethiol oxidase*, *selenoprotein J*, *selenoprotein T1a*, *serotransferrin*) and iron (*iron-sulfur protein 4*, *iron-sulfur protein 2*, *ferritin heavy subunit*, *ferrochelatase*, *ferroxidase*, *transferrin receptor*, *iron-sulfur cluster assembly enzyme*, *cytosolic iron-sulfur assembly component 2B*) in response to the Poly I:C and LPS challenges suggests they may have an essential role in immune defence against both bacterial and viral proxies.

The duodenum was the most responsive tissue to Poly I:C followed by the skin and liver, with a high similarity of response between duodenum and liver. Overall, the transcriptional response of all tissues partially recapitulated the response to a virus reported in mammals [32]. The core biological processes modified in the skin and duodenum resembles what has been shown in other fish and were associated with inflammation and enhanced leukocyte transendothelial migration into tissues [33]. Some of the same pathways enriched in the skin and duodenum were also observed in the intestine of zebrafish (*Danio rerio*) challenged with LPS [34]. The Poly I:C treatment provoked a typical core response in the four tissue transcriptomes, with both humoral and cellular immune components, including cell-cell adhesion, signalling and metabolism. Similar findings have been previously reported in *N. coriiceps* [19]. A requirement for partitioning of resources, when confronted by an immune challenge, may explain the abundance of DEGs related to

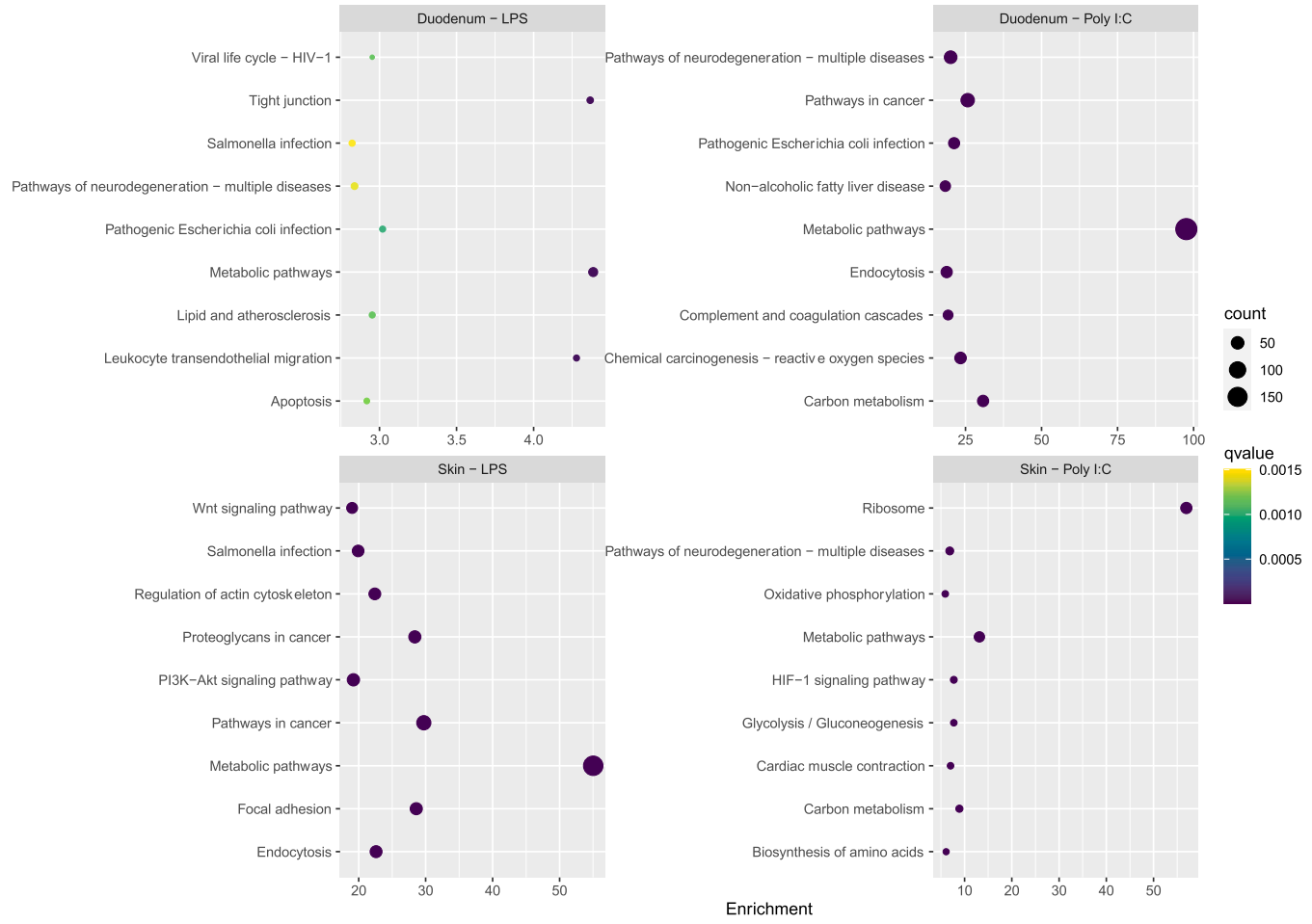

Fig. 5. The ten highly enriched KEGG pathways in duodenum and skin between Control versus LPS (LPS) and Control versus Poly I:C (Poly I:C) in *N. rossii*. The values are represented by adjusted p-value (qvalue) and negative log10 of adjusted p-value (enrichment). The pathways linked to human diseases were not considered for the top ten.

metabolic pathways in our study. Interestingly, metabolism was also modified in mammals exposed to viruses and suggested to be a consequence of the biosynthesis of new viral copies [35]. This was the case in human cells infected by herpes simplex virus (HSV), in which the tricarboxylic acid cycle (TCA) was upregulated, justified by the need for energy to produce the bases necessary for viral genome construction [36]. Our results with a non-replicating viral proxy, Poly I:C, suggest that other factors may also contribute to the recruitment of metabolism-related transcripts. Interestingly, mainly Poly I:C compared to control also caused suppression of apoptotic processes in the liver and duodenum, resulting in immune defects which is a typical response to viral particles in vertebrates including teleost fish, possibly regulated by microRNA [37].

The liver revealed DEGs mostly related to ribosomes (e.g., *rpl14*, *rpl4*), mineral reabsorption, particularly sodium (Na^{+}) and calcium (Ca^{2+}) (e.g., *atp1b33*, *fm*) and cGMP-PKG signalling (e.g., *slc25a5*). The ribosome pathway was also enriched in *N. coriiceps* exposed to heat-killed bacteria (HKEB O11:B4), antigen processing and presentation and MHC processes [9].

The complex tissue-specific transcriptomic response makes identifying genes directly linked to the immune response challenging. For example, the highly modified transcriptional response in signalling pathways related to cell adherence in the skin and duodenum (e.g., *ep300*, *mapk3*, *baiap2*) is in keeping with the importance of these tissues in osmoregulation and as a core multi-functional immune barriers [33]. Several studies across vertebrates have shown the gut, a mucosal lymphoid tissue, has modified permeability when exposed to LPS or directly to pathogens, e.g., channel catfish (*Ictalurus punctatus*) [38] and after LPS exposure in rainbow trout (*Oncorhynchus mykiss*) [39].

The spleen had a mild response to both challenges, although a few genes linked to fatty acid metabolism and signalling (e.g., PPAR and long-chain-fatty-acid-CoA ligase) were modified. Such changes have previously been reported to have an immunomodulatory function primarily in the sturgeon (*Acipenser dabryanus*) [40]. In contrast, bacterial infections in olive flounder (*Paralichthys olivaceus*) and rainbow trout (*Oncorhynchus mykiss*) stimulated the coagulation and complement cascade pathways and several immune gene transcripts - *tumour necrosis factor-alpha* (*TNF-α*), *interleukin 1-beta* (*IL-1β*) and *interferon* (*IFN-β*), possibly a response after the primary innate immune barriers were breached [41,42]. The differing results between studies may be related to the challenge (LPS, Poly I:C, live bacteria), route of administration (immersion vs i.p.) and the time from exposure and sample collection (from 8 h to 48 h).

4.1. The immune-specific response

Immune-related DEGs were almost exclusively detected in the skin (Fig. 6A) and the duodenum (Fig. 6B). The strongest response was in the duodenum stimulated by poly I:C and in the skin by LPS. Although very much the same class of immune genes were modified by the two stimuli, the main difference was the exclusive upregulation of immunoglobulins and IFN (a few of the latter also downregulated) by control compared to

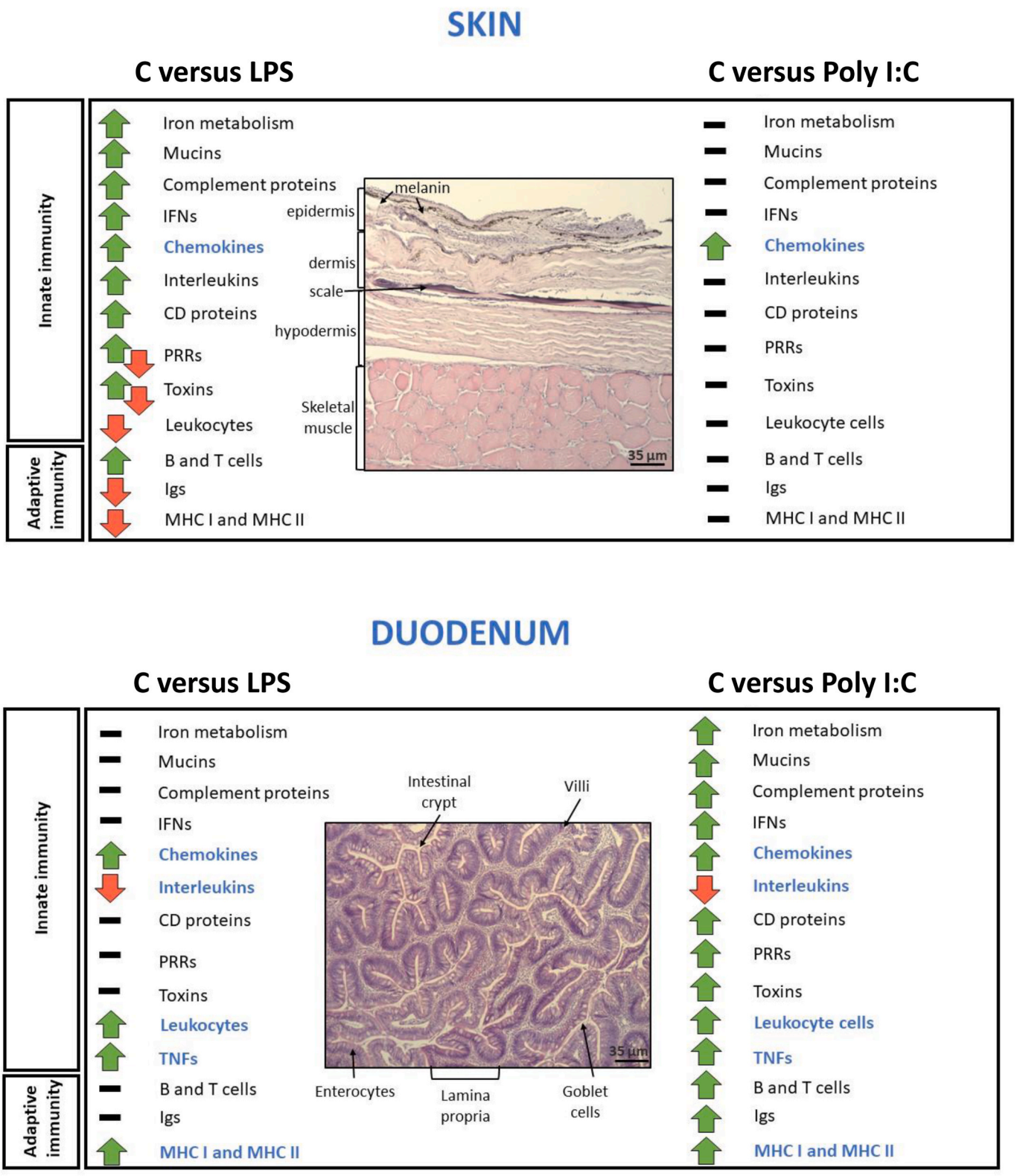

Fig. 6. The immune components modified in A) skin and B) duodenum transcriptomes of *N. rossii* after LPS and poly I:C challenges. The statistical analysis threshold was based on FDR <0.05; up-regulation by the green arrow, down-regulation by the red arrow and the absence of DE gene expression by a black line represent up-regulation gene expression.

Poly I:C (Supplementary Table S3). The common response included MCH I and II systems, PRRs linked to NOD-like and scavenger receptors (*ssc4d*, *nlrc3*, *clec11a*, *mrc2*), cytokines, chemokines, leukocytes, toxins, other proteins (beta(β)-2-microglobulin, IFNs, *TGF-β*, *prss1*, *lyzg*) and cytokine-related factors and receptors and lymphocytes B and T. These immune markers were also identified in other teleost fish, including *N. coriiceps* [12,19] (Fig. 6A). An essential role for other PRRs is also suggested. For example, the membrane-bound C-type like receptors

(*galactase-specific lectin attention, c-type lectin domain family 11 member A*), RIG-I-like receptors (*adiponectin receptor protein 2*) and, *NACHT, LRR and PYD domains-containing protein 3* (*nlrp3*) and 14 (*nlrp14*). Similar observations have been reported in Atlantic salmon (*Salmo salar*, C-type lectin genes), Barramundi (*Lates calcarifer*, C-type lectin genes), Japanese flounder (*Paralichthys olivaceus*, *nlrc3* genes) and Miiuy croaker (*Miichthys miiuy*, *nod1* genes) [43–45]. In the skin transcriptome, the PRRs CLRs (*clec13a*, *clec4a*) and NLR (*nlrp1*, *nlrc3*) genes seem to be important for Poly I:C recognition and these have also been identified in other teleosts stimulated with viral agonist [46]. A surprising absence from the response was observed in toll-like receptors (Tlrs) previously characterised in *N. rossii* and other Antarctic fish species [47].

Despite the relatively modest LPS transcriptional response in the duodenum, cellular innate immunity genes, including gene transcripts for proteins involved in leukocyte migration (e.g., *cl4*, *cxcr4*, *itg1b*, *myl2*, *ezra*) and phagocytosis (e.g., *hb24*, *ctss2.2*, *hla-dpa1*, *eea1*) were highly modified. Recent studies in brown marbled grouper (*Epinephelus fuscoguttatus*), goldfish (*Carassius auratus*), yellowhead catfish (*Pelteobagrus fulvidraco*), carp (*Schizothorax prenanti*), Nile tilapia (*Oreochromis niloticus*) and Atlantic salmon revealed the modification of several humoral and cellular immune components depending of the route of LPS administration, either i.p. [48] or immersion [5]. Pathways linked to leukocyte transendothelial migration in *Danio rerio* [49] and TGF-β signalling in *Oncorhynchus mykiss* [50] have also been linked to LPS challenge.

The spleen had a few immune-related DEGs, among them *interleukin (IL)-17*, a cytokine, which has been suggested to possess antiviral activities against reovirus infection [51]. More immune genes were observed in the liver transcriptome of *N. rossii* that included TNFs (*ccl20*, *kat6b*, *creb3l3*), Th17 (*stambpl1*, *stat3*) and the PRR (*Tlr3*), which was previously shown to be changed by Poly I:C in teleost fish including the phylogenetically closely related *N. coriiceps* species [52].

The differential response to LPS and Poly I:C challenges according to tissue is indicative of immune specialisation towards pathogens of the duodenum and the skin. Of note was the activation of PRRs compared to the control, which was dominated by NOD-like receptors, scavenger receptors and C-type lectin receptors, suggesting that in *N. rossii,* these may be more important for pathogen recognition but also inhibition of some that may avoid the host from sepsis since overactivation could be dangerous. Those PRRs presumably are more significant than toll-like receptors, which, although present in the skin and duodenum were not significantly modified by LPS and Poly I:C exposure (except *Tlr3*) by immersion in *N. rossii*. These findings suggest a transcriptional response specialisation in response to pathogens in these two immune barriers.

Validation of the results of the transcriptome study was obtained by comparing them with the results of RT-qPCR of selected genes. Of the seven genes analysed by RT-qPCR four and two, respectively had a significantly modified expression in the duodenum and skin of the immune challenged fish. The different results obtained for RT-qPCR and RNA sequencing was unsurprising since the DE of the genes chosen varied by tissue and RNA seq is more sensitive and accurate [26]. Nonetheless, a positive correlation was obtained between the results of RT-qPCR and sequencing for DE genes that were up- or down-regulated. The absence of a robust transcriptional response at the level of the liver and spleen to both LPS and Poly I:C challenges by immersion may be indicative of the efficacy of the response at the level of the two innate immune barriers, skin, and the duodenum. If the response of the skin and duodenum to the immune challenge is effective this may render the response of other tissues unnecessary. However, it should be noted that the immersion challenge was only 8 h, and it is possible that the stimulation by LPS and Poly I:C may have been insufficient to provoke an immune response in the liver and spleen. Further experimental work will be required using different doses and challenges to better characterise the immune response in *N. rossii*.

5. Conclusion

The results of the present study showed a clear and specific response of the skin and duodenum of *N. rossii* to a challenge with, LPS and Poly I:C, not previously reported in other teleost fishes. This may suggest that these findings are linked to the adaptation of the *N. rossii* immune system to LPS and Poly I:C exposure in the cold-extreme environment of Antarctica. The novel observations in our study are of general interest for understanding teleost immune system evolution and physiology. However, further experiments are needed to confirm the efficacy of the barriers to a real-life infection.

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Ethics statement

Fish collection and experimental protocols were approved by the Portuguese Environmental Agency, under the regulations set by the Treaty of Madrid for scientific investigation in Antarctica. The experiments performed complied with European Union and Portuguese regulations for animal experimentation.

CRediT authorship contribution statement

Cármen S.V. Sousa: Conceptualization, Investigation, Validation, Writing – original draft, Funding acquisition, Formal analysis, Laboratory experiments. **Maoxiao Peng:** Formal analysis. **Pedro M. Guerreiro:** Conceptualization, Investigation, Validation, Writing – original draft, Funding acquisition, Writing – review & editing, Funding acquisition. **João C.R. Cardoso:** Formal analysis. **Liangbiao Chen:** Funding acquisition, Project administration, Validation, Writing – review & editing. **Adelino V.M. Canário:** Conceptualization, Investigation, Validation, Writing – original draft, Funding acquisition. **Deborah M. Power:** Conceptualization, Investigation, Validation, Writing – original draft, Funding acquisition.

Conflicts of interest

The authors declare no competing interests.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2024.109516.

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