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Transcriptomic Response to Immune Challenge in Zebra Finch (Taeniopygia Guttata) Using RNA-SEQ

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TRANSCRIPTOMIC RESPONSE TO IMMUNE CHALLENGE IN ZEBRA FINCH (*TAENIOPYGIA GUTTATA*) USING RNA-SEQ

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TRANSCRIPTOMIC RESPONSE TO IMMUNE CHALLENGE IN ZEBRA FINCH (*TAENIOPYGIA GUTTATA*) USING RNA-SEQ

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I dedicate this thesis to my parents for always being supportive in my advancements, and to Basal and Link for their unending entertainment.

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TRANSCRIPTOMIC RESPONSE TO IMMUNE CHALLENGE IN ZEBRA FINCH (*TAENIOPYGIA GUTTATA*) USING RNA-SEQ

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Despite the convergence of rapid technological advances in genomics and the maturing field of ecoimmunology, our understanding of the genes that regulate immunity in wild populations is still nascent. Previous work to assess immune function has relied upon relatively crude measures of immunocompetence. However, with next-generation RNA-sequencing, it is now possible to create a profile of gene expression in response to an immune challenge. In this study, captive zebra finch (*Taeniopygia guttata*; adult males) were challenged with bacterial lipopolysaccharide (2 mg/Kg BW; dissolved in 0.9% saline) or vehicle (0.9% saline) to stimulate the immune system. Two hours after injection, birds were euthanized and hypothalami, spleen, and red blood cells (RBCs) were collected. Taking advantage of the fully sequenced genome of zebra finch, total RNA was isolated, sequenced, and partially annotated in these tissue/cells. The data show 628 significantly upregulated transcripts in the hypothalamus, as well as 439 and 121 in the spleen and RBCs, respectively, relative to controls. Also, 134 transcripts in the hypothalamus, 517 in the spleen, and 61 in the RBCs were significantly downregulated. More specifically, a number of immunity-related transcripts (e.g., IL-1β, RSAD2, SOCS3) were upregulated among tissues/cells. Additionally, transcripts involved in metabolic processes (APOD, LRAT, RBP4) were downregulated, suggesting a potential trade-off in expression of genes that regulate immunity and metabolism. Unlike

mammals, birds have nucleated RBCs, and these results suggest a novel transcriptomic response of RBCs to immune challenge. Lastly, molecular biomarkers could be developed to rapidly screen bird populations by simple blood sampling in the field.

1. INTRODUCTION

Resources are not always ubiquitous within the environment. This is especially true for organisms that migrate and/or experience seasonal shifts. The energetically costly activities of reproduction and metabolic physiology are often cyclic within these resource shifts or seasons (Ebling, 2014). In birds, seasonal cycles are not only involved in courting and mating, but there exists a need for seasonally available food and the shift of energy for molting and migratory functions (Dawson et al., 2001). Resources are also needed to combat pathogens, and immune defense is allocated strategically among life-history activities (e.g., territory defense, reproduction, growth; Sheldon and Verhulst, 1996; Norris and Evans, 2000). These trade-offs of immune defense with other behavioral and physiological activities can serve as proximate underpinnnings that shape life-history decisions (Ricklefs and Wilkelski, 2002).

The innate immune system involves rapid, indiscriminate responses (Demas et al., 2011), initiated by pattern recognition receptors that are highly conserved among vertebrates (Owen-Ashley and Wingfield, 2007; Suffredinin et al., 1999). The acute phase response (APR) is the first level of defense against infection as part of the innate immune system (Owen-Ashley and Wingfield, 2007). This rapid response, within hours of infection, is characterized by behavioral and physiological alterations within the organism. These stereotypical behavioral changes are known as “sickness behaviors” and include lethargy, somnolence, reduced food and water intake, and decreased activities such as grooming (Hart, 1988; Gruys et al., 2005; Ashley and Wingfield, 2012). Physiological changes occur through upregulation of genes that lead to translation of

proteins that regulate fever and inflammation (interleukin-1β (IL-1β), interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF-α)), and downregulation of genes associated with reproduction and growth (Gruys et al., 2005; Ashley et al., 2012). The APR can also be triggered by exposure to lipopolysaccharides (LPS) derived from gram-negative bacteria in a dose-dependent manner (Sköld-Chiriac et al., 2014). Importantly, use of LPS avoids the confounding effect of pathogen manipulation upon the immune system, such that measurement of host immune responses can be accurately quantified (Ashley and Wingfield, 2012).

Most studies of the immune system have focused on the adaptive immune system, which takes longer to develop than innate immunity (Janeway and Medzhitov, 2002; Hedrick, 2004). Of the studies that have examined the innate immune system, and more specifically the APR, most have used mammals and domesticated birds (Bayne and Gerwick, 2001; Owen-Ashley and Wingfield, 2007; Cray et al., 2009). Studies on free-living organisms are uncommon. The interdisciplinary field of ecoimmunology has developed to understand how immune responses are related to host fitness, along with environmental and genetic variability, in non-model organisms (Demas and Nelson, 2012). However, ecoimmunology studies have typically used few markers to measure immunocompetence (Demas et al., 2011). With newer technology, analysis of gene expression in these non-model organisms can give further understanding into the APR and trade-offs that occur within other aspects of life.

The relatively new high-throughput DNA sequencing technology known as RNA-sequencing (RNA-seq) presents advantages and uses in non-model systems in conjunction with those model systems already in use. This method allows for the

mapping and quantifying of whole transcriptomes, and does not depend on an existing genomic sequence (Wang et al., 2009). RNA-seq technology can be used to compare expression levels in different tissues as well as to facilitate the identification of genes that regulate the response to infection. The relatively low cost and increased sensitivity of RNA-seq compared to other sequencing methods (Wang et al., 2009) also makes it a viable option for non-model system studies.

Previous studies using RNA-seq on zebra finch (*Taeniopygia guttata*) found many immune-related genes, such as the major histocompatibility complex, to be constitutively expressed in a tissue-specific manner (Ekblom et al., 2010). The zebra finch is one of the first birds with a fully sequenced genome (Warren et al., 2001). One study has assessed the effect of West Nile virus (WNV) infection, in which transcriptomes were analyzed 2 days post-inoculation (Newhouse et al., 2017). However, no study to date has measured rapid transcriptomic responses of birds to an immune challenge that is isolated from pathogen manipulation. The previous work in the zebra finch makes it an ideal candidate organism to pinpoint functionally important immune system genes.

This study examines the transcriptome of zebra finch following challenge of the immune system with LPS, using RNA-seq. It is hypothesized there will be an upregulation in expression of immune-regulated genes and a corresponding down-regulation of genes associated with growth and reproduction, which would suggest a molecular mechanism that could lead to a trade-off between immune defense and other life-history activities.

2. METHODS

2.1 **Animals**

Male zebra finch (*Taeniopygia guttata*) taken from our breeding colony were housed in individual cages (16.5x11.8x22, Petsmart Co.) in the animal facility at Western Kentucky University for 7 days. Food (Finch and Canary Breeding and Molting Seed Blend, Lady Gouldian Finch, Irvine, CA; lettuce/cabbage leaves) and water were provided *ad libitum*. Animals were exposed to a 12 h light: 12 h dark photoperiod (lights on at 0700) and an ambient temperature of 23 ± 1°C. Two hours before tissue collection (between 4 and 5 hours after lights on), birds were given an i.p. injection of either bacterial lipopolysaccharide (LPS; 2mg/kg of BW; n=8 *Escherichia coli*; serotype 026:B6; Sigma-L8274) dissolved in 0.9% saline (vehicle) or vehicle alone (n=8). Animals were euthanized using isoflurane until unconscious and then rapidly decapitated. Brain and spleen were collected from half of the treatment group (n=4) and half of the control group (n=4) and immediately placed in RNA*later* (AM7021; Thermo Fischer Scientific). Tissues were stored in RNA*later* at -20°C until processed. The hypothalamus was dissected out of the brain before RNA isolation (see below). Whole trunk blood was collected from the remaining four treatment and four control birds and placed into tubes containing EDTA and kept on ice until processed (see "Blood" below).

All experimental procedures were approved by the Institutional Animal Care and Use Committee at Western Kentucky University (#16-01).

2.2 **Blood**

EDTA-treated blood was first carefully layered on top of a single step density gradient medium (PolymorphPrep; Axis-Shield) consisting of 1-part blood: 2-parts

gradient medium and then further processed following the manufacturer's protocol. Layers of peripheral blood mononuclear cells (PBMC), polymorphonuclear leukocytes (PMN), and white blood cells (WBC) were extracted and pooled per individual animal. Slides were prepared from erythrocyte (red blood cells; RBCs) and PBMC/PMN samples and stained using Hemacolor Rapid (Millipore Sigma) following the manufacturer's protocol. Each slide was surveyed under 400 x magnification for 10 minutes for contamination before RNA isolation.

2.3 **RNA Isolation**

RNA was isolated from PBMC/PMNs and RBCs using Trizol (Ambion) lysing and a RNeasy Mini Kit (Qiagen). The hypothalamus and spleen were homogenized and RNA was extracted using a RNeasy Mini Kit (Qiagen) following the manufacturer's instructions. RNA yield and purity were assessed using a NanoDrop N-D 2000 spectrophotometer (Thermo Fischer Scientific) and an Agilent 2100 Bioanalyzer (Agilent), respectively. Unfortunately, PBMC/PMNs yielded RNA concentrations that were too low for sequencing purposes. Low RNA yield can be attributed to the low concentration of WBCs in whole blood and the small volume of whole blood we were able to obtain from zebra finch. (typically < 250 µl).

2.4 **Library Preparation and Sequencing**

Samples were sent to the University of Louisville Genomics Core (Louisville, KY) for library preparation and sequencing. SMART-Seq v4 Ultra Low Input RNA kits (Takara Bio USA, Inc.) were used to prepare cDNA libraries. Samples were barcoded with Illumina TruSeq Adapters. After library clean-up using Agencourt AMPure XP

Beads, quality was assessed on an Agilent Bioanalyzer using the Agilent DNA 1000 Kit. This confirmed the final fragment size for all samples to be approximately 400bp, as expected from the protocol. Samples were sequenced twice on an Illumina NextSeq 500 sequencer, with four biological replicates and four lanes per replicate.

2.5 **Bioinformatics Analysis**

Bioinformatics analysis was performed by the Kentucky Biomedical Research Infrastructure Network (KBRIN) Bioinformatics Core. Raw sequencing files were downloaded from Illumina's BaseSpace. Quality scores for raw sequences were sufficiently high, above the recommended score of 20 (base call accuracy of 99%), to proceed with analysis (Ewing and Green, 1998; Ewing et al., 1998). The Tuxedo Suite pipeline was used for analysis of data (Trapnell et al., 2012). High quality single-end reads (152 million for hypothalamus, 140 million for spleen, and 326 million for RBCs) were successfully mapped to the zebra finch reference genome (taeGut3.2.4.84) from Ensembl (www.ensembl.org; Kersey et al., 2018) using the TopHat2 (v. 2.0.13) tool. Differential expression between LPS and saline conditions was determined from fragments per kilobase million (FPKM) normalized read counts using Cuffdiff2 (v. 2.2.1) of the Tuxedo Suite programs and log base 2 transformed. All fold change expression values presented from this study are provided as log base 2. Genes with Benjamini-Hochberg false discovery rate (FDR) (q-value) ≤ 0.05 and a log base 2 transformed fold change ≥ 1 were considered significantly differentially expressed.

2.6 **Gene Ontology Analysis**

Gene annotations came from Ensembl BioMarts as part of the KBRIN Bioinformatics Core analysis (Ashburner et al., 2000; Kinsella et al., 2011; The Gene Ontology Consortium, 2017). Transcripts with differential expression from sequence code but no known gene ID were excluded from further analysis. Gene ontology (GO) enrichment analysis was performed on differentially expressed genes (DEGs) using the ShinyGO web-based tool (http://ge-lab.org:3838/go/)(v. 0.1). GO functional categories falling under biological processes, with a q-value ≤ 0.05 following hypergeometric testing, were considered significantly overrepresented. In the hierarchy of GO, a gene can be represented in more than one category because of the functional versatility of genes, but only once within each category. Likewise, genes appear in the categories represented by both the specific (child term) and broad (parent term) categories under these biological processes.

3. RESULTS

3.1 **Transcriptome and Gene Ontology Analysis**

Analysis of cDNA library reads for differential expression resulted in 628 significantly upregulated genes in the hypothalamus, 439 in spleen, and 121 in RBCs (Figure 1A) among LPS-treated birds compared to control samples. Additionally, 134 genes in the hypothalamus, 517 in the spleen, and 61 in RBCs were shown to be significantly downregulated in LPS birds relative to control birds (Figure 1B). These numbers include overlap in the DEGs (Figure 1A, B). GO analysis was performed on

these significantly DEGs and returned functional categories being overrepresented, based on GO terms annotated to our gene sets, with a *q*-value cutoff ≤ 0.05.

3.2 **Hypothalamus**

The top 20 significantly upregulated genes of the hypothalamus in response to LPS challenge are shown in Table 1 with each gene's transformed fold change in expression compared to controls. GO analysis of all 628 upregulated DEGs in the hypothalamus showed 92 to be involved in the stress response (Table 2). Several of the most upregulated genes appear in this overexpressed category: radical S-adenosyl methionine domain containing 2 (RSAD2), suppressor of cytokine signaling 3 (SOCS3), connective tissue growth factor (CTGF), G protein-coupled receptor 75 (GPR75), interferon regulatory factor 1 (IRF1), and eukaryotic translation initiation factor 4E binding protein 1 (EIF4EBP1).

Differential expression analysis from the transcriptomic response resulted in 134 downregulated genes with Table 3 detailing the 20 most significantly downregulated. GO analysis of the total downregulated DEGs indicates an overrepresentation of metabolic processes, involving genes from the most downregulated genes, such as calcitonin related polypeptide alpha (CALCA), retinol binding protein 4 (RBP4), carboxyl ester lipase (CEL), acetylserotonin O-methyltransferase (ASMT), phosphoenolpyruvate carboxykinase 1 (PCK1), apolipoprotein D (APOD), and tyrosine hydroxylase (TYH) (Table 3; Table 4). Pro-melanin-concentrating hormone (PMCH), in addition to CALCA and TYH, were some of the genes present in the overrepresented function of feeding behavior. The defense gene, avian beta defensin 9 (AvBD9) was also downregulated.

3.3 **Spleen**

Tables 5 and 6 show the 20 most up- and downregulated genes, respectively, from analysis of transcript expression in the spleen. The serine peptidase inhibitor, kazal type 4 (SPINK4) gene was the most upregulated of all genes in our global results (Table 5; 14.71-fold change). From GO analysis, functions associated with the stress response, including a response to LPS and external stimulus, are overrepresented by our 439 upregulated spleen genes (Table 7). An overall immune response was also overrepresented. Some of the top 20 DEGs, such as ankyrin repeat and BTB (POZ) domain containing 2 (ABTB2), activating transcription factor 3 (ATF3), and aldehyde dehydrogenase 1 family, member A3 (ALDH1A3), are shown to be involved in the above stress response categories. Several other DEGs are associated with more than one function, demonstrating the multi-functionality of genes. Interleukin-1 beta (IL-1β), interleukin-8 (IL-8), C-C motif chemokine 4 homolog (CCL4), and DEXH (Asp-Glu-X-His) box polypeptide 58 (DHX58) are involved in the stress response, immune function, and cytokine production, while SOCS3 is involved in the stress response and cytokine production, and 2'-5'-oligoadenylate synthase-like protein 1-like (OASL) is involved in the stress response and immune function.

Functions resulting from GO analysis of the 517 downregulated genes show a gross overrepresentation in metabolic processes within the 30 most significantly overrepresented functional categories (Table 8). Lecithin retinal acyltransferase (LRAT), hydroxyacid oxidase 2 (HAO2), tyrosine aminotransferase (TAT), dihydropyrimidinase (DPYS), fatty-acid amide hydrolase 1-like (FAAH), serpin family C member 1 (SERPINC1), and RBP4 are DEGS associated with metabolic process and found within

the top 20 downregulated genes in response to LPS. Additionally, the LRAT gene experienced the largest fold-change in downregulation (-9.51-fold change) of all DEGs across all tissues analyzed (Table 6).

3.4 **Red Blood Cells**

From the GO analysis on the 121 upregulated DEGs of the RBCs, an overrepresentation of immune and cytokine functions (e.g. innate immune response, leukocyte activation, cytokine production, type I interferon production), as well as stress response, is shown (Table 9). Of those 121 upregulated genes, the top 20 are presented in Table 10, with C-C motif chemokine ligand 5 (CCL5), toll-like receptor 3 (TLR3), leucine-rich repeat kinase 1 (LRRK1), serum amyloid A like 1 (SAAL1), mitogen-activated protein kinase kinase kinase 8 (MAP3K8), and interferon induced with helicase C domain 1 (IFIH1) associated with the overrepresented immune and cytokine functions.

There were 61 significantly downregulated genes in the RBCs, with the 20 most downregulated shown in Table 11. An overrepresented function of interest from GO analysis is the negative regulation of interleukin 8 (IL-8) (Table 12). Annexin A1 isoform p37-like (ANXA1) and arrestin beta 1 (ARRB1) are among the most downregulated genes that contribute to this negative regulation of IL-8 (Table 11; Table 12). It should be noted that the lymphocyte antigen 86 gene (LY86), which mediates the LPS signaling pathway, was also downregulated.

3.5 **Shared Genes**

Five genes were significantly upregulated in all three tissues tested (Table 13). Two of the five common genes— CCL5 and RSAD2—are found among the top 30

overrepresented functional categories (Table 14). Additionally, a single gene, glutathione S-transferase class-alpha variant 2 (GSTA2), was downregulated in all three tissues (Table 15). The GSTA2 gene is involved in the metabolic process according to its GO annotation.

4. DISCUSSION

The aim of this study was to determine the transcriptome of the zebra finch following an lipopolysaccharide (LPS) challenge to the immune system using RNA sequencing (RNA-seq). Gene expression was assessed in three tissues or cells: hypothalamus, spleen, and red blood cells (RBCs). I was particularly interested in documenting the transcriptomic response during an acute phase response (APR) - the initial behavioral and physiological changes that occur during infection (Ashley and Wingfield, 2012; Gruys et al., 2005). 1,741 genes were differentially expressed, with 1,056 upregulated and 685 downregulated across all three tissues. RNA-seq analysis also allowed for the quantification of the response to LPS beyond that of other sequencing methods (Wang et al., 2009).

Genes involved with the stress response are upregulated within the hypothalamus in response to LPS challenge. This response is consistent with activation of the hypothalamic-pituitary-adrenal (HPA) axis that occurs during the APR, including in birds (Ashley and Demas, 2017; Owen-Ashley and Wingfield, 2007). Given the well-known function of the spleen in regulating immunity, it was not surprising that immune function and cytokine production genes were overrepresented in the spleen. However, the overrepresentation of immune-related and cytokine production-related genes in avian

RBCs indicates a novel function of nucleated RBCs. Most studies to date have assumed that RBCs play little or no role in regulating immunity, but only a few have examined non-mammalian RBCs (Morera and MacKenzie, 2011; Morera et al., 2011). Increased cytokine production plays a key role in triggering the APR and associated sickness behaviors (Gruys et al., 2005).

The downregulated transcripts of the hypothalamus and spleen imply a reduction in metabolism based on the Gene Ontology (GO) analysis (Table 4). Although many endotherms exhibit fever when challenged with LPS, in small songbirds, such as the zebra finch, hypothermia results, with a concurrent decrease in metabolic rate (Sköld-Chiriac et al., 2015). It is hypothesized that in small endotherms, the heat loss from a fever response would be more detrimental than the purported advantages (Jones et al., 1983). Hyperthermia, experienced by most of the larger endotherms (pigs, goats, and chickens), and hypothermia in small birds, is thought to be augmented by the same inflammatory and stress signals that trigger fever (Romanovsky et al., 2005). In the hypothalamus, feeding behavior and hormone-related processes are also overrepresented as downregulated functions. There was also a downregulation in genes related to the negative regulation of IL-8 production found in the RBCs (detailed below).

Of the total number of transcripts differentially expressed in each tissue, only six were shared among all three, and included five upregulated genes and one downregulated gene (Figure 1A, B). The majority of differentially expressed genes (DEGs) were tissue specific, with the hypothalamus experiencing the largest shift in unique upregulated DEGS and spleen having the most downregulated. These data suggest that different tissues have a specialized role when responding to an immune challenge. The most DEGs

within overrepresented GO functional categories are the most informative for the study and were chosen for further examination within those functions.

4.1 **Hypothalamus**

The hypothalamus plays an integral role in homeostasis by regulating body temperature, appetite, reproductive behaviors, and circadian rhythms (Saladin, 2010a). The APR, triggered by LPS treatment, activates the hypothalamic-pituitary-adrenal (HPA) axis and suppresses the hypothalamic-pituitary-gonadal (HPG) axis via stimulation by inflammatory cytokines such as IL-1β (Tsigos and Chrousos, 2002; Owen-Ashley and Wingfield, 2007).

4.1.1 *Upregulated Gene Functions*

Of the top 20 most upregulated hypothalamus genes (Table 1), six are involved in the stress response. RSAD2 was the most upregulated (4.045-fold change) gene in this category based on transcript abundance in the hypothalamus. This gene, also known as viperin, has been shown to be upregulated in response to H5N1 avian flu (Ranaware et al., 2016) and dengue infections (Schoggins, 2014) through involvement in interferon and cytokine signaling pathways. RSAD2 is upregulated in chicken kidney samples following avian infectious bronchitis virus exposure (Cong et al., 2013), suggesting that RSAD2 might be induced during early infection.

Of the other five genes in this functional category, SOCS3 and EIF4EBP1 are associated with the inhibition of interferon production via a negative feedback loop that prevents interferon and other inflammatory cytokines from inducing immunopathology (Chaves de Souza et al., 2013). SOCS3 was upregulated by our LPS challenge with a

3.462-fold change increase compared to control samples (Table 1). Ranaware *et al.* (2016) saw a 4.2-fold change increase following H5N1infection in chicken lung tissue. It is also possible that a virus, such as H5N1 or influenza A, may evade the host's immune response by inducing cytokine suppressors (SOCS1 and SOCS3), that in turn inhibit interferon and toll-like receptor signaling (Smith et al., 2004; Pothlichet et al., 2008). SOCS3 has also been implicated in the inhibition of leptin and insulin signaling (Piekarski-Welsher et al., 2016), supporting the metabolic inhibition reported from GO analysis.

EIF4EBP1 augments the innate immune system as a translational repressor of interferon production (Nehdi et al., 2014). EIF4EBP1 acts by repressing expression of interferon regulatory factor 7 (IRF7), thereby repressing interferon production (Colina et al., 2008). Without EIF4EBP1 repression, IRF7 triggers interferon production, creating a positive feedback loop for further IRF7 and interferon production. IRF7 was not significantly upregulated in these samples (0.964-fold change, *q*-value 0.191), and interferon was not present. However, there was upregulation in IRF1 (2.975-fold change; Table 1). The products of IRF1 also trigger an interferon response during early infection. A moderate upregulation in IRF1 transcription was seen in chicken lung following H9N2 infection (Ranaware et al., 2016) and IRF1 knock-out mice cannot survive West Nile virus infection (Brien et al., 2011).

The other two upregulated stress response genes found in the hypothalamus are cytokine receptor GPR75 and the pro-fibrotic CTGF genes. GPR75 is restricted to the central nervous system and retina, and upon stimulation by the chemokine, CCL5, a gene upregulated in all three of our tissues (Table 13), activates downstream pathways

involving MAP kinases (Cartier et al., 2005; Pease, 2006). The CTGF gene is involved in early wound repair through modulating the activities of several growth factors and other cytokines (Wick et al., 2010; Alfaro et al., 2013). In pancreatic alcohol injury CTGF stimulates pro-inflammatory cytokine gene expression, specifically IL-1β (Charrier et al., 2014). Wang *et al.* (2010b) demonstrated the role of CTGF in transforming growth factor beta (TGF-β) signaling. In the hypothalamus samples, IL-1β was not significantly upregulated (*q*-value of 0.053), but this does not discount a possible biological significance, especially when examining the fold change (5.132) and results found in the other tissues examined (fold change value 7.184 in spleen and 2.129 in RBC) (Table 5; Table 10).

4.1.2 *Downregulated Gene Functions*

Seven of the 20 most downregulated genes in the hypothalamus are involved in metabolic processes (Table 3; Table 4). The CALCA gene encodes several peptide hormones, including calcitonin and calcitonin-gene-related peptide (CGRP), that are linked to glucose and lipid metabolism (Bartelt et al., 2017). CGRP is also involved in controlling inflammation through anti-inflammatory actions (Assas et al., 2014; Russell et al., 2014). In chronic inflammation disorders, such as inflammatory response syndrome in dogs, CALCA is upregulated (Giunti et al., 2010), where it inhibits tumor necrosis factor (TNF) and interleukin 12 (IL-12) cytokines (Assas et al., 2014). Tsujikawa *et al.* (2007) found this anti-inflammatory effect 24 hours after LPS treatment in mice, where cytokine levels were highest 3-6 hours post-treatment.

Another downregulated gene that influences both metabolism and inflammation is RBP4. RBP4 is described as a negative acute phase inflammatory reactant; decreased

transcription in favor of the amino acids being used for producing positive acute phase reactants, such as IL-1β and TNF, that also inhibit RBP4 (Rosales et al., 1996; Moraes-Vieira et al., 2014; Zabetian-Targhi et al., 2015). Metabolic GO terms associated with this gene include 'gluconeogenesis' and 'positive regulation of insulin secretion,' and it has been linked with human obesity (Kotnik et al., 2013). The RBP4 protein is needed for the transport and utilization of retinol (vitamin A) from the liver to peripheral tissues (Racke et al., 1995) where retinol derivatives are involved in lipid metabolism (Klör et al., 2011). However, retinol has been shown to enhance recovery from infection via actions in lymphoid tissues and its role in immune cell development (neutrophils, macrophages, natural killer cells; Stephensen, 2001). During the APR, already circulating retinol can be utilized, but less will be transported from the liver due to the downregulation of RBP4 transcription (Racke et al., 1995). Consequently, the immune system of malnourished individuals, with less circulating retinol before infection, may have a decreased ability to combat illness, based upon the above.

Among the five other downregulated genes involved in metabolic processes, CEL and APOD are involved in lipid and lipoprotein metabolism (Hui and Howles, 2002; Do Carmo et al. 2007), and PCK1, also known as PEPCK, regulates decarboxylation of oxaloacetate to pyruvate (Noce and Utter, 1975; Duan et al., 2013), thus reducing energy transport and utilization. The fourth of these genes, ASMT, encodes an enzyme that metabolizes N-acetylserotonin to melatonin, but is shown to be suppressed by IL-1β in sheep during inflammation, regardless of photoperiod (Herman et al., 2016). Lastly, the TYH gene regulates catecholamine metabolism (Daubner et al., 2011).

There were also downregulated genes involved with mediating feeding behavior. PMCH encodes melanin-concentrating hormone (MCH), with confirmed activity in appetite control by the use of MCH agonists and antagonists (Naufahu et al., 2013). Saito *et al.* (2001) and Adams *et al.* (2011) demonstrated the role of this hormone in olfaction, where the downregulation or deletion of PMCH impaired food-seeking behaviors. CALCA and TYH are also in this category for their metabolic cascades driving energy homeostasis by CALCA (Bartelt et al., 2017) and brain function products by TYH (Daubner et al., 2011).

The AvBD9 gene, with a primary role in the innate immune system, was downregulated. The expressed protein usually destroys bacteria through actions such as membrane and intracellular component damage (Wang et al., 2010a). It may be downregulated in the hypothalamus because of its tissue specific expression. AvBD9 is typically expressed in epithelial cells of the skin, kidneys, and trachea-brachial lining (Yang et al., 1999), where it exerts actions on the innate immune system (Wang et al., 2010b). However, other beta-defensin genes are expressed in other tissues, such as mouse beta-defensin 41 and 42, expressed in the male urogenital tract epithelium (Jalkanen et al., 2005), and grouper beta-defensins in the pituitary and testes of fish (Jin et al., 2010). More research is needed to understand the regulation of AvBD9 in the hypothalamus of zebra finch.

4.1.3 *Hypothalamus Conclusions*

The differing roles of the genes being up- or downregulated in the hypothalamus following LPS treatment reflects a potential trade-off between functions. Several of the most upregulated genes code for proteins that interact in cytokine pathways. Control of

cytokine activity is evident in our results through the upregulation of the cytokine inhibitors SOCS3 and IEF4EBP1. All of the downregulated genes discussed here participate in metabolism and/or feeding behaviors. CALCA has anti-inflammatory actions (Assas et al., 2014; Russell et al., 2014), which are depressed during the APR in this study. The reduction in feeding behaviors by the downregulation of CALCA, PMCH, and TYH may lower the risk of being a predatory target when searching for food when the animal is not in optimal health. The TYH enzyme product facilitates catecholamine synthesis (dopamine, epinephrine, norepinephrine), further affecting brain functions such as attention, memory, and cognition (Daubner et al., 2011). The above results suggest a physiological shift towards combating illness, and a reduction in unnecessary energy expenditure and behaviors that would increase risks. The downregulation of AvBD9 is not well understood in these results, based on its reported upregulation in studies in other avian species: chicken (*Gallus gallus domesticus*; Xiao et al., 2004; van Dijk et al., 2007), mallard duck (*Anas platyhynchos*; Lynn et al., 2007), and goose (*Anser cygnoides*; Ma et al., 2012).

4.2 **Spleen**

One of the largest organs of the lymphatic system, the spleen filters the fluids in the body, including blood (Saladin, 2010b). During filtration, excess blood can be stored, old RBCs removed, and iron recycled (Mebius and Kraal, 2005). The spleen also plays a prominent role in the peripheral immune system. When macrophages in the spleen interact with bacterial or viral components, the innate immune system is triggered (Gordon, 2002). Additionally, the spleen is involved in the adaptive immune system through B cell production of antibodies and T cell activation (Balázs et al., 2002).

4.2.1 *Upregulated Gene Functions*

In response to immune challenge by LPS, the spleen experienced an upregulation in genes involved in the stress response, immune function, and cytokine production (Table 8). It is also noteworthy that across all tissues in this study, the SPINK4 gene in the spleen exhibited the highest fold change increase (Table 5; 14.710-fold change). Although SPINK4 was not shown in the GO functional categories, it is considered a defense gene. SPINK4 is a member of a family of serine protease inhibitors that protects epithelial and mucosal tissues from proteolytic degradation, including by bacterial actions (Wapenaar et al., 2007). The exclusion of this gene from the GO categories is likely due to the GO terms associated with it, 'serine-type endopeptidase inhibitor activity' and 'negative regulation of endopeptidase activity,' not mapping to stress, immune, or cytokine functions; rather it is involved in negative regulation of metabolism.

Three of the most upregulated genes are involved in the stress response only. One of these, ABTB2, produces enzymes that catalyze the degradation of tumorigenic proteins (Roy and Pahan, 2013), and is upregulated in lymph node metastasis (Yasui et al., 2004) and during bronchial infections (Islam et al., 2010). Interestingly, in silkworm, this gene encodes an inhibitor of Bruton tyrosine kinase involved in male genital development of certain insects (Cheng et al., 2014). More investigation is needed to examine this in vertebrates. The second of these stress genes is ATF3, with products serving as a translational repressor when bound to DNA, or as an activator when heterodimerized with JUN proteins (Jadhav and Zhang, 2017). ATF3 can be induced by, as well as create a negative feedback loop, with toll-like receptor stimulation (Hashimoto et al., 2002; Jadhav and Zhang, 2017).

ALDH1A3, also known as RALDH3, is the last of the three of these stress response genes upregulated in the spleen. ALDH1A3 produces the rate limiting dehydrogenase enzyme for converting retinol (vitamin A) to retinoic acid, a hormone-like metabolite with the ability to modulate immune responses by promoting inflammation (Peck, 1984; Broadhurst et al., 2012). Retinoic acid activates and increases cytokine production in the innate immune system and is used by dendritic, B, and T cells in the adaptive immune system (Pino-Lagos et al., 2008). Increased conversion of retinol by the upregulation of ALDH1A3 also decreases the limited serum retinol resulting from the downregulation in transcription of its transporter, RBP4. Without this transporter, retinol remains stored in the liver and unusable in other parts of the body (Stephenson, 2001). The limit on retinol is further compounded by the fact RBP4 is downregulated in both the hypothalamus and spleen.

The potent cytokine gene, IL-1β, is involved in the stress response, immune function, and cytokine production GO function categories. IL-1β is one of the most studied pro-inflammatory cytokines for its crucial role in initiating and regulating the immune system, and has been well documented in chickens by LPS injection (Weining et al., 1998), infectious bursal disease (Heggen et al., 2000), and different bacterial infections (Kogut et al., 2005; Lavrič et al., 2008). In wild birds that act as natural reservoirs for disease and encounter domesticated animals, IL-1β is less understood. Park *et al.* (2017) showed that house finch splenocytes had several cytokines significantly induced by IL-1β, including interleukin 2 (IL-2), interleukin 10 (IL-10), and chemokine C-X-C motif ligand 1 (CXCL1).

Two chemokines, IL-8 and CCL4, were also upregulated. IL-8 products induce lysosomal enzyme release from neutrophils and chemotactic activity for basophils and adaptive T cells (Mukaida et al., 1998). IL-8 gene is greatly stimulated by IL-1β, but not by interferon (John et al., 1998), and is elevated in respiratory distress syndrome in humans (Harada et al., 1994). CCL4 also encodes products with chemotactic activity. CCL4 attracts natural killer cells, monocytes, and in later adaptive responses, T cells (Cheung et al., 2009). Of interest is the change in expression levels of CCL4 with H5N1 infection. Compared to seasonal influenza viruses, such as H1N1 and H3N2, CCL4 is more strongly induced by H5N1 (Peiris et al., 2009). Cheung *et al.* (2002) even reported the downregulation of CCL4 in human samples with H1N1 infection. This relationship of H5N1 and CCL4 may have additional implications for wild and domestic avian species.

Although DHX58, also known as retinoic acid inducible gene 1-like receptor 3 (RLR3), is involved in the stress response, immune function, and cytokine production in our data, the role of this gene within these functions is not clear. The encoded protein, laboratory of genetics and physiology 2 (LGP2), is reported as inhibiting RIG-I signaling and inducing IFIH1, otherwise known as melanoma differentiation-associated gene 5 (MDA5) (Zhu et al., 2014). DHX58 has some involvement in the fine tuning of interferon signaling in response to viral products (Sato et al., 2015). Expression of the LGP2 protein in plasmid vectors inhibits RIG-I signaling, but DHX58 knockout mice are more susceptible to infection by polio virus and picornaviruses, both of which are recognized by RIG-I and MDA5 elements (Bruns et al., 2014). DHX58 gene is also upregulated with Newcastle disease virus (NDV) infection, which can be fatal to many species of birds, including chickens, that lack RIG-I, but for which ducks and some geese have natural

resistance attributed to having RIG-I genes (Alexander, 2000; Chen et al., 2013). Further studies into the interaction of DHX58 in RIG-I signaling are required, and may provide valuable information on the treatment of economically detrimental diseases, such as NDV.

Interestingly, some of the actions of the upregulated OASL gene products work in contrast to DHX58. These proteins help in the recognition of and response to picornaviruses, but will enhance, rather than inhibit, RIG-I signaling (Choi et al., 2015). OASL has previously been used as a biomarker in predicting the response of patients with rheumatoid arthritis to treatment with the drug tocilizumab (Choi et al., 2015). Its expression is upregulated during influenza A infections (Zhu et al., 2015). The contrasting control of RIG-I signaling by DHX58 and OASL is likely another way in which the immune system is optimized to efficiently combat infection without excess.

SOCS3, involved in both the stress response and cytokine production, was upregulated by 3.462-fold change in the hypothalamus and a 4.421-fold change in the spleen (Table 1 and 5, respectively). SOCS3 helps regulate the immune system response by preventing the induction of immunopathology from over-abundant cytokine presence (Chaves de Souza et al., 2013). More cytokine genes were among the most upregulated of the spleen compared to the hypothalamus, so the increased expression of SOCS3 may be required to better control the inflammatory response from cytokines within the spleen.

4.2.2 *Downregulated Gene Functions*

GO analysis showed the suppression of metabolic processes in the spleen, represented by our downregulated genes (Table 6; Table 8). One of these, LRAT, showed the greatest downregulation of all genes across all three tissues, with a fold change

decrease of -9.510. LRAT is involved in retinol metabolism. Specifically, it encodes the predominant enzyme for esterification of retinol (O'Byrne et al., 2005; Zhao et al., 2017). LRAT negatively regulates retinoic acid biosynthesis (Ghosh, 2009). The downregulation of LRAT, along with the upregulation of ALDH1A3 (see above), can result in increased biosynthesis of retinoic acid. The increased presence of retinoic acid further suggests the importance of retinoic acid in inflammation, as previously discussed. As was seen in the hypothalamus, RBP4 was also downregulated in the spleen.

Among the other downregulated genes involved in metabolism, HAO2 encodes an enzyme that mediates the oxidation of fatty acids (Jones et al., 2000; Barawkar et al., 2011). Likewise, the hydrolase encoding gene, FAAH, is involved in the hydrolysis of 2-arachidonoylglycerol, a lipid that stimulates phagocytic activities in macrophages during a defense response (Lee et al., 2017). Also, by the downregulation of TAT, the amino acid tyrosine is not catabolized (Grossman and Mavrides, 1967; Iynedjian et al., 1985), possibly making the amino acid further available for cytokine production. Another downregulated gene, DPYS, encodes a rate-limiting enzyme that plays a role in pyrimidine catabolism (Kikugawa et al., 1994). Inhibiting these genes reduces degradation of materials needed by the immune response, such as amino acids and immune system mediators.

SERPINC1 provides another case of downregulation in genes with anti-inflammatory activity during the APR. The SERPINC1 gene encodes a serine protease inhibitor that inhibits the blood clotting enzyme, thrombin (Geng et al., 2013). The normal functions of SERPINC1 within the coagulation system limits neutrophil interactions with endothelial cells, reduced platelet aggregation, and pro-inflammatory

cytokine production (Warren et al., 2001). Downregulation of SERPINC1 also occurs in chicken embryonic cells infected with avian infectious laryngotracheitis (ILT) herpesvirus, a disease that can result in large economic losses by mortality and reduced egg production (Li et al., 2016).

4.2.3 *Spleen Conclusions*

As was shown with the hypothalamus, the spleen experienced polarized expression changes between immune involved genes and metabolic genes. Out of the 20 most upregulated genes, more cytokine genes were present as DEGs in the spleen compared to the hypothalamus. The spleen shared the upregulation in the cytokine suppressor, SOCS3, with the hypothalamus. The expression level of SOCS3 in the spleen experienced a higher fold change, compared to what was found in the hypothalamus (4.421, Table 5; 3.462, Table 1, respectively). RBP4, the negative acute phase reactant, was a downregulated gene shared with the hypothalamus, in the metabolic functional category. From specific functions of genes in the metabolic category, there is a reduction in catabolic processes of fatty acids, amino acids, and pyrimidines. The most differentially expressed gene in either direction was also found within the spleen: SPINK4 (14.710-fold change; Table 5) and LRAT (-9.510-fold change; Table 6). The downregulation of LRAT, which normally inhibits retinoic acid biosynthesis, along with the upregulation of ALDH1A3, further suggests the importance of retinoic acid to the immune system. The known functions of the spleen, compared to the hypothalamus, and the absence of behavioral-based genes is expected.

4.3 **Red Blood Cells**

In contrast to mammalian RBCs, avian, reptilian, and amphibian RBCs contain a nucleus (Zhang et al., 2011). The best reported defense that anucleated RBCs have against pathogens is the release of reactive oxygen species from hemoglobin when the cell is lysed, breaking down lipids, proteins, and DNA of nearby pathogens (Jiang et al., 2007). Little attention has been given to the function of nucleated RBCs. It has been assumed these nucleated RBCs merely participate in gas exchange, with little or no protein synthesis, like mammalian anucleated RBCs (Heegaard and Brown, 2002; Saladin, 2010c). Studies using avian whole blood show an immune response (Videvall et al., 2015; Watson et al., 2017), but the results cannot be parsed out between RBC and leukocyte activity.

More recently, a few studies have started to examine the participation of nucleated RBCs in immune function. There is now evidence of active transcriptional machinery within these cells that do react to various stimuli. A differential response of genes involved in the immune system and metabolic processes was shown in trout and chicken cultured RBCs (Morera et al., 2011). To my knowledge this is the first study to use RNA-seq for quantitative analysis of nucleated RBCs to immune challenge in a model for wild avian species.

4.3.1 *Upregulated Gene Functions*

The reaction of the nucleated RBCs in the zebra finch to LPS involve genes associated with the immune response. Genes that regulate inflammation, cytokine production, and the stress response are upregulated within top DEGs (Table 10). One of these, the chemokine CCL5, is expressed early in an immune response, activated through

signaling by the presence of pro-inflammatory cytokines and by contact with pathogens (Cartier et al., 2005; Marques et al., 2013). Elevated expression of CCL5 has been noted in hantavirus, reovirus, adenovirus, and influenza virus (H5N1) infections, but severe acute respiratory syndrome (SARS) coronavirus is an especially strong inducer in human lung tissue (Marques et al., 2013).

Three upregulated genes are involved in a cascade of signaling; MAP3K8, TLR3, and IFIH1. MAP3K8, activated by IL-1β, is critically involved in TLR signaling (Miekle et al., 2009). TLR3 propagates signals after being induced by interferons and cytokines, and is involved in RIG-I and MDA5 pathways (Chen et al., 2013). Upregulation of TLR and IFIH1 (MDA5) is documented in chickens infected with H5N1, low pathogenic avian influenza (LPAIV) H7N1, and avian Tembusu virus (ATMUV) that cause great economic loss and present a danger to humans (Chen et al., 2016). Once activated, the gene encoding MDA5 initiates further signal transduction for cytokine secretion. MDA5 is the primary influenza A virus sensor for chickens (Chen et al., 2013). The upregulation of TLR3 is one gene that can be used to assess RIG-I or MDA5 expression, depending on the avian species.

Other upregulated genes included in the immune response and cytokine production are LRRK1 and SAAL1, also known as synoviocyte proliferation-associated in collagen-induced arthritis 1 (SPACIA1). LRRK1 and its paralog LRRK2 are thought to have similar functions due to redundant expression profiles (Biskup et al., 2007). Both are part of the receptor-interacting protein kinase family (RIPK), that mediate signaling in the inflammatory response (Dzamko and Halliday, 2012). However, LRRK1 has its own unique functions apart from LRRK2. LRRK1 has roles in intracellular trafficking of

epidermal growth factors, osteoclast differentiation, and regulation of autophagy, which are not known as functions of LRRK2 (Morimoto et al., 2016). SAAL1 encodes an acute phase protein and is another gene induced by IL-1β, as well as other cytokines and immune stimulants (Zhang et al., 2008; Revathy et al., 2012).

4.3.2 *Downregulated Gene Functions*

Few functional categories were produced by GO analysis of the downregulated genes of the RBCs, and few genes represented these functions. Out of 61 downregulated genes, the functional category of regulation of cellular component organization only contained 12 genes from the DEG list. This was the most overrepresented category, of eight, and is a departure from the other analyses of the 30 most overrepresented categories. Nonetheless, this does not discount the importance of the functions of genes that were not produced into categories from GO analysis of these downregulated genes. Two genes were involved in the negative regulation of IL-8. IL-8 has chemotactic activities for basophils, as well as stimulating phospholipase enzymes that interact with neutrophils (L'Heureux et al., 1995; Mukaida et al., 1998). Both downregulated genes in this category have anti-inflammatory activity. ANXA1 inhibits phospholipase, interfering with IL-8 downstream mediation of inflammation (Flower, 1988). ARRB1 inhibits upstream processes in TLR signaling that lead to IL-8 expression (Drewniak et al., 2010; Anjum et al., 2013).

Although not included in a functional GO category, the LPS signaling gene, LY86, was downregulated. LY86 encodes a helper molecule to the toll-like receptor homolog toll-like receptor 4 (TLR4), CD180, found on cell surfaces (Gorczynski et al., 2000; Nagai et al., 2002). This CD180/LY86 complex inhibits the binding of LPS to toll-

like receptor 4 (TLR4; Gorczynski et al., 2006). The downregulation of LY86 would allow for early signaling by TLR4.

4.3.3 *Red Blood Cell Conclusions*

These novel findings show there are transcriptome adjustments during the APR of the immune response. In fact, the six most upregulated DEGs are involved in the immune system and/or cytokine production. The upregulation of CCL5 in this these cells may be advantageous because of the gene product's role in leukocyte recruitment. Despite there being few downregulated genes represented in the GO functional categories, ANXA1 and ARRB1 are involved in the negative regulation of IL-8. With dampened expression of ANXA1 and ARRB1, IL-8 involved signaling is not inhibited. Additionally, the downregulation of LY86 reduces inhibition of TLR's binding to LPS. More can be elucidated about the complete transcriptome changes from immune challenge by examining the other 115 upregulated and 58 downregulated genes.

4.4 **Shared Genes**

Of the five genes shared between all three tissues, only two contributed to the functions produced by GO analysis (Table 13; Table 14). These are the CCL5 and RSAD genes. While not within the top most upregulated in all tissues, they were both statistically significantly expressed. The first of these genes, CCL5, as discussed, is a chemokine that regulates leukocyte trafficking (Marques et al., 2013). It has also been highly conserved during evolution, with sequence homology in mammals, fish, and birds (Cartier et al., 2005). The second, RSAD2, regulates interferon and cytokine signaling pathways (Schoggins, 2014; Ranaware et al., 2016). The exclusion of the other genes was

based on their associated GO terms. In the case of ZNFX1, the only GO term associated with it is ‘poly(A) RNA binding,’ which is considered under GO molecular function. This study concentrated on the biological processes affected by DEGs.

The single downregulated gene that was shared, GSTA2, has the biological process GO term: ‘metabolic process.’ The encoded enzyme is reported to be involved in cellular detoxification and excretion of several xenobiotic substances, with strong implications for ingestion of mycotoxin aflatoxin B1 in turkeys (Kim et al., 2010). Turkeys are especially susceptible to the toxin because GSTA2 and the enzyme product are unresponsive to aflatoxin B1. In human hepatocytes, Klein *et al.* (2014) found GSTA2 to be downregulated by treatment of interleukin 6 (IL-6). IL-6 was significantly upregulated in the spleen compared to controls (1.834-fold change, *q*-value 0.030). This is further explained by GSTA2’s anti-inflammatory properties and activation by glucocorticoids (Ki et al., 2005). Interestingly, GSTA2 is associated with plumage dichromatism; GSTA2 and APOD are involved with carotenoid uptake, binding, and deposition in pheasants (Guang-Qi et al., 2016). In this study, APOD was downregulated in the hypothalamus (-3.050-fold change, *q*-value 0.002). The role of GSTA2 in carotenoid processes suggests a downregulation much like with RBP4 and LRAT, by reducing interference in the use of retinoic acid.

4.5 **Ecological Impact**

Given these results we have a better understanding of the global transcriptomic changes in the hypothalamus, spleen, and nucleated RBCs of zebra finch following activation of the APR by the bacterial component LPS. Because the zebra finch is a model for free-living avian species, the highly DEGs can be used to screen wild birds for

the same altered genomic profiles. This can be invaluable in monitoring the spread of particularly devastating diseases. In the past decade over 800 cases of the highly pathogenic H5N1 influenza virus have been reported, with almost half of those cases being fatal (www.who.int/csr/disease/avian_influenza/en/). H5N1 is spread easily from migratory or trafficked birds to domestics and humans (Kilpatrick et al., 2006). It should also be noted that diseases with high mortality, like H5N1, also impact conservation efforts due to the easy transmission of wild to captive birds (Roberton et al., 2006).

Likewise, this study provides insight into physiological changes during the APR, with many genes involved in the immune system being upregulated and genes in metabolic pathways being downregulated. Because the environment of free-living organisms is widely variable in resources and conditions, it is important to understand these potential trade-offs and implications on seasonally important functions, such as migration, reproduction, and growth. For example, parental care has been shown to decrease in house sparrows during illness (Bonneaud et al., 2003), as well as LPS injection reducing territorial displays in white-crowned sparrows (Owen-Ashley et al., 2006).

4.6 **Future Work**

4.6.1 *White Blood Cells*

Comparing transcriptome changes of RBCs and WBCs will give further insight into the APR of zebra finch. However, complications with obtaining an appropriate amount of RNA for sequencing prevented that analysis in this study. Although RNA-seq requires much less RNA than other methods (Wang et al., 2009), the concentration of

WBCs in whole blood is low. Combined with the size of the zebra finch, other methods or techniques will be needed to accomplish this in the future.

4.6.2 *Biomarker Creation*

The creation of biomarkers from these data can be used in proposed screenings of avian spread illness. We provide here several significantly expressed genes during the APR of the innate immune system in different tissues. It is possible that blood samples can be used for routine screenings without destruction of the organism.

4.6.3 *RT-PCR Validation*

Further validation of these results can be done through RT-PCR. This would be best accomplished with multiple gene primers based on the sheer number of DEGs in our data. Custom primer design is recommended.

4.7 **General Conclusions**

In conclusion, this study provides further insight into the early molecular adjustments during immune system activation, using RNA-seq on a non-model organism, the zebra finch. The APR is an evolutionarily conserved rapid response to eliminate and control infection, and within two hours of LPS administration we show an upregulation in immune genes in all three tissues of interest, and a downregulation in genes involved in metabolic pathways in the hypothalamus and spleen. The upregulation in immune related genes in the hypothalamus, spleen, and red blood cells supports part of the hypothesized transcriptomic response to immune challenge in zebra finch. However, the current results can only suggest a trade-off in immune function and other important aspects of life.

Further research is needed to examine this potential trade-off using experimental methods.

By using LPS, a component of gram-negative bacteria, pathogen manipulation is avoided. Tissue specificity is also shown by the DEGs not shared between and among tissues (Figure 1A, B). Of the total DEGs, only five upregulated and one downregulated gene were shared. We additionally present novel insight into transcriptional changes in nucleated RBCs of a non-model avian organism. These findings on the immunological response of avian RBCs will lay a framework for further investigations into the function of nucleated RBCs, a topic that to date has been poorly studied.

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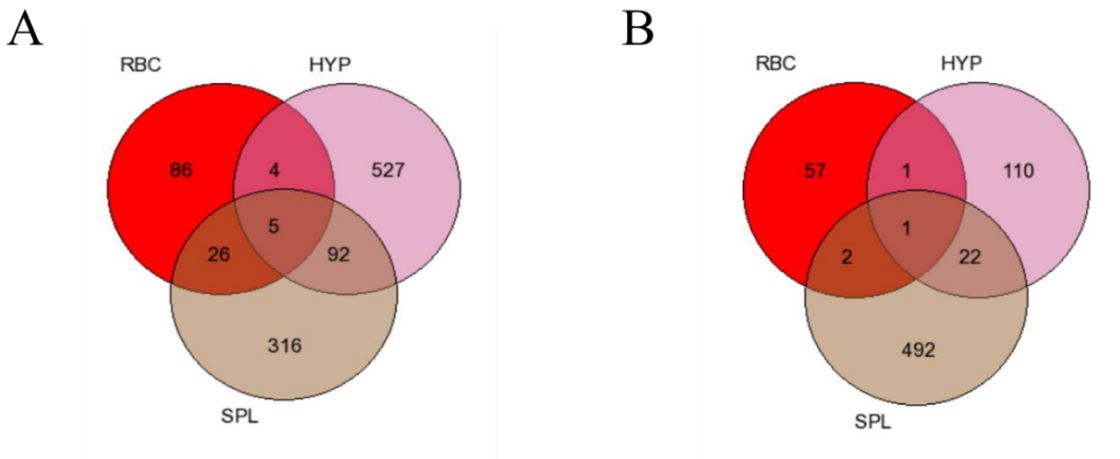

Figure 1. Venn diagram of number of differentially expressed genes (A) upregulated and (B) downregulated in hypothalami (HYP), spleen (SPL), and red blood cells (RBC) after lipopolysaccharide challenge. Overlapping sets show differential expression in comparison of two or three tissues.

Table 1. List of the top 20 upregulated transcripts in the hypothalamus following lipopolysaccharide challenge. Column headers show the Ensembl transcript ID tag, gene symbol, full gene name as description, the log base 2 transformed fold change (FC) for the hypothalamus (HYP) compared to controls, and the corresponding *q*-value of the gene.

Ensemble Transcript ID	Gene Symbol	Gene Description	HYP $\log_2$(FC)	*q*-value
ENSTGUT00000008217	DPEP1	dipeptidase 1	8.79228	0.0016993
ENSTGUT00000014026	COA4	cytochrome C oxidase assembly factor 4	8.79228	0.0042524
ENSTGUT00000008416	BCL2A1	BCL2-related protein A1	5.17096	0.0442345
ENSTGUT00000004130	CPLX3	complexin 3	4.34958	0.0016993
ENSTGUT00000013531	RSAD2	radical S-adenosyl methionine domain containing 2	4.04599	0.0106243
ENSTGUT00000004220	TNFRSF4	TNF receptor superfamily member 4	3.54403	0.0385857
ENSTGUT00000003480	SOCS3	suppressor of cytokine signaling 3	3.4621	0.0016993
ENSTGUT00000012111	CTGF	connective tissue growth factor	3.27628	0.0016993
ENSTGUT00000007545	GPR75	G protein-coupled receptor 75	3.0548	0.0016993
ENSTGUT00000001431	IRF1	interferon regulatory factor 1	2.9748	0.0016993
ENSTGUT00000000121	RNM7	RNA binding motif protein 7	2.93391	0.0072173
ENSTGUT00000004906	EIF4EBP1	eukaryotic translation initiation factor 4E binding protein 1	2.92841	0.0016993
ENSTGUT00000012331	COCH	coagulation factor C homolog, cochlin (*Limulus polyphemus*)	2.90484	0.0016993
ENSTGUT00000010886	VHL	von Hippel-Lindau tumor suppressor, E3 ubiquitin protein ligase	2.89828	0.0016993
ENSTGUT00000009878	PARS2	prolyl-tRNA synthetase 2	2.88749	0.0016993
ENSTGUT00000012522	SSTR1	somatostatin receptor 1	2.87423	0.0016993
ENSTGUT00000004620	SH2B2	SH2B adaptor protein 2	2.86192	0.0016993
ENSTGUT00000004296	STAMBP	STAM binding protein	2.84901	0.0031694
ENSTGUT00000002291	FBXL20	F-box and leucine rich repeat protein 20	2.82315	0.0016993
ENSTGUT00000006806	ZNF503	zinc finger protein 503	2.81819	0.0106243

Table 2. List of 30 most significantly overrepresented Gene Ontology (GO) functional categories enriched ($q \leq 0.05$) by the upregulated genes of the hypothalamus following lipopolysaccharide challenge. Columns show GO term descriptors, enrichment *q*-values, number of genes in our differentially regulated gene list that contribute to overrepresented GO categories, and total number of genes in the zebra finch genome annotated with GO terms from the category.

GO Functional Category	*q*-value	Diff. genes	Total genes in category
Response to stress	5.04E-06	92	1392
Macromolecule localization	5.04E-06	83	1195
Cellular localization	5.04E-06	80	1118
Protein localization	5.04E-06	74	1043
Cellular protein localization	5.04E-06	59	753
Intracellular transport	5.04E-06	54	653
Protein catabolic process	5.04E-06	40	410
Cellular macromolecule localization	5.34E-06	59	757
Organic substance transport	1.70E-05	71	1022
Macromolecule catabolic process	1.70E-05	44	509
Intracellular protein transport	4.57E-05	40	458
Protein exit from endoplasmic reticulum	4.57E-05	8	20
Organelle organization	4.94E-05	99	1659
Cellular response to stress	5.08E-05	59	824
Catabolic process	5.08E-05	58	806
Establishment of protein localization	5.08E-05	57	787
Organic substance catabolic process	5.08E-05	57	788
Protein transport	5.08E-05	52	689
Proteolysis involved in cellular protein catabolic process	5.08E-05	31	315
Cellular protein catabolic process	6.99E-05	32	337
Retrograde protein transport, ER to cytosol	0.000115907	7	17
Endoplasmic reticulum to cytosol transport	0.000115907	7	17
Mitochondrion organization	0.000116611	29	297
Cellular macromolecule catabolic process	0.000121994	36	418
Negative regulation of response to stimulus	0.000128254	48	642
Response to endoplasmic reticulum stress	0.000138692	16	108
Establishment of localization in cell	0.000148164	58	845
Cellular response to chemical stimulus	0.000344313	67	1057
Regulation of localization	0.000392033	69	1105
Proteasomal protein catabolic process	0.000392033	19	160

Table 3. List of the top 20 downregulated transcripts in the hypothalamus following lipopolysaccharide challenge. Column headers show the Ensembl transcript ID tag, gene symbol, full gene name as description, the log base 2 transformed fold change (FC) for the hypothalamus (HYP) compared to controls, and the corresponding *q*-value of the gene.

Ensemble Transcript ID	Gene Symbol	Gene Description	HYP $\log_2$(FC)	*q*-value
ENSTGUT00000005337	SH3BGR	SH3 domain binding glutamate rich protein	-7.94579	0.0016993
ENSTGUT00000019084	NDUFB3	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3, 12kDa	-7.94579	0.0016993
ENSTGUT00000008854	CALCA	calcitonin related polypeptide alpha	-6.46883	0.0016993
ENSTGUT00000002980	NPVF	neuropeptide VF precursor	-5.76127	0.0090084
ENSTGUT00000009132	RBP4	retinol binding protein 4, plasma	-4.56235	0.0098398
ENSTGUT00000008312	TMEM27	transmembrane protein 27	-4.22706	0.0143428
ENSTGUT00000005495	FGG	fibrinogen gamma chain	-3.77963	0.0031694
ENSTGUT00000005528	CEL	carboxyl ester lipase (bile salt-stimulated lipase)	-3.69424	0.017685
ENSTGUT00000011763	PMCH	pro-melanin-concentrating hormone	-3.63824	0.0272501
ENSTGUT00000012782	SIM1	single-minded homolog 1 (Drosophila)	-3.48072	0.0063246
ENSTGUT00000011180	TTR	transthyretin-like	-3.46811	0.0090084
ENSTGUT00000006350	HPD	4-hydroxyphenylpyruvate dioxygenase	-3.37179	0.0016993
ENSTGUT00000009218	TPH1	tryptophan hydroxylase 1	-3.36684	0.0016993
ENSTGUT00000011189	ASMT	acetylserotonin O-methyltransferase	-3.33173	0.0016993
ENSTGUT00000012324	NECAB1	N-terminal EF-hand calcium binding protein 1	-3.32378	0.0143428
ENSTGUT00000008609	PCK1	phosphoenolpyruvate carboxykinase 1 (soluble)	-3.28267	0.0063246
ENSTGUT00000009362	APOD	apolipoprotein D	-3.0502	0.0016993
ENSTGUT00000013835	AvBD9	avian beta-defensin 9	-2.97032	0.0016993
ENSTGUT00000009709	TYH	tyrosine hydroxylase	-2.92504	0.0016993
ENSTGUT00000011992	C10orf90	chromosome 10 open reading frame 90	-2.86064	0.0016993

Table 4. List of 30 most significantly overrepresented Gene Ontology (GO) functional categories enriched ($q \leq 0.05$) by the downregulated genes of the hypothalamus following lipopolysaccharide challenge. Columns show GO term descriptors, enrichment *q*-values, number of genes in our differentially regulated gene list that contribute to overrepresented GO categories, and total number of genes in the zebra finch genome annotated with GO terms from the category.

GO Functional Category	*q*-value	Diff. genes	Total genes in category
Small molecule metabolic process	5.26E-06	25	880
Regulation of hormone levels	5.26E-06	13	211
Feeding behavior	0.00012138	6	38
Response to chemical	0.00014978	32	1653
Cell-cell signaling	0.00017307	17	554
Hormone transport	0.00017307	9	137
Hormone metabolic process	0.000468136	7	84
Small molecule biosynthetic process	0.000690636	10	215
Hormone secretion	0.00070793	8	130
Regulation of biological quality	0.00086916	28	1587
Organonitrogen compound metabolic process	0.00086916	21	994
Single-organism biosynthetic process	0.00086916	16	595
Response to oxygen-containing compound	0.00086916	14	482
Behavior	0.00086916	11	290
Nitrogen compound transport	0.00086916	11	287
Signal release	0.00086916	9	184
Organic hydroxy compound metabolic process	0.00086916	8	147
Glial cell differentiation	0.00086916	7	100
Cellular hormone metabolic process	0.00086916	5	44
Positive regulation of nucleotide metabolic process	0.00086916	5	42
Positive regulation of hormone secretion	0.00086916	5	43
Oligodendrocyte differentiation	0.00086916	5	42
Positive regulation of purine nucleotide metabolic process	0.00086916	5	42
Regulation of purine nucleotide metabolic process	0.001098616	6	78
Regulation of nucleotide metabolic process	0.001215574	6	81
Positive regulation of cAMP metabolic process	0.001215574	4	25
Positive regulation of cAMP biosynthetic process	0.001215574	4	25
Gliogenesis	0.001290233	7	121
Response to nitrogen compound	0.001494132	10	273
Gluconeogenesis	0.001739481	4	28

Table 5. List of the top 20 upregulated transcripts in the spleen following lipopolysaccharide challenge. Column headers show the Ensembl transcript ID tag, gene symbol, full gene name as description, the log base 2 transformed fold change (FC) for the spleen (SPL) compared to controls, and the corresponding *q*-value of the gene.

Ensemble Transcript ID	Gene Symbol	Gene Description	SPL $\log_2$(FC)	*q*-value
ENSTGUT00000001524	SPINK4	serine peptidase inhibitor, kazal type 4	14.7096	0.00109134
ENSTGUT00000002493	CELA2A	chymotrypsin-like elastase family member 2A-like	12.997	0.00109134
ENSTGUT00000005528	CEL	carboxyl ester lipase (bile salt-stimulated lipase)	11.5494	0.00109134
ENSTGUT00000005040	AMY1A	pancreatic alpha-amylase-like	9.18748	0.00109134
ENSTGUT00000013728	TRY1	trypsin I-P1-like	9.016	0.00109134
ENSTGUT00000003515	IL-1β	interleukin-1 beta-like	7.18436	0.00109134
ENSTGUT00000001605	IL-8	interleukin-8-like	6.99345	0.00109134
ENSTGUT00000003333	CCL4	C-C motif chemokine 4 homolog	6.89018	0.00109134
ENSTGUT00000012249	C6orf58	chromosome 3 open reading frame, human C6orf58	6.464	0.00109134
ENSTGUT00000008415	TMED6	transmembrane emp24 protein transport domain containing 6	6.20836	0.0107874
ENSTGUT00000019201	MMP7	matrix metallopeptidase 7	5.87498	0.00109134
ENSTGUT00000007368	TNFRSF6B	TNF receptor superfamily member 6B	5.86711	0.00109134
ENSTGUT00000006824	OASL	2'-5'-oligoadenylate synthase-like protein 1-like	4.97508	0.00109134
ENSTGUT00000005928	ABTB2	ankyrin repeat and BTB (POZ) domain containing 2	4.69453	0.00109134
ENSTGUT00000001478	STEAP4	STEAP family member 4	4.52199	0.00109134
ENSTGUT00000003480	SOCS3	suppressor of cytokine signaling 3	4.42141	0.00109134
ENSTGUT00000002624	DHX58	DEXH (Asp-Glu-X-His) box polypeptide 58	4.41334	0.0464056
ENSTGUT00000003175	ATF3	activating transcription factor 3	4.28426	0.00109134
ENSTGUT00000009265	ALDH1A3	aldehyde dehydrogenase 1 family, member A3	4.28246	0.00109134
ENSTGUT00000008692	IFIT5	interferon-induced protein with tetratricopeptide repeats 5	4.21115	0.00109134

Table 6. List of the top 20 downregulated transcripts in the spleen following lipopolysaccharide challenge. Column headers show the Ensembl transcript ID tag, gene symbol, full gene name as description, the log base 2 transformed fold change (FC) for the spleen (SPL) compared to controls, and the corresponding *q*-value of the gene.

Ensemble Transcript ID	Gene Symbol	Gene Description	SPL $\log_2$(FC)	*q*-value
ENSTGUT00000005505	LRAT	lecithin retinol acyltransferase	-9.51031	0.00109134
ENSTGUT00000009017	SLC35G1	solute carrier family 35 member G1	-9.51031	0.00697467
ENSTGUT00000007476	ZP4	zona pellucida glycoprotein 4	-9.51031	0.0413816
ENSTGUT00000002938	G6PC	glucose-6-phosphatase, catalytic subunit	-8.51031	0.00109134
ENSTGUT00000003904	ABCG8	ATP binding cassette subfamily G member 8	-5.80867	0.00283554
ENSTGUT00000013902	HAO2	hydroxyacid oxidase 2 (long chain)	-5.72502	0.00283554
ENSTGUT00000010888	HRG	histidine rich glycoprotein	-5.59108	0.00109134
ENSTGUT00000008728	TAT	tyrosine aminotransferase	-5.58552	0.00109134
ENSTGUT00000012685	DPYS	dihydropyrimidinase	-5.57705	0.00109134
ENSTGUT00000011180	TTL	transthyretin-like	-5.54955	0.0449671
ENSTGUT00000006350	HPD	4-hydroxyphenylpyruvate dioxygenase	-5.54451	0.00109134
ENSTGUT00000008658	FAAH	fatty-acid amide hydrolase 1-like	-5.4819	0.00109134
ENSTGUT00000004246	SERPINC1	serpin family C member 1	-5.45677	0.00109134
ENSTGUT00000004418	APOH	apolipoprotein H (beta-2-glycoprotein I)	-5.44738	0.0170742
ENSTGUT00000011686	KMO	kynurenine 3-monooxygenase (kynurenine 3-hydroxylase)	-5.42767	0.00109134
ENSTGUT00000010889	KNG1	kininogen 1	-5.40218	0.00109134
ENSTGUT00000011237	ACSL5	acyl-CoA synthetase long-chain family member 5	-5.37228	0.00109134
ENSTGUT00000002586	AMBP	protein AMBP-like	-5.37017	0.00109134
ENSTGUT00000007485	LGR5	leucine rich repeat containing G protein-coupled receptor 5	-5.35556	0.0457017
ENSTGUT00000009132	RBP4	retinol binding protein 4, plasma	-5.30538	0.0304134

Table 7. List of 30 most significantly overrepresented Gene Ontology (GO) functional categories enriched ($q \leq 0.05$) by the upregulated genes of the spleen following lipopolysaccharide challenge. Columns show GO term descriptors, enrichment *q*-values, number of genes in our differentially regulated gene list that contribute to overrepresented GO categories, and total number of genes in the zebra finch genome annotated with GO terms from the category.

GO Functional Category	*q*-value	Diff. genes	Total genes in category
Immune system process	2.93E-14	71	916
Response to external biotic stimulus	3.40E-13	35	260
Response to other organism	3.40E-13	35	260
Response to biotic stimulus	1.48E-12	35	275
Response to external stimulus	1.63E-12	61	788
Immune response	5.87E-12	42	416
Response to stress	7.70E-12	84	1392
Defense response	7.70E-12	42	422
Regulation of immune system process	2.41E-10	43	490
Regulation of response to stimulus	1.04E-09	86	1585
Response to lipopolysaccharide	1.21E-09	17	77
Cell death	2.01E-09	55	801
Positive regulation of response to stimulus	2.01E-09	55	800
Response to molecule of bacterial origin	2.80E-09	17	82
Regulation of response to stress	4.22E-09	42	521
Negative regulation of cellular process	6.07E-09	97	1973
Regulation of cell death	9.53E-09	47	651
Positive regulation of immune system process	9.53E-09	30	294
Cytokine production	9.53E-09	27	239
Response to bacterium	1.32E-08	19	118
Cellular response to chemical stimulus	1.34E-08	63	1057
Cell activation	1.34E-08	32	338
Multi-organism process	1.44E-08	45	617
Programmed cell death	2.90E-08	50	750
Regulation of intracellular signal transduction	4.01E-08	50	758
Regulation of signaling	5.20E-08	74	1398
Regulation of cytokine production	5.55E-08	24	210
Regulation of programmed cell death	1.04E-07	43	613
Response to organic substance	1.26E-07	60	1045
Regulation of cell communication	1.50E-07	72	1380

Table 8. List of 30 most significantly overrepresented Gene Ontology (GO) functional categories enriched ($q \leq 0.05$) by the downregulated genes of the spleen following lipopolysaccharide challenge. Columns show GO term descriptors, enrichment *q*-values, number of genes in our differentially regulated gene list that contribute to overrepresented GO categories, and total number of genes in the zebra finch genome annotated with GO terms from the category.

GO Functional Category	*q*-value	Diff. genes	Total genes in category
Small molecule metabolic process	1.44E-42	118	880
Organic acid metabolic process	1.03E-31	72	423
Carboxylic acid metabolic process	1.03E-31	69	382
Oxoacid metabolic process	1.03E-31	69	384
Oxidation-reduction process	1.45E-24	78	639
Small molecule catabolic process	3.75E-24	35	111
Single-organism catabolic process	2.89E-22	54	335
Monocarboxylic acid metabolic process	4.87E-21	43	218
Organonitrogen compound catabolic process	2.24E-19	31	113
Alpha-amino acid metabolic process	7.56E-19	29	100
Organic acid catabolic process	1.11E-18	27	85
Single-organism biosynthetic process	1.65E-18	66	595
Small molecule biosynthetic process	6.53E-17	38	215
Organic acid biosynthetic process	1.44E-16	30	130
Alpha-amino acid catabolic process	8.19E-16	16	28
Lipid metabolic process	3.68E-15	60	585
Cellular lipid metabolic process	1.56E-14	49	417
Carboxylic acid biosynthetic process	1.56E-14	27	121
Organonitrogen compound metabolic process	6.13E-14	79	994
Fatty acid metabolic process	1.76E-13	27	133
Carboxylic acid catabolic process	3.26E-13	21	76
Cellular amino acid catabolic process	5.63E-13	15	33
Catabolic process	4.26E-12	66	806
Dicarboxylic acid metabolic process	1.06E-11	15	39
Cellular amino acid metabolic process	2.07E-11	25	137
Organic substance catabolic process	1.38E-10	62	788
Aromatic amino acid family metabolic process	5.46E-10	11	22
Cellular catabolic process	7.92E-10	53	638
Alcohol metabolic process	1.40E-09	19	92
Lipid homeostasis	4.53E-09	13	40

Table 9. List of 30 most significantly overrepresented Gene Ontology (GO) functional categories enriched ($q \leq 0.05$) by the upregulated genes of the red blood cells following lipopolysaccharide challenge. Columns show GO term descriptors, enrichment *q*-values, number of genes in our differentially regulated gene list that contribute to overrepresented GO categories, and total number of genes in the zebra finch genome annotated with GO terms from the category.

GO Functional Category	*q*-value	Diff. genes	Total genes in category
Immune system process	1.43E-05	24	916
Immune system development	1.46E-05	16	423
Hemopoiesis	1.46E-05	15	372
Hematopoietic or lymphoid organ development	2.82E-05	15	400
Leukocyte differentiation	8.52E-05	11	223
Cytokine production	0.000140984	11	239
Immune response	0.000160706	14	416
Lymphocyte activation	0.000199514	11	255
Regulation of cytokine production	0.000223344	10	210
Cellular nitrogen compound catabolic process	0.000241921	8	126
Heterocycle catabolic process	0.000241921	8	126
Regulation of cytokine biosynthetic process	0.00024865	5	33
Leukocyte activation	0.000311795	11	287
Innate immune response	0.000311795	8	135
Organic cyclic compound catabolic process	0.000311795	8	136
Cytokine biosynthetic process	0.000311795	5	36
Cytokine metabolic process	0.000315313	5	37
Cellular response to interferon-gamma	0.000345965	4	18
Regulation of immune system process	0.000394806	14	490
Response to stress	0.00040824	25	1392
Response to interferon-gamma	0.000693871	4	22
Cellular response to mechanical stimulus	0.000771638	4	23
Interferon-alpha production	0.000771638	3	8
Cell differentiation	0.000889564	27	1669
Positive regulation of macromolecule metabolic process	0.000889564	23	1320
Cell activation	0.000889564	11	338
Myeloid cell differentiation	0.000889564	8	178
Cytokine-mediated signaling pathway	0.000889564	7	129
Aromatic compound catabolic process	0.000889564	7	126
Type I interferon production	0.000889564	4	27

Table 10. List of the top 20 upregulated transcripts in the red blood cells following lipopolysaccharide challenge. Column headers show the Ensembl transcript ID tag, gene symbol, full gene name as description, the log base 2 transformed fold change (FC) for the red blood cells (RBC) compared to controls, and the corresponding *q*-value of the gene.

Ensemble Transcript ID	Gene Symbol	Gene Description	RBC $\log_2$(FC)	*q*-value
ENSTGUT00000001478	STEAP4	STEAP family member 4	7.52763	0.0038457
ENSTGUT00000019281	SCARNA13	small cajal body-specific RNA 13	7.52763	0.0038457
ENSTGUT00000002574	CNP	2',3'-cyclic nucleotide 3' phosphodiesterase	6.07946	0.0038457
ENSTGUT00000009670	H2B-I	histone H2B 1/2/3/4/6-like	5.37972	0.0239506
ENSTGUT00000009190	PLSCR1	phospholipid scramblase 2-like	5.32366	0.0038457
ENSTGUT00000010708	CD82	cluster of differentiation 82 molecule	5.30416	0.0239506
ENSTGUT00000009254	RAB3B	RAB3B, member RAS oncogene family	5.25133	0.0383759
ENSTGUT00000003334	CCL5	C-C motif chemokine ligand 5	5.03947	0.0168508
ENSTGUT00000011025	LGALS2	galectin 2	4.56388	0.0038457
ENSTGUT00000007158	TLR3	toll-like receptor 3	4.41933	0.0064357
ENSTGUT00000005910	TRANK1	tetratricopeptide repeat and ankyrin repeat containing 1	4.33291	0.0038457
ENSTGUT00000007732	DLG3	discs, large homolog 3 (Drosophila)	3.91696	0.0425722
ENSTGUT00000009301	LRRK1	leucine-rich repeat kinase 1	3.7414	0.0298556
ENSTGUT00000009227	SAAL1	serum amyloid A like 1	3.59486	0.0038457
ENSTGUT00000005906	MLH1	mutL homolog 1, colon cancer, nonpolyposis type 2 (E. coli)	3.55976	0.0038457
ENSTGUT00000001291	SLC46A2	solute carrier family 46, member 2	3.55046	0.0038457
ENSTGUT00000012962	BPGM	bisphosphoglycerate mutase-like	3.52653	0.0257833
ENSTGUT00000001098	MAP3K8	mitogen-activated protein kinase kinase kinase 8	3.4143	0.0038457
ENSTGUT00000001477	SRI	sorcin-like	3.378	0.0038457
ENSTGUT00000007216	IFIH1	interferon induced with helicase C domain 1	3.37215	0.0038457

Table 11. List of the top 20 upregulated transcripts in the red blood cells following lipopolysaccharide challenge. Column headers show the Ensembl transcript ID tag, gene symbol, full gene name as description, the log base 2 transformed fold change (FC) for the red blood cells (RBC) compared to controls, and the corresponding *q*-value of the gene.

Ensemble Transcript ID	Gene Symbol	Gene Description	RBC $\log_2$(FC)	*q*-value
ENSTGUT00000001730	PINLYP	phospholipase A2 inhibitor subunit gamma B-like	-8.9848	0.00384573
ENSTGUT00000009842	EMP1	epithelial membrane protein 1	-8.9848	0.00384573
ENSTGUT00000011087	AIFM3	apoptosis-inducing factor, mitochondrion-associated, 3	-8.9848	0.00384573
ENSTGUT00000012224	B3GLCT	beta 3-glucosyltransferase	-8.9848	0.00384573
ENSTGUT00000014134	STARD10	StAR related lipid transfer domain containing 10	-8.9848	0.00643571
ENSTGUT00000012200	ALOX5AP	arachidonate 5-lipoxygenase activating protein	-8.9848	0.0107812
ENSTGUT00000002394	LY86	lymphocyte antigen 86	-4.34823	0.00844688
ENSTGUT00000019084	NDUFB3	NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 3, 12kDa///NDUFB3	-3.52895	0.00384573
ENSTGUT00000013418	GSTA2	glutathione S-transferase class-alpha variant 2	-3.50615	0.0383759
ENSTGUT00000014129	WDR73	WD repeat-containing protein 73-like	-3.10134	0.00844688
ENSTGUT00000000826	ANXA1	annexin A1 isoform p37-like	-2.98367	0.00643571
ENSTGUT00000006738	SEMA3F	semaphorin 3F	-2.79417	0.033661
ENSTGUT00000014131	ARRB1	arrestin beta 1	-2.75381	0.00384573
ENSTGUT00000002148	INTU	inturned planar cell polarity effector homolog (Drosophila)	-2.55811	0.0286682
ENSTGUT00000000804	RPUSD4	RNA pseudoridylate synthase domain containing 4	-2.53762	0.0150167
ENSTGUT00000002031	LIMD2	LIM domain containing 2	-2.53527	0.0107812
ENSTGUT00000005362	MST1	macrophage stimulating 1	-2.34264	0.0311683
ENSTGUT00000010961	H1F0	H1 histone family member 0	-2.31436	0.0168508
ENSTGUT00000010500	MRPL43	mitochondrial ribosomal protein L43	-2.27312	0.00384573
ENSTGUT00000005610	ANXA2	annexin A2	-2.22297	0.00384573

Table 12. List of most significantly overrepresented Gene Ontology (GO) functional categories enriched ($q \leq 0.05$) by the downregulated genes of the red blood cells following lipopolysaccharide challenge. Columns show GO term descriptors, enrichment *q*-values, number of genes in our differentially regulated gene list that contribute to overrepresented GO categories, and total numbers of genes of zebra finch annotated with GO terms of category.

GO Functional Category	*q*-value	Diff. genes	Total genes in category
Regulation of cellular component organization	0.02926416	12	1098
Receptor metabolic process	0.02926416	4	92
Regeneration	0.02926416	3	42
Regulation of vesicle fusion	0.02926416	2	7
Positive regulation of vesicle fusion	0.02926416	2	5
Negative regulation of interleukin-8 production	0.02926416	2	6
Intermediate filament organization	0.02926416	2	9
Negative regulation of cellular component organization	0.036804127	6	300

Table 13. List of upregulated transcripts found in all three tissues, following lipopolysaccharide challenge. Column headers show the Ensembl transcript ID tag, gene symbol, full gene name as description, the log base 2 transformed fold change (FC) for hypothalami (HYP), spleen (SPL), and red blood cells (RBC) compared to controls, respectively, and the corresponding *q*-value of the gene for each tissue.

Ensembl Transcript ID	Gene Symbol	Gene Description	HYP $\log_2$(FC)	*q*-value	SPL $\log_2$(FC)	*q*-value	RBC $\log_2$(FC)	*q*-value
ENSTGUG00000003209	CCL5	C-C motif chemokine ligand 5	2.41279	0.0042524	1.38202	0.0311043	5.03947	0.0168508
ENSTGUG00000012992	RSAD2	radical S-adenosyl methionine domain containing 2	4.04599	0.0106243	3.85512	0.0010913	2.88651	0.0038457
ENSTGUG00000001423	STEAP4	STEAP family member 4	1.5343	0.0449547	4.52199	0.0010913	7.52763	0.0038457
ENSTGUG00000004451	TOR1B	torsin family 1 member B	1.38827	0.0098398	2.18621	0.0010913	2.77455	0.0038457
ENSTGUG00000005628	ZNFX1	zinc finger NFX1-type containing 1	1.86888	0.0063246	2.76895	0.0010913	1.78621	0.0226722

Table 14. List of 30 most significantly overrepresented Gene Ontology (GO) functional categories enriched ($q \leq 0.05$) by the differentially upregulated genes found in all three tissues following lipopolysaccharide challenge. Columns show GO term descriptors, enrichment *q*-values, number of genes in our differentially regulated gene list that contribute to overrepresented GO categories, and total numbers of genes of zebra finch annotated with GO terms of category.

GO Functional Category	*q*-value	Diff. genes	Total genes in category
Negative regulation of viral genome replication	0.000504245	2	12
Viral genome replication	0.001127605	2	39
Regulation of viral genome replication	0.001127605	2	32
Negative regulation of viral process	0.001127605	2	39
Negative regulation of viral life cycle	0.001127605	2	26
Negative regulation of multi-organism process	0.001878551	2	55
Regulation of viral life cycle	0.002321921	2	66
Viral life cycle	0.005437736	2	108
Protein secretion	0.006536614	2	188
Response to virus	0.006536614	2	136
T cell activation	0.006536614	2	174
Regulation of multi-organism process	0.006536614	2	188
Regulation of symbiosis, encompassing mutualism through parasitism	0.006536614	2	154
Innate immune response	0.006536614	2	135
Regulation of protein secretion	0.006536614	2	147
Regulation of viral process	0.006536614	2	145
Protein oligomerization	0.006536614	2	188
Leukocyte aggregation	0.006536614	2	178
T cell aggregation	0.006536614	2	174
Lymphocyte aggregation	0.006536614	2	175
Leukocyte cell-cell adhesion	0.006691672	2	195
Viral process	0.008540128	2	238
Response to external biotic stimulus	0.008540128	2	260
Symbiosis, encompassing mutualism through parasitism	0.008540128	2	252
Interspecies interaction between organisms	0.008540128	2	252
Multi-organism cellular process	0.008540128	2	240
Lymphocyte activation	0.008540128	2	255
Response to other organism	0.008540128	2	260
Regulation of secretion by cell	0.008540128	2	243
Regulation of secretion	0.008570074	2	265

Table 15. The downregulated transcript found in all three tissues, following lipopolysaccharide challenge. Column headers show the Ensembl transcript ID tag, gene symbol, full gene name as description, the log base 2 transformed fold change (FC) for hypothalami (HYP), spleen (SPL), and red blood cells (RBC) compared to controls, respectively, and the corresponding *q*-value of the gene for each tissue.

Ensembl Transcript ID	Gene Symbol	Gene Description	HYP $\log_2$(FC)	*q*-value	SPL $\log_2$(FC)	*q*-value	RBC $\log_2$(FC)	*q*-value
ENSTGUT00000013418	GSTA2	glutathione S-transferase class-alpha variant 2	-1.23069	0.0031694	-1.30786	0.0494673	-3.50615	0.0383759