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Transcriptomic Down-Regulation of Immune System Components in Barrier and Hematopoietic Tissues after Lipopolysaccharide Injection in Antarctic *Notothenia coriiceps*

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Abstract: The environmental conditions and isolation in the Antarctic have driven the evolution of a unique biodiversity at a macro to microorganism scale. Here, we investigated the possible adaptation of the teleost *Notothenia coriiceps* immune system to the cold environment and unique microbial community of the Southern Ocean. The fish immune system was stimulated through an intraperitoneal injection of lipopolysaccharide (LPS 0111:B4 from *E. coli*) and the tissue transcriptomic response and plasma biochemistry were analyzed 7 days later and compared to a sham injected control. Gene transcription in the head-kidney, intestine and skin was significantly modified by LPS, although tissues showed different responsiveness, with the duodenum most modified and the skin the least modified. The most modified processes in head-kidney, duodenum and skin were related to cell metabolism (up-regulated) and the immune system (comprising 30% of differentially expressed genes). The immune processes identified were mostly down-regulated, particularly interleukins and pattern recognition receptors (PRRs), nucleotide-binding oligomerization domain-like receptors and mannose receptors, unlike the toll-like receptors response commonly described in other teleost fish. The modified transcriptional response was not mirrored by a modified systemic response, as the circulating levels of enzymes of innate immunity, lysozyme and antiproteases, were not significantly different from the untreated and sham control fish. In conclusion, while the *N. coriiceps* immune system shares many features with other teleosts there are also some specificities. Further studies should better characterize the PRRs and their role in Antarctic teleosts, as well as the importance of the LPS source and its consequences for immune activation in teleosts.

Keywords: Antarctic fish; head-kidney; innate immunity; duodenum; lipopolysaccharide; skin; transcriptomics

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1. Introduction

The dominant teleost fish fauna in the Southern Ocean belong to the perciform suborder Notothenioidei, which evolved from a benthic ancestor through adaptive radiation modulated by a largely stable cold thermal environment [1–3]. The nototheniids have developed unique physiological adaptations, including antifreeze glycoproteins [4–7] and the loss of hemoglobin and myoglobin in the *Channichthyidae* [8]. Additional physiological adaptations include buoyancy modifications through reduced bone density and higher

whole body lipid levels [9], cold-efficient cellular microtubule assembly [10,11], high mitochondrial densities [12] and the absence of a heat shock response [11,13,14]. The impact of the rapid speciation and isolation of the Antarctic nototheniids on the immune system, however, has been surprisingly little explored. Nonetheless, the frigid Antarctic waters, inhabited by a unique microbial community [8,15], have presumably influenced their immune repertoire and response to pathogens. Furthermore, increased human activity and climate change have been associated with the intrusion of alien organisms into Antarctic waters [16–18], raising the possibility of their unwitting exposure to new pathogens not far into the future.

Teleost fish, in common with other vertebrates, possess both innate and acquired components of the immune system [19–21]. However, innate immunity in teleost fish is proposed to have a more important role than the acquired immune response presumably because of the constant contact of fish epithelia or mucosal barriers (e.g., gills, skin and intestine) with a micro-organism rich aquatic environment [20,22–24].

Studies to assess how water temperature affects the immune response in temperate teleost fish indicate that lower water temperatures generally suppress the immune response [10,20,25–31]. In contrast, in vitro studies of fish immune cells maintained under reduced temperatures revealed enhanced cytotoxic activity [32] and an enhanced macrophage response [33] and respiratory burst [34]. Low temperatures did not inhibit phagocytosis by macrophages [35] or the inflammatory response in nototheniids [36–38]. In the bullhead (*Notothenia coriiceps*), damage repair of skin occurred at a slower pace than in teleosts from warmer waters with no sign of contamination by pathogens, which was attributed to the protective action of the mucous layer or the low pathogenicity of microorganism in Antarctic seawater [39]. Studies taking a candidate gene approach revealed that the exposure of *N. coriiceps* and *N. rossii* to bacterial infection agonist lipopolysaccharide (LPS) modified toll-like receptor gene expression in liver, kidney and spleen [40,41] and modulated iron-related immune genes, including *hepcidin 4* (*hp4*) [42]. A novel hepcidin (hepcidin type II) has been identified in the Antarctic Notothenioids *Dissostichus mawsoni* and *Notothenia angustata* and was shown to be under positive selection [43]. Liver of Antarctic bullhead exposed to heat killed bacteria or polyinosinic:polycytidylic acid (poly I:C) was enriched in antigen processing and presentation and bacterial ligand exposure transcripts, which use antigen presentation against bacterial infection, but it may also use other defense mechanisms, such as TNF-mediated apoptosis, against viral infection [44]. Several toll-like receptor genes within the Notothenioid clade were shown to be under positive selection in pathogen recognition domains suggesting adaptation to the specific Antarctic microbiota [41]. A proteome study of the head kidney between white blood fish, *Chionodraco hamatus*, and two red blood fish, *Trematomus bernacchii* and *N. coriiceps*, revealed differences in erythropoiesis, heme biogenesis, leucocyte, and platelet development, with upregulation of lymphoid and megakaryocytic lineage marker proteins in ice fish [45].

The present study was designed to investigate the possible adaptation of the *N. coriiceps* immune system to the cold environment and unique microbial community of the Southern Ocean. Considering that adaptations might include gene retention and loss and/or response to potential pathogens, the bacterial agonist liposaccharide (LPS), a widely used teleost fish immunogen, was used as an immune challenge. LPS is ubiquitous (including in Antarctica) in the cell-wall of gram-negative bacteria of the *Enterobacteriaceae* family, although the host response may vary with chemical differences that exist in LPS from different Gram-negative species [46–48]. The transcriptional response to LPS was analyzed in the head-kidney, the main hematopoietic organ in fish, and in two mucosal-associated lymphoid tissues, the skin and intestine. The activity of serum enzymes associated with the innate immune response was also analyzed.

2. Materials and Methods

Fish sampling and experimentation were based on a permit issued by the Portuguese Environmental Agency under the regulations of the Madrid Protocol. *N. coriiceps* specimens

(30 ± 2.4 cm total length and 384 ± 93 g weight) were captured using hook-and-line between 5 m and 30 m deep, near the Great Wall Station, King George Island in the Antarctic Peninsula (GPS coordinates: 62°12′57″ S, 58°57′42″ W) in January and February (the Antarctic summer) of 2017. Fish were acclimated for 5 days and maintained indoors in 200 L tanks in a flow-through circuit with seawater pumped from the surrounding bay. They were fed twice daily (morning and evening) with a mixture of limpets, salps, amphipods and fish muscle. Water temperature (2.0 ± 0.8 °C), salinity (28 ± 0.2 psu) and oxygen (11 ± 2 mg/L) were monitored three times a day (7.00 h, 14.00 h and 21.00 h) and were stable throughout the experiments.

LPS from the outer layer of 0111:B4 Gram-negative bacteria (L2630, Sigma-Aldrich, Madrid, Spain), which interacts with toll-like receptors (TLRs) and stimulates cytokine and acute phase protein release in vertebrates (including fish) [49,50] was used in the challenge experiments. Three days before the start of the experiments, fish were randomly allocated between three experimental tanks to create groups corresponding to: (i) the non-injected control (n = 7); (ii) the saline injected sham control (n = 7); and (iii) the LPS-injected treatment (n = 7). The LPS dosage was estimated on the basis of previous studies on teleost fish [29,51–54]. For injections, fish were lightly anaesthetized in 0.2 mL/L 2-phenoxyethanol (Sigma-Aldrich, Madrid, Spain) and weighed. The fish received an intraperitoneal (i.p.) injection of saline (1.1% NaCl, 2% fish wet weight) in the saline injected control (sham), or an i.p. injection of 1.5 mg/mL LPS in saline (LPS treatment). A second saline and LPS injection was administered to the sham control and to the LPS-treated group, respectively, 48 h after the first injection. Five days after the second injection was administered (day 7 of the experiment), fish were deeply anaesthetized (2-phenoxyethanol, 2.0 mL/L), and the blood was collected by caudal puncture using a heparinized 21-G needle fitted into a 1-mL syringe before sacrificing by cervical section. The blood plasma was separated by centrifugation and frozen at −80 °C until analysis. The head-kidney, skin, and anterior intestine (duodenum region) were dissected out and stored in RNA later (Sigma-Aldrich). Two fish died during the experiment only in the LPS treatment group.

Total plasma protein was quantified using a Quick Start™ Bradford Protein assay kit (Bio-Rad, Portugal) optimized for a 96-well plate [55]. Absorbance was measured at 590 nm at 25 °C in a spectrophotometer (Agilent Technologies, Santa Clara, CA, USA).

The lysozyme and antiprotease activities in blood plasma were measured based on previously described methods [56,57] with modifications [41]. Lyophilized *Micrococcus luteus* cells, hen egg white lysozyme, trypsin from porcine pancreas, azocasein and buffers were purchased from Sigma-Aldrich. Spectrometric measurements were made at 450 nm in a spectrophotometer (Agilent Technologies).

Plasma cortisol was measured by radioimmunoassay (RIA) using cortisol antiserum 20-CR50 (Fitzgerald Industries International, Concord, USA) as previously described [58]. The tritiated cortisol was purchased from GE Healthcare Europe GmbH (Carnaxide, Portugal). Cross-reactivities of the cortisol RIA were 54% for 11-desoxycortisol, 10% for cortisone, 16% for 17,21-dihydroxy-5β-pregnan-3,11,20-trione, 5% for 11β,17,21-trihydroxy-5β-pregnan-3,20-dione, 0.05% for 11β-hydroxytestosterone and less than 0.001% for testosterone.

Differences in biochemical parameters between the control, sham and LPS-treated groups were assessed using one-way analysis of variance (ANOVA) after verifying that normality and homoscedasticity assumptions were met using SigmaPlot v12.5 (Systat Software Inc, Palo Alto, CA, USA). Graphs were generated using GraphPrism v6.01 (GraphPad Software, San Diego, CA, USA). The threshold for significance was set at $p < 0.05$ (Figure S1).

Total RNA was extracted from approximately 25 mg of head-kidney, skin and anterior intestine (duodenum region) of five specimens of *N. coriiceps* per experimental group using an E.Z.N.A. Total RNA Kit I (Omega Bio-Tek, Norcross, GA, USA). Total RNA was treated with RNase-free DNase I (Omega Bio-Tek) to remove contaminating genomic DNA before poly(A)+ messenger RNA (mRNA) was purified for sequencing using a DynaBead mRNA Purification Kit (Life Technologies, Carlsbad, CA, USA). Samples with an RNA integrity

number of at least eight were used to construct 45 paired-end complementary DNA (cDNA) libraries with an average insert size of 250 base pairs (bp) using a VAHTS stranded mRNA-*seq* Library Prep Kit from Illumina following the manufacturer's protocol (Vazyme Biotech Co., Ltd., Nanjing, China). Sequencing library quality was monitored using an Agilent Bioanalyzer DNA 1000 Kit #5067-1504 (Agilent Technologies, Santa Clara, CA, USA). All libraries were sequenced using an Illumina Hiseq X Ten instrument (Illumina, Inc., San Diego, CA, USA) between 30 and149 read length.

A total of 298 million paired-end (PE) raw reads were generated by RNA-seq. Quality control and editing of raw reads to trim adapter sequences and low-quality bases was performed using a Trimgalore wrapper script v0.4.5 and output FastQC quality reports were obtained [59,60]. Mitochondrial and ribosomal reads were removed by aligning reads against the transcripts annotated as ribosomal gene products and the *N. coriiceps* mitochondrial genome (accession number NC_015653.1) using Bowtie2 v2.3.4 mapping software [61]. One sequencing library (L5) from the head-kidney of LPS challenged *N. coriiceps* did not produce enough reads to pass the FastQC and was excluded from the analysis (data not shown). Clean reads were quantified using the RSEM package v1.3.1 [62]. The raw sequence reads were deposited to the NCBI Sequence Reads Archive with BioProject accession ID PRJNA822876.

Two different analyses were made: one based on the mapping of clean reads against the *N. coriiceps* genome (NCBI reference genome NC01) [4], the other based on a reference transcriptome produced from the *de novo* assembly of all reads from the different treatment groups.

The Galaxy platform v22.01 [63] was used to map reads to the reference genome using Hisat2 v2.2.1 [64], Stringtie v2.1.7 [65] was used to count mapped reads and Deseq2 v1.34.0 [66] to determine differential gene expression.

Transcriptome assembly was performed in Trinity v2.5.1 with the "-normalize reads" parameter defined [67]. TransRate v 1.0.3 was used for quality filtering using default parameters [68]. EdgeR package v3.14.0 was used to determine differentially expressed genes [69]. The contigs were initially automatically annotated against *Danio rerio* and *Homo sapiens* genomes available in the Ensembl database using Trinotate v3.1.1 [70] and integrated into a SQLite database v3.34.0 to allow for fast efficient searching for terms with biological meaning. Annotations were based on the best deduced open reading frame (ORF) obtained with Transdecoder v1.03 [68]. Further manual annotation of non-annotated differentially expressed contigs interrogated Notothenioidei (*N. coriiceps* reference genome NC01, *Trematomus bernacchii* reference genome fTreBer1.1, *Pseudochaenichthys georgianus* reference genome fPseGeo1.1, *Gymnodraco acuticeps* reference genome fGymAcu1.1, *Dissostichus mawsoni* reference genome KU_Dm_1.0 and *Cottoperca gobio* reference genome fCotGob3.1), *Homo sapiens* (reference genome GRCh38.p13) and *Danio rerio* (reference genome GRCz11) genomes available at the NCBI.

Differentially expressed genes (DEGs) were identified using pairwise comparisons between the three treatment groups (non-injected control, sham and LPS) for head-kidney, duodenum and skin (FDR < 0.05), based on mapped transcripts per million (TPM). A heatmap of DEGs was generated with the "heatmap" function from RStudio v1.0.143. Gene Ontology (GO) enrichment was analysed with GOrilla [71] and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways with KOBAS (FDR < 0.05) [72]. GO terms were summarized using ReViGO [73] and graphical outputs were represented in Rstudio v1.0143.

The benchmarking universal single-copy orthologues (BUSCO) v5.3.2 [74] were used to assess the completeness of the NC01 genome and transcriptome assembly against the eukaryota, vertebrata and actinopterygii databases.

3. Results

3.1. Blood Biochemistry

Plasma protein, plasma total antiprotease activity, plasma lysozyme activity and plasma cortisol levels were not significantly modified between any of the treatment groups (Figure S1).

3.2. Transcriptome Assembly and Differentially Expressed Genes

The analysis using the reference genome yielded few annotated differentially expressed genes (duodenum 33, head kidney eight and skin one) and therefore we resorted to the *de novo* transcriptome assembly from which we could map more reads and annotate a higher number of differentially expressed genes (see below). Although tissue transcriptomes are unlikely to contain the full set of genes represented in the genome, the BUSCO analysis showed that over 80% of the eukaryote core genes were present in our transcriptomes (Table S1). The average mapping of reads to genome and reference transcriptome were 78% and 84% respectively (Table S2). The assembly of reads from the 45 sequencing libraries (1.1 Gbp) generated 51,506 contigs in the head-kidney, 58,916 in the duodenum and 66,329 in the skin with a N50 of 643 bp (Table S3).

Clustering revealed that individual samples clustered separately according to treatment, but the control and sham samples formed treatment specific sub-clusters separated from the LPS treated group (Figure 1a–c). The percentage annotation of DEGs varied between 47% and 100% (Table S4).

3.3. Control Versus Sham Differentially Expressed Genes

Pairwise comparison of the control versus sham, contigs representing the effect of handling and injection, resulted in 329 DEGs. Of these, 178 DEGs were from the head-kidney (129 up- and 49 down-regulated), 138 from the duodenum (77 up- and 61 down-regulated) and three from the skin (two up and one down-regulated). The skin shared one DEG with head-kidney and duodenum, while duodenum and head-kidney shared six DEGs and one DEG was shared between the three tissues (Figure 2a, Tables S3 and S4).

In head kidney, the five annotated most upregulated genes were macrophage mannose receptor 1 (*mrc1*), creatine kinase, muscle a (*ttpa*), GTPase IMAP family member 7 (*gimap7*), NLR family, the CARD domain containing 3 (*nlrc3*) and the kinesin family binding protein (*kifbp*), and the most downregulated were shootin 3 (*shtn3*), low affinity immunoglobulin gamma Fc region receptor II (*fcgr2b*), SLAM family member 7 (*slamf7*), nuclear factor 7, brain-like (*nf7bl*), and ciliogenesis associated TTC17 interacting protein (*catip*). In the duodenum the five annotated most upregulated genes were Acyl-CoA dehydrogenase family, member 11 (*acad11*), *nlrc3*, electron transfer flavoprotein dehydrogenase (*etfdh*), trichohyalin-like (*tchhl1*) and ladderlectin-like (name *n/a*), and the five most downregulated genes were *gimap7*, zinc finger protein 37 (*zfp37*), interferon-induced very large GTPase 1 (*gvinp1*), elastase 2-like (*ela2l*) and C2 calcium-dependent domain containing 2 (*c2cd2*). In skin only *gimap8* was upregulated and *gimap7* was downregulated. *nlrc3* and sodium-dependent multivitamin transporter *slc5a6*, were shared between the head-kidney and duodenum, *gimap8* was shared between head-kidney and skin and *gimap7* was shared by the three tissue transcriptomes (Figure 2a, Table S5).

Functional annotation identified significant enrichment of GO terms in the head kidney and the biological processes (BP) were related to endoplasmic reticulum, translation, and catabolic process (Table S6).

Enriched KEGG pathways of DEGs in the head-kidney were stress and immune response related, including ribosome, adrenergic signaling in cardiomyocytes, cardiac muscle contraction, phagosome, and calcium signaling pathway. In the duodenum, mainly digestive system pathways were represented, including protein digestion and absorption, pancreatic secretion and apoptosis. No KEGG pathway enrichment was obtained in skin (Table S7).

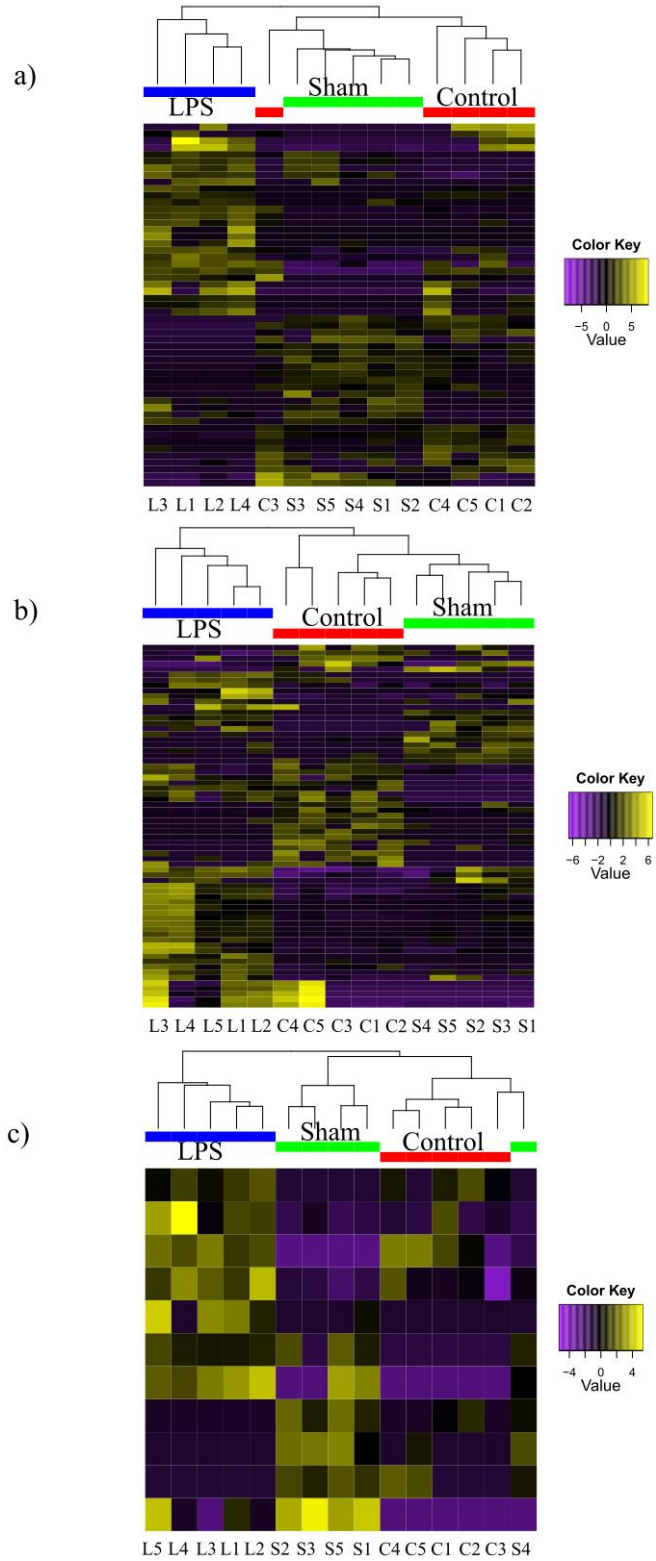

Figure 1. Heatmap generated from DEGs identified in the (**a**) head-kidney, (**b**) duodenum and (**c**) skin transcriptomes of control and LPS-treated fish. The heatmap of clustered DEGs (log2 expression) between control (C1–C5), sham (S1–S5) and LPS (L1–L4) challenged fish in the three tissue transcriptomes (FDR < 0.05). The yellow color gradient indicates high relative abundance (up-regulation), the purple color gradient indicates low relative abundance (down-regulation) and black indicates equal abundance.

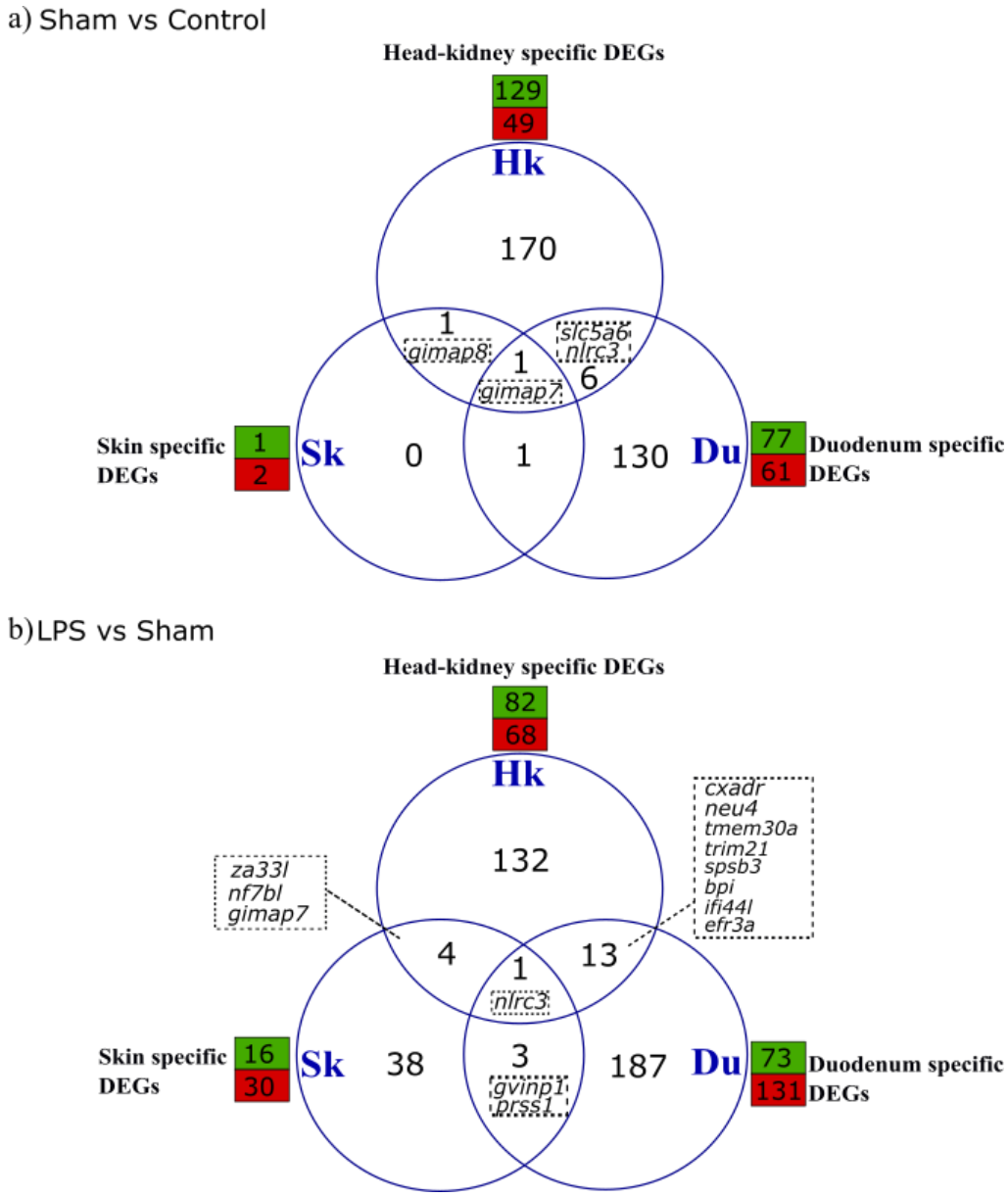

Figure 2. Venn diagram representing the number of unique and common DEGs in head-kidney (Hk), duodenum (Du) and skin (Sk) in comparisons of (**a**) Sham versus Control and (**b**) LPS versus Sham. The green and red boxes represent the total number of genes up- or down-regulated (FDR < 0.05). The dashed boxes contain the name of shared annotated gene transcripts.

3.4. LPS versus Sham Differentially Expressed Genes

Pairwise comparison of LPS versus sham yielded 401 DEGs, of which 150 were from the head-kidney (82 up- and 68 down-regulated), 204 from the duodenum (73 up- and 131 down-regulated) and 46 from the skin (16 up- and 30 down-regulated) (Figure 2b, Tables S4 and S8). While ca. 30% of DEGs were immune related genes, of these a large number were downregulated: 50% in duodenum, 44% in head kidney and 75% in skin.

In head kidney, the five topmost upregulated genes annotated in response to LPS were *shtn3*, zinc-binding protein A33-like (*za33l*), *gimap8*, disheveled-associated activator of morphogenesis 1 (*daam1*) and methyltransferase-like protein 12, mitochondrial (*cskmt*), and the most downregulated were *nf7bl*, *gimap2*, EFR3 homolog A (*efr3a*), *gimap7* and aldehyde dehydrogenase 5 family, member A1 (*aldh5a1*) (Table 1). In duodenum, the 5 most upregulated genes were trypsin-2 (*prss2*), butyrophilin-like protein 1 (*btnl1*), trypsin-3

(*prss3*), chymotrypsin-like elastase family, member 1 (*cela1*) and high choriolytic enzyme 1-like (*hce2l2*), and the most downregulated were adaptor related protein complex 5 subunit sigma 1 (*ap5s1*), bactericidal permeability-increasing protein(bpi), proton-coupled amino acid transporter 1 (*slc36a1*), protein mono-ADP-ribosyltransferase PARP14 (*parp14*), cytochrome P450, family 19, subfamily A, polypeptide 1b (*cyp19a1*). In skin the 5 most upregulated were serine and arginine rich splicing factor 2a (*srsf2a*), cationic trypsin (*prss1*), POU domain, class 6, transcription factor 1-like (*pou6d1*), *nf7bl*, zinc finger protein 524 (*znf524*) and the most downregulated were sushi domain containing 2 (*susd2*), gimap7, *nlrc3*, cytochrome P450 2K1-like (*cy2k1l*) and homeobox B9a (*hxb9a*). The shared DEGs between the head-kidney and skin after LPS challenge group included *za33l*, *nf7bl* and *gimap7* (Figure 2b, Table 1). The DEGs shared between the head-kidney and duodenum were cell cycle control protein 50A-like (*tmem30A*), E3 ubiquitin-protein ligase TRIM21-like (*trim21*), SPRY domain-containing SOCS box protein 3-like (*spsb3*), bactericidal permeability-increasing protein-like (*bpi*), interferon-induced protein 44-like (*if44l*), coxsackievirus and adenovirus receptor (*cxadr*), sialidase 4 (*neu4*) and *efr3a* (Figure 2b, Table 1). *Nlrc3* was modified by LPS in the three tissue transcriptomes and *gvinp1* and *prss1* were differentially expressed in duodenum and skin (Figure 2b, Table 1).

Table 1. Top 10 most upregulated and top 10 most downregulated differentially expressed genes between LPS and sham-injected fish in each tissue (head-kidney, duodenum and skin).

TRINITY ID	logFC	UniProtKB ID	Gene Definition	Gene Symbol
Head-kidney				
TRINITY_DN159360_c4_g1	9.78	F1RAW5	Shootin 3	*shtn3*
TRINITY_DN158576_c1_g3	8.91	N/A	Zinc-binding protein A33-like	za33l
TRINITY_DN163380_c1_g1	8.84	Q8ND71	GTPase IMAP family member 8	*gimap8*
TRINITY_DN159230_c9_g2	8.51	Q9Y4D1	Disheveled-associated activator of morphogenesis 1	*daam1*
TRINITY_DN142659_c1_g6	8.40	A8MUP2	Methyltransferase-like protein 12, mitochondrial	*cskmt*
TRINITY_DN159639_c0_g1	8.38	Q01130	Serine and arginine rich splicing factor 2a	*srsf2*
TRINITY_DN144517_c5_g2	8.25	Q9Y3Z3	SAM domain and HD domain 1	*samhd1*
TRINITY_DN137183_c4_g1	8.19	Q8WWR8	Sialidase 4	*neu4*
TRINITY_DN122607_c0_g1	7.94	Q9NV96	Cell cycle control protein 50A-like	*tmem30a*
TRINITY_DN155301_c3_g7	7.86	Q6ZSJ9	Protein shisa-6	*shisa6*
TRINITY_DN148291_c0_g2	−2.18	P62241	40S ribosomal protein S8	*rps*
TRINITY_DN143420_c2_g1	−2.43	O60462	Neuropilin 2	*nrp2*
TRINITY_DN155536_c2_g1	−2.94	Q53G44	Interferon-induced protein 44-like	*ifi44l*
TRINITY_DN175530_c0_g1	−3.21	Q969X5	Endoplasmic reticulum-golgi intermediate compartment 1	*ergic1*
TRINITY_DN162708_c0_g2	−3.34	O95398	Rap guanine nucleotide exchange factor (GEF) 3	*rapgef3*
TRINITY_DN142663_c1_g1	−3.86	N/A	Peptidoglycan-recognition protein LB-like	*pgrp-lbl*
TRINITY_DN149357_c4_g1	−3.89	N/A	Galactose-specific lectin nattectin-like	*lgals3l*
TRINITY_DN140144_c4_g1	−4.14	Q6IMW7	Parvalbumin 4	*pvalb4*
TRINITY_DN156139_c0_g1	-1.07×10	P17213	Bactericidal permeability-increasing protein	*bpi*
TRINITY_DN157841_c3_g1	-1.44×10	Q7RTR2	NLR family CARD domain-containing protein 3	*nlrc3*
Duodenum				
TRINITY_DN153528_c0_g2	9.89	P07478	Trypsin-2	*prss2*
TRINITY_DN160991_c2_g2	9.70	Q7TST0	Butyrophilin-like protein 1	*btnl1*
TRINITY_DN153528_c0_g3	9.66	P35030	Trypsin-3	*prss3*
TRINITY_DN123020_c0_g1	9.47	Q9UNI1	Chymotrypsin-like elastase family, member 1	*cela1*
TRINITY_DN134620_c1_g2	9.39	E7F0X0	High choriolytic enzyme 1-like	*hce2l2*
TRINITY_DN151930_c0_g3	9.36	P01833	Polymeric immunoglobulin receptor	*pigr*
TRINITY_DN152124_c5_g2	9.15	Q9BQ24	Zinc finger, FYVE domain containing 21	*zfyve21*
TRINITY_DN140296_c3_g2	9.13	P08217	Chymotrypsin-like elastase family member 2A	*cela2a*
TRINITY_DN153528_c0_g1	9.09	P07477	Trypsin 1	*prss1*
TRINITY_DN138531_c1_g2	8.88	Q7Z2Y8	Interferon-induced very large GTPase 1	*gvinp1*
TRINITY_DN155812_c1_g1	−2.07	Q7Z6B7	SLIT-ROBO Rho GTPase activating protein 1	*srgap1*
TRINITY_DN162540_c4_g1	−2.68	Q16653	Myelin-oligodendrocyte glycoprotein	*mog*
TRINITY_DN92606_c0_g1	−2.94	P09382	Beta-galactoside-binding lectin	*lgals1*
TRINITY_DN153065_c2_g2	−2.97	P18847	Activating transcription factor 3	*atf3*
TRINITY_DN6238_c0_g1	−3.02	P01344	Insulin-like growth factor 2	*igf2*
TRINITY_DN135989_c1_g5	−3.07	Q9NPI9	Inward rectifier potassium channel 16	*kcnj16*
TRINITY_DN139165_c1_g1	−3.37	Q01995	Transgelin	*tagln*

Table 1. *Cont.*

TRINITY ID	logFC	UniProtKB ID	Gene Definition	Gene Symbol
Skin				
TRINITY_DN159639_c0_g1	8.87	U3JA80	Serine and arginine rich splicing factor 2a	*srsf2a*
TRINITY_DN150109_c2_g2	8.44	P07477	Cationic trypsin	*prss1*
TRINITY_DN141237_c0_g7	7.65	Q14863	POU domain, class 6, transcription factor 1-like	*pou6d1*
TRINITY_DN137203_c7_g3	7.10	A0A1S3L397	Nuclear factor 7, brain-like	*nf7bl*
TRINITY_DN139257_c0_g1	4.05	Q96C55	Zinc finger protein 524	*znf524*
TRINITY_DN162280_c2_g2	3.26	Q96A28	SLAM family member 9	*slamf9*
TRINITY_DN129892_c0_g1	3.15	Q7Z2Y8	Interferon-induced very large GTPase 1	*gvinp1*
TRINITY_DN158576_c1_g3	−3.94	N/A	Zinc-binding protein A33-like	*za33l*
TRINITY_DN159712_c1_g3	−7.06	Q96P20	NACHT, LRR and PYD domains-containing protein 3	*nlrp3*
TRINITY_DN143097_c2_g4	−7.44	Q13216	Excision repair cross-complementation group 8	*ercc8*
TRINITY_DN159613_c2_g1	−7.60	Q14258	E3 ubiquitin/ISG15 ligase TRIM25	*trim25*
TRINITY_DN158530_c2_g1	−7.63	Q6BDS2	UHRF1 binding protein 1	*uhrf1bp1*
TRINITY_DN149926_c4_g3	−7.68	Q9PWM2	Homeobox B9a	*hxb9a*
TRINITY_DN151272_c3_g5	−7.81	N/A	Cytochrome P450 2K1-like	*cy2k1l*
TRINITY_DN151209_c9_g1	−7.85	Q7RTR2	NLR family CARD domain-containing protein 3	*nlrc3*
TRINITY_DN156424_c1_g2	−8.13	Q8NHV1	GTPase IMAP family member 7	*gimap7*
TRINITY_DN137852_c4_g3	−8.71	Q9UGT4	Sushi domain containing 2	*susd2*

Note: TRINITY ID = transcript reference; logFC = log2 fold change; UniProtKB ID = Uniprot unique reference.

Functional annotation identified significant enriched GO terms mostly in duodenum and head kidney with little representation in the skin. (Figure 3a–c, Table S9). Enriched GO terms were only identified in duodenum (Figure 3a). The main biological processes were related to the immune system, extracellular matrix disassembly and the regulation of activated T cell differentiation.

The main KEGG pathways associated with LPS treatment were identified in the duodenum and included secretory and digestive processes related to protein digestion and absorption, viral responses (viral myocarditis, influenza A) and the immune and hormonal related responses focal adhesion, natural killer cell mediated cytotoxicity, apoptosis, oxytocin signaling pathway (Figure 4a, Table S10). The skin was enriched with viral infection pathway (influenza A) (Figure 4b, Table S10). In the head kidney, no enriched pathways were identified.

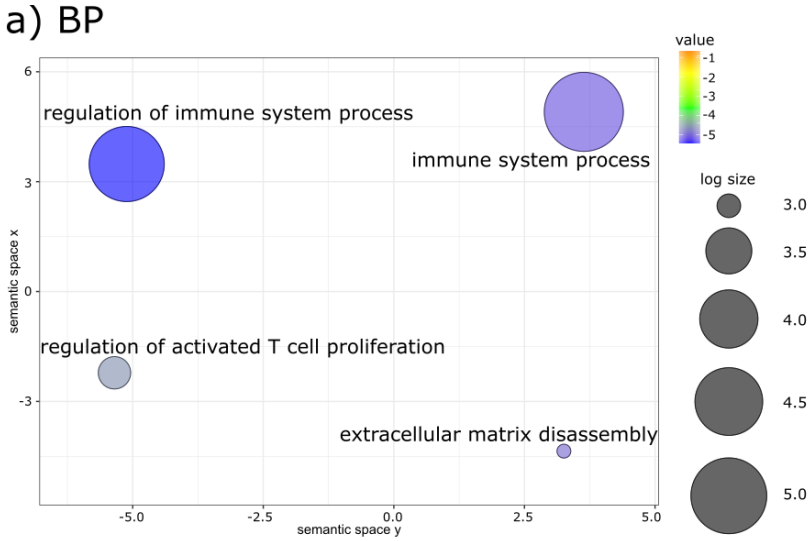

Figure 3. *Cont.*

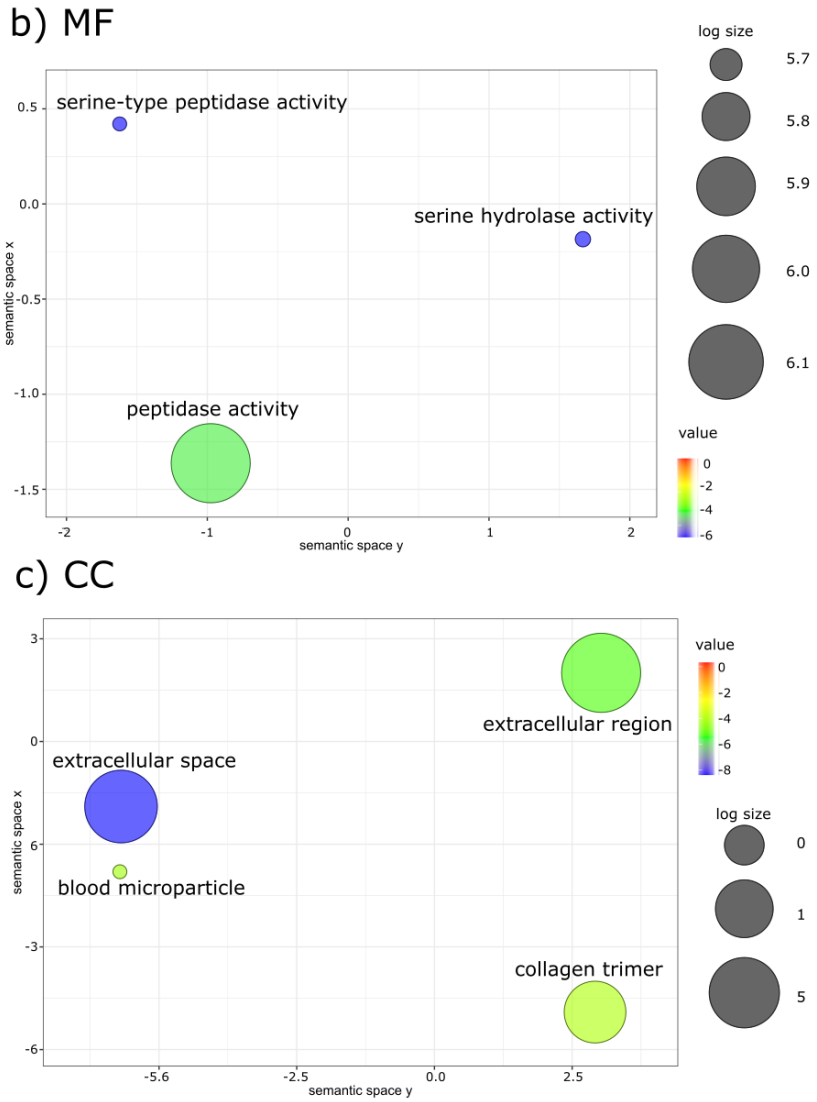

Figure 3. Enriched GO terms of LPS versus Sham DEGs in Duodenum (**a**) Biological Process (BP) (**b**) Molecular Function (MF) and (**c**) Cellular Component (CC). The sizes of the circles represent the relative abundance, and the color gradient represents values of the log10 *p*-value.

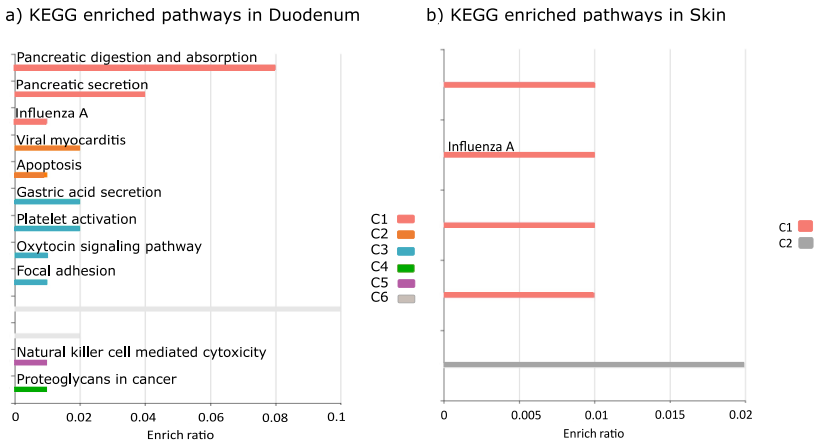

Figure 4. KEGG enriched pathways of LPS versus Sham DEGs. (**a**) duodenum, (**b**) skin. The enriched KEGG pathways that are closely related are grouped in the same cluster (C1–C6) represented by the same color. Different clusters have a different color.

4. Discussion

A challenge with an endotoxin from the cell wall of Gram-negative bacteria (LPS) caused significant modifications of gene transcription 7 days later in the head-kidney, duodenum and skin of *N. coriiceps*, mostly downregulation, but had no effect on the activity of blood plasma circulating enzymes. However, each tissue showed different responsiveness to LPS, with the duodenum the most modified and the skin the least modified, presumably because of tissue specificities and the route of administration (i.p.) of the LPS that simulated an internal infection, rather than a pathogenic contact with the fishes' surfaces, as would have been the case if administered through exposure in seawater.

The most modified processes in head-kidney, duodenum and skin in response to LPS were related to cell metabolism (up-regulated) and immune system (comprising 30% of DEGS, many downregulated). The main transcriptional modifications in the immune process category were related to pathogen recognition via receptors such as nucleotide-binding oligomerization domain-like (NOD) receptors, and expression of proteins against bacterial pathogens such as interleukin 6. The modified transcriptional response was not mirrored by a modified systemic response, as the circulating levels of enzymes of innate immunity, lysozyme and antiproteases, were not significantly different between the untreated, sham control and LPS treated fish.

The pairwise comparisons and clustering of the transcriptomes showed a clear effect of the treatments separating saline injected from non-injected controls and these two from LPS. However, although handling and injection modified gene transcription this was not translated in differential cortisol response, possibly because the cortisol peak had subsided after 7 days post injection [75]. The lack of cortisol and plasma protein responses to LPS are also consistent with observations in Atlantic salmon (*Salmo salar*) exposed to LPS for 1 and 19 days [51]. Based on studies of immunity in fish and shellfish, in which an LPS challenge was administered, we had anticipated a change in plasma lysozyme and antiprotease activity after LPS treatment in *N. coriiceps*. However, a lack of response of antiprotease activity to LPS has also been reported in gilthead seabream (*Sparus aurata*) larvae exposed to LPS for 24 h and in blood sera lysozyme activity in rohu (*Labeo rohita*) injected to LPS for 7–15 days [76,77]. It has been hypothesized that the high resistance and low response of some fish to LPS injections is due to the lack of a TLR4 recognition mechanism that in mammals mediates the down-stream immune activation, including TNF-α- and IL-1β-mediated inflammation, although zebrafish possess TLR4 and not activated by LPS [50,78–80]. Taken together, the results suggest that LPS may not always be an effective stimulus of immunity in teleost fish although the specific reasons are not clear.

Although no changes were observed in the activity of plasma circulating enzymes, it was notable the low basal level of blood lysozyme activity in *N. coriiceps* (50–90 U.mL^{-1} at 22 °C) compared to other teleosts, including the European sea bass (*Dicentrarchus labrax*) used as positive control in the present study (270–350 U.mL^{-1} at 22 °C) [81,82] and the Mozambique tilapia (*Oreochromis mossambicus*) (770–1000 U.mL^{-1} at 22 °C) [83]. The reason for the low levels of plasma lysozyme detected in *N. coriiceps* was not established but it is consistent with the absence of differentially expressed gene transcripts for lysozyme. The observation that the cold-water fishes cusk (*Brosme brosme*) and Atlantic cod (*Gadus morhua*) have low levels of lysozyme in the kidney (40 and 150 U.mL^{-1}, respectively) [84] may indicate that lysozyme activity is less important in fishes from colder waters. Furthermore, in the Siberian sturgeon (*Acipenser baerii*), water temperature and physiological condition (e.g., health status, diet and age) modulate basal lysozyme activity [85]. The relative contribution of water temperature and physiological status to the low basal lysozyme activity in wild captured *N. coriiceps* remains to be further explored.

The constancy of the plasma biochemical parameters in *N. coriiceps* exposed to LPS could be related to the timeframe of the experiment (7 days post-i.p. injection), although the source of LPS may also have been a contributing factor. LPS is composed of an O-antigen polysaccharide and lipid A layers. The lipid A moiety varies with the bacterial species [86] and variations in O-antigen affect the virulence and infection process [87]. Temperature has

a substantial impact on the structure of LPS and therefore the origin of the bacterial species' source, from warm, temperate or cold waters, determines its conformation [88], as shown with bacterial LPS from the Siberian permafrost [89,90]. Since innate immune activation occurs through the recognition of microbe associated molecular patterns (MAMPs) [91] changes in LPS structure may interfere with host-bacteria recognition. We hypothesize that since the LPS used in the present study was from *E. coli*, which colonizes the warm gut of mammals, the relatively low biochemical and transcriptional response of *N. coriiceps* might be linked to the structure acquired by *E. coli* LPS 0111:B4 at low temperature making it less recognizable by the immune system of Antarctic fish. This notion seems to be supported by the absence of a serum lysozyme response and should also be reflected in a lower efficacy of humoral mediator recognition [92]. In several temperate fish challenged with LPS at different water temperatures, fish at lower temperatures tended to be more susceptible to pathogen infections, e.g., *Paralichthys olivaceus*, *Oreochromis niloticus*, *Epinephelus coioides* and *Danio rerio* [93–97]. Furthermore, it has been suggested that cold water fish such as Atlantic cod may have a slower as well as a suppressed immune response [98]. Interestingly, *N. coriiceps* exposed to fragmented heat killed *E. coli* 0111:B4, showed a strong transcriptional response of toll-like receptor genes 6 and 12 h post-challenge in the spleen but not in the liver or kidney [40]. Overall, a combination of the duration of the experiment, form of LPS, route of administration, water temperature, and species may have contributed to the absence of a plasma biochemical immune response.

The importance of bacterial infections in aquaculture means that transcriptomics has been used to describe the global immune response to LPS in a range of fish species: e.g., *Lates calcarifer*, *Pelteobagrus fulvidraco*, *Salmo salar*, *Ictalarus punctatus* [54,99–101]. However, there are few immune challenge studies of wild fish such as the present one and those that exist have focused on a single immune tissue [29,99].

In the present study, the head-kidney and duodenum had a strong transcriptional response and much lower in the skin. In the head kidney biological processes and pathways related energy generation and protein metabolism were mainly modified, consistent with observations in rainbow trout (*Oncorhynchus mykiss*) after 24 to 72 h after i.p. LPS injection [29] and likely reflect metabolic demand associated with the immune response, since any stress effects of injection and handling have been considered in the comparisons. There was a conserved core response of immune-related DEGs in the head kidney and duodenum, which encompassed PRRs like *nlrc3*, an intracellular sensor of PAMPs, and *cxadr*, a viral receptor that also is involved in leukocyte transepithelial migration [102,103], *tmem30a*, that stimulates phagocytosis and humoral defense-related factors [104,105], bactericidal permeability-increasing protein-like (*bpi*) and type I interferon stimulated gene (*ifi44l*). Head kidney-specific DEGs falling within the same general categories were mannose receptors (*mrc1*) and the humoral response genes pyrin-like (*mefv*) and peptidoglycan recognition proteins (*pgrp-lbl*). In *Scophthalmus maximus* L. [106] and *Larimichthys crocea* [107] exposed to bacteria, *nlrc*3 and *mrc1* were also modified. Furthermore, in *Miichthys miiuy*, *Paralichthys olivaceus* and *Cirrhinus mrigala*, exposed to pathogen, *nod1* expression was also modified [52,108–110] and the common response with *N. coriiceps* suggests it is part of the core antibacterial defence response of teleost fish.

In the duodenum responses were related to blood vessel formation, mitochondrial function, and immune system. Modifications of VEGF signaling were reported in studies with mammals exposed to LPS, an effect linked to the known effects of LPS on angiogenesis [111,112]. Interestingly, in vitro studies reported modifications in *IL-1β* expression in lumpfish leukocytes [113] and in gilthead seabream macrophages after 24 h bacterial exposure [114] and in *IL-6* expression in LPS treated trout IgM+ B cells [115].

The response of immune function related genes to LPS was tissue-specific, compared to the other tissues. Nevertheless, changes in the humoral response due to bacterial pathogen exposure was reported in *Ictalurus punctatus* [116] and *Megalobrama amblycephala* [117] and *bpi*, one of the modified gene transcripts, was also differentially expressed in *N. coriiceps*. Similarly, peptidoglycan recognition proteins (e.g., *pgrp-lbl*), that bind multiple components

of bacteria, a well characterized part of the innate humoral response in *Danio rerio* [118] and *Cyprinus carpio* L. [119], were changed in *N. coriiceps*. *Ifi44l*, typically associated with a viral challenge in *Danio rerio* [120] was differentially expressed in both HK and duodenum of *N. coriiceps* suggesting it also responds to a bacterial stimulus.

The large percentage of down-regulated immune genes in *N. coriiceps* were mainly related to the inflammatory response and included the IL-6 receptor and PRRs (NLR and MRC receptors) particularly in the head-kidney and duodenum. *IL-6*, a pleiotropic cytokine in mammals, is not well-studied in teleosts, but a recent study in *Oreochromis niloticus* indicated it promoted an inflammatory response to bacterial infection and antibody production [121]. The PRR response to LPS in *N. coriiceps* diverged from the more commonly described TLR in other teleost fish [122] and instead NLR and MRC receptor genes were more responsive. The lack of TLR response could be linked to the recent observation that TLR5, TLR8, TLR21, TLR22 and TLR23 are under positive selective pressure in Antarctic Notothenioids [41]. Furthermore, a study of deep-sea bacteria revealed pathogen associated molecular patterns (PAMPs) that failed to interact with human TLR, and this was explained by the absence of selective pressure for their recognition during pathogen-recognition receptors (PRR) evolution and so they were "immune silent" [123]. Nonetheless, although the transcriptional response of *N. coriiceps* to i.p. LPS was not identical to that of other teleosts, the general recruitment of PRRs for pathogen recognition and humoral factors for pathogen neutralization was a well-conserved response.

The DEGs identified in the mucosa-associated lymphoid tissue (MALT), the duodenum and skin, had little in common except for *nlrc3*. Interestingly this gene was downregulated by LPS in all three tissues suggesting it was important for LPS-mediated bacterial recognition. The duodenum had the highest number of DEGs of all the tissue analyzed, probably due to a more direct and continuous exposure to i.p. LPS. The most notably modified process in *N. coriiceps* duodenum was mitochondrial depolarization, which has not previously been reported in fish under LPS exposure. It is possible that this reflects the higher cellular mitochondrial density in the Antarctic fish [12], but it may also be related to the upregulation in the duodenum of inflammasome immune related genes, such as *nlrp3*, which have been associated with mitochondrial depolarization and mitophagy in mammals [124]. In *Danio rerio*, Nlrp3 is involved in cytokine processing and secretion [125], and a similar function probably exists in *N. coriiceps* as an elevated number of DEGs for cytokines occurred in the LPS exposed duodenum, as well as a gene for signal transduction (*il6st*) that respond to a bacterial challenge [126]. Curiously, in the skin gene transcripts linked to mitochondrial function and more specifically the generation of reactive oxygen species (ROS), a process linked with the inflammatory response in fish, were modified [127,128]. In line with this, increased ROS generation was observed in *Lobeo rohita*, after 7- and 15-days exposure to LPS [77,127,128].

A characteristic transcriptional response to bacterial exposure in a variety of teleost fish is the upregulation of complement [54,122,129] and this was evident in DEGs of the duodenum of *N. coriiceps*. In *Ctenophcuyngodon idellus'* exposure to LPS i.p. increased the expression of adiponectin receptors in the liver [130] and in *Trachinotus, ovatus keap1* was upregulated [131]. The hypoferraemic response in response to LPS is also conserved irrespectively [42,51,132].

The results of the present study contrasted with that of Ahn and collaborators who found several TLR genes up-regulated in liver, spleen and kidney after *N. coriiceps* were immersed in heat killed *E. coli* (HKEB) for 6–12 h [40]. They identified 567 DEGs in liver and the most enriched immune related process was the antigen processing and presentation pathway and TLR related pathways—TNF pathway, T-cell receptors, B-cells, interleukins and chemokines [44]. Although there was little overlap in the DEGs identified between the previous and the present study, some common processes were identified: antigen processing and presentation in skin, chemokines in the head-kidney and interleukins in both skin and duodenum. Altogether, the results from the present and previous transcriptome studies revealed that, despite its unique evolution and habitat, *N. coriiceps* respond

rapidly to an immune challenge and that the general pattern of immune response is largely similar to teleosts from warmer waters, even taking into consideration some very divergent experimental protocols.

In our study, we selected the results of mapping transcripts to the *de novo* transcriptome, rather than the available *N. coriiceps* genome [4] because more genes were mapped and more core genes were annotated. However, we are aware of inherent bias of *de novo* transcriptome assemblies compared to map-to-reference estimates both in terms of diversity underestimation and positive bias at low expression levels, although these can be minimized using filtering methods, such as we those have used, and other computational methods [133]. It is therefore important that future work should be directed at improving genomes of key species for environmental and evolutionary studies such as *N. coriiceps* so as to maximize the power of transcriptomics.

5. Conclusions

Among the most striking features of the *N. coriiceps* response to LPS i.p injection was the unmodified plasma biochemical indicators and the marked difference in the quantitative and qualitative transcriptomic response between tissues. Skin and duodenum are barrier tissues and their difference in response may be related both to immune differences and effective exposure. A unique facet of the response to LPS in *N. coriiceps* was the up-regulation of genes linked to mitochondria and it is unclear if this is a consequence of adaptation to life in sub-zero conditions or if it is directly linked to the immune response. The immune processes identified were mostly down-regulated, particularly interleukins and pattern recognition receptors NLR and MRC, and the typical TLR response commonly described in other teleost fish was absent. This could have been because of the long period after stimulation (7 days) and/or to specificities derived from very specific pathogens and the cold marine environment of Antarctica. Further studies will be required to better characterize the PRRs and their role in Antarctic teleosts. Moreover, the importance of the LPS source and its consequences for immune activation in teleosts needs to be better understood.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fishes7040171/s1, Figure S1: Biochemical analysis parameters in blood plasma.; Table S1: BUSCO analysis results from genome and reference transcriptome; Table S2: Alignment rate of the sequenced samples of the control and LPS-treated fish in Head-kidney (Hk), Duodenum (Du) and Skin (Sk) tissues; Table S3: Assemblies input and output data; Table S4: Differential expressed genes annotation statistics; Table S5: Differential expressed genes of Sham versus Control groups in different tissues; Table S6: Gene Ontology enriched terms derived from the analysis of the differentially expressed genes in Sham versus Control groups in different tissues; Table S7: KEGG enriched pathways represented in the sham versus control group in different tissues; Table S8: Differential expressed genes of LPS versus Sham groups in different tissues; Table S9: Gene Ontology enriched terms derived from the analysis of the differentially expressed genes of LPS versus Sham groups in different tissues; Table S10: KEGG enriched pathways represented in the LPS versus Sham group in different tissues.

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Data Availability Statement: The data presented in this study are available in Supplementary Figure S1 and Supplementary Tables S1–S10.

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