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EDITED BY
Çağan H. Şekercioğlu,
The University of Utah, United States

REVIEWED BY
M. Çisel Kemahlı Aytekin,
Koç University, Türkiye
Morteza Naderi,
Koç University, Türkiye

*CORRESPONDENCE
Carlos W. Esperanza
cesperanza@sfsu.edu

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Comparative gene expression responses to *Babesia* infection and oil contamination in a seabird

Carlos W. Esperanza[1*], Rachel C. Quock[1,2], Rebecca S. Duerr[3], Scott W. Roy[1] and Ravinder N. M. Sehgal[1]

[1]Department of Biology, San Francisco State University, San Francisco, CA, United States, [2]Department of Herpetology, California Academy of Science, San Francisco, CA, United States, [3]International Bird Rescue, Fairfield, CA, United States

The common murre (*Uria aalge*) is a species of seabird particularly vulnerable to several environmental stressors, including parasitic infection and oil contamination. However, the molecular response to these stressors is severely understudied. This study investigated the common murre's transcriptomic responses to these stressors. Blood samples were collected from common murres undergoing rehabilitation at International Bird Rescue in Fairfield, CA. Total RNA was extracted from these samples, followed by library preparation and Illumina sequencing to generate whole transcriptome data. Differential gene expression analysis was conducted using DeSeq2 to identify genes significantly altered in response to oil contamination and parasitic infection. Differential gene expression analysis revealed 194 genes shared between oil-contaminated and infected birds, including key immune-related genes, such as *ANXA2*, *LY96*, and *LY86*. These genes play vital roles in mediating the production of pro-inflammatory cytokines. Gene Set Enrichment Analysis indicated significant alterations in stress, immune, and inflammatory responses, with additional lipid metabolism changes in contaminated birds. Our findings highlight the detrimental effects that these stressors have on wild birds. These findings suggest a generalized stress response and specific metabolic adaptations to oil exposure, providing insights for seabird conservation.

KEYWORDS

seabird, RNA sequencing, differential expression analysis, oil contamination, *Babesia*, gene expression, environmental stressors

1 Introduction

Seabirds are highly vulnerable to many major environmental stressors such as pollution, oil spills, habitat loss, and harmful algal blooms (Phillips et al., 2011; Orgeret et al., 2022). They are also increasingly susceptible to disease from parasitic infections, with host–parasite interactions expected to significantly shift as a direct result of climate change

(Khan et al., 2019). A seabird's chance of exposure to these conditions may be increased when compared to other taxa because they have long lifespans and can traverse various continents throughout a single annual cycle (Boulinier et al., 2016). Behaviors such as migration, foraging, and dispersal have been shown to be important factors in the spread of infectious diseases and parasite transmission (Boulinier et al., 2016). The common murre (*Uria aalge*) is a representative of the seabirds in their susceptibility to the effects of climate change and other stressors (Phillips et al., 2011; Piatt et al., 2020). At wildlife rehabilitation centers, common murres are regularly admitted exhibiting symptoms and injuries related to oil contamination, anemia, disease, and starvation (Hampton et al., 2003; Henkel et al., 2014; Duerr et al., 2016). These environmental stressors can have significant health consequences for seabirds, including increased susceptibility to diseases like babesiosis.

Babesia is a protozoan parasite that is transmitted by ticks of the genus *Ixodes*, and infects the erythrocytes of mammalian, reptile, and avian hosts, resulting in the disease babesiosis (Puri et al., 2021; Zaki et al., 2021). Infection can lead to malaria-like symptoms such as fever, anemia, jaundice, and death in severe cases (Ristic and Kreier, 1984; Peirce et al., 2003; Vannier et al., 2015). Historically, babesiosis has had devastating impacts on the cattle industry and domestic animals, and a majority of *Babesia* research has focused on humans, mice, cattle, and dogs (Igarashi et al., 1999; Brown et al., 2006; Miles et al., 2012; Iwasaki and Medzhitov, 2015; Torina et al., 2020; Kumar et al., 2023), leaving a large gap in knowledge on avian species' response to *Babesia* or apicomplexan parasites in general. The few investigations into the host's response to infections have presented evidence of the innate and adaptive immune pathways playing a major role, with differential expression of genes relating to IL-1β, TNF-α, and IFN-γ (Brown et al., 2006; Beletic et al., 2021; Smith et al., 2021). The species *Babesia uriae* was first identified in 2009 in common murres undergoing rehabilitation and has been identified by light microscopy of blood smears in approximately 30% of murres admitted to International Bird Rescue's California Wildlife hospital annually since 2016 (International Bird Rescue, unpublished; Yabsley et al., 2009). This parasite is closely related to *B. poelea* which infects brown boobies (*Sula leucogaster*) (Yabsley et al., 2009; Quillfeldt et al., 2011). Currently, there are 15 known species of avian *Babesia* which infect seabirds, and *B. uriae* is the first to be identified in a seabird in the Alcidae family (Peirce and Feare, 1978; Earle et al., 1993; Work and Rameyer, 1997; Merino, 1998; Peirce et al., 2003; Criado et al., 2006; Yabsley et al., 2006, 2009; Jefferies et al., 2008). Like other haemosporidian parasites in birds, acute infection is short and, if the individual survives this stage, results in chronic infection with lower parasitemia, regulated by the host's immune system (Valkiūnas, 2005). Understanding how common murres respond to *Babesia* infection is crucial, as this parasite is becoming increasingly prevalent in seabird populations. Existing research on avian babesiosis has primarily focused on other species, but studies like Videvall et al. (2015) on *Plasmodium* infection in siskins provide valuable insights into potential immune system responses.

Plasmodium species and *Babesia* are similar in their evolutionary history, infection dynamics, and disease outcomes (Clark and Jacobson, 1998; Djokic et al., 2021; Inoue et al., 2013), so a similar response to both parasites in birds would be expected. Videvall et al. (2015) investigated the avian transcriptome changes to *P. ashfordi* infection in Eurasian siskins (*Spinus spinus*) at different stages of parasitemia and found that differential gene expression correlated with infection time. Moreover, gene set enrichment analysis (GSEA) was performed and found that gene ontology (GO) terms such as "immune system process," "response to stress," and "cell death regulation" were significantly overrepresented during peak infection (Videvall et al., 2015). Studies on the immune response to *Babesia* in mice have found further evidence of the innate immune response to mediate disease pathogenesis (Hemmer et al., 2000). Coupled with additional stressors that can activate an immune response, disease and symptoms can be dramatically exacerbated (Clark and Jacobson, 1998; Ye et al., 2020). In a recent study on Hawaiian honeycreepers, Hawai'i 'Amakihi (*Chlorodrepanis virens*) were inoculated with *P. relictum*, the species of *Plasmodium* that led to the extinction of several species of honeycreepers (Paxton et al., 2023). This study focused on the host's immune response to avian malaria and found that differences in adaptive and innate immune responses led to differences in host outcome to infection (Paxton et al., 2023). Similar to *Babesia* infection, oil contamination also poses a significant health threat to common murres.

In addition to pathogens, common murres also face the effects of oil spills, either from anthropogenic sources or from natural seepages (Hampton et al., 2003; Henkel et al., 2014). Seabirds exposed to petroleum oil risk feather contamination and ingestion, leading to severe health problems with direct impacts to the animal's kidneys, liver, leukocyte populations, and blood cell physiology (Leighton et al., 1983; Fry and Lowenstine, 1985; Briggs et al., 1996). Feather contamination can cause a disruption of the bird's outer plumage, which is crucial to their survival in the cold marine environment and leaves the individual at risk of hypothermia and death (Jenssen, 1994). Previous clinical studies in common murres have shown that ingestion of oil led to consistent decreases in body weight despite normal food consumption, indicating metabolic and malabsorption issues (Khan and Ryan, 1991). Another study on the immunosuppressive effects of oil and duck plague herpesvirus found that mallard ducks dosed with cyclophosphamide (CY) exhibited an immune response like that of duck plague herpesvirus, and a combination of both CY ingestion and duck plague herpesvirus resulted in higher death tolls than those with either CY or herpesvirus (Goldberg et al., 1990). Investigations into the transcriptome changes in response to oil contamination have primarily been performed on marine microbial species, coral, mice, and plants (Rivers et al., 2013; Alvarez et al., 2018; Liu et al., 2020; DeLeo et al., 2021). However, one transcriptomic study on seaside sparrows (*Ammospiza maritima*) that were indirectly exposed to petroleum in the Deep-Water Horizon (DWH) oil spill found significant upregulation of genes relating to hepatocellular proliferation and liver regeneration, while genes relating to necrosis, liver steatosis, and apoptosis were inhibited (Bonisoli-Alquati et al., 2020).

These two stressors, oil contamination and disease, are anticipated to increase in prevalence and severity due to anthropogenic climate change. In the case of oil contamination, changing ocean temperatures can lead to the release of previously trapped oil reserves by way of melting permafrost. In the case of *Babesia*, rising temperatures can expand the geographic range and increase the abundance of the invertebrate vectors of protozoan parasites, including *Ixodes* ticks. Investigating how a marine organism responds to these stressors can provide valuable insight into the potential impacts of climate change on marine organisms and can lead to long-term studies that can help inform conservation efforts, as well as develop strategies to protect vulnerable populations. The goal of this study was to map the changes in gene expression in response to differing environmental conditions that common murres, as well as other marine organisms face in the wild. To accomplish this broader goal, we identified differentially expressed genes in common murres infected with *Babesia*, as well as those contaminated with petroleum oil. We then performed GSEA to explore which biological pathways were enriched by differentially expressed genes in each condition. We hypothesized that both *Babesia* infection and oil contamination would lead to distinct transcriptional responses in Common murres, with each condition inducing expression changes of genes associated with biological pathways relating to immune functions. This work represents the first step in understanding the commonalities, differences, and underlying biological processes that occur in response to parasitic infection and oil contamination in seabirds.

2 Materials and methods

2.1 Sample collection

Biological samples used in this study were selected from a larger set of 261 Common murre samples collected by the staff at International Bird Rescue (IBR) in Fairfield, CA between May 13, 2021, to August 30, 2022 (Supplementary Table S1). Because of the cost of RNA sequencing, the number of biological replicates chosen for each condition was limited to three, including three samples from Common murres either infected with *Babesia* or contaminated with oil, used as a control group. While more replicate numbers are recommended for this type of analysis (ideally 12 per condition), DeSeq2 has been shown to be suitable for 3 or fewer replicated when the fold-change threshold is set to 0.5, and limma when the fold-change threshold is set to 2.0 (Schurch et al., 2016).

While it is difficult to obtain samples of healthy adult seabirds, several factors were taken into consideration to ensure there was as much similarity between the samples as possible. Even though all samples were collected over the duration of roughly a year, samples chosen for our control group were collected during the summer months: June and July of 2021, and June of 2022. All birds that were sampled were found beached in Santa Cruz County, California, and did not exhibit any major visible bodily injuries. They were listed in the IBR database as emaciated and dehydrated. Further, although one sample was aged as a hatch-year and the other two were identified as after hatch-year, all three samples were roughly similar in weight (600–800 g). All samples had relatively similar packed cell volume (PCV) values (>30%), normal total protein (TP) values (>4.0 g/dL), and normal buffy coat (BC) values (0–1%) (Newman et al., 1997). All birds were hydrated with a 5% by body weight 0.9% NaCl solution by the IBR staff before blood samples were taken. Any medication, supplements, and anti-parasitic were administered after sampling had taken place. Birds chosen for the oil contamination group were visually diagnosed with petroleum oil feather contamination and were reported to the Oiled Wildlife Care Network (OWCN) by IBR staff. To limit the amount of variability of each sample, it was ensured that samples chosen were not also later diagnosed with other major ailments that would also affect gene expression (i.e. fractured bones, joint luxation, etc.). Each bird was sampled at the time of entry to the rehabilitation center before any treatment was given.

For each sample, whole blood was collected using a 25-gauge needle attached to a 1 mL syringe from the medial metatarsal vein. Approximately 0.1 mL of blood was drawn from each bird, enough to be stored in Queen's lysis buffer for DNA extraction (Longmire et al., 1997), in Invitrogen TRIzolTM LS ReagentTM (Thermo Fisher Scientific, Waltham, MA) for RNA preservation, as well as to make two blood smears for microscopy. The two blood smears were immediately set in methanol for fixation, and stained using JorVet Dip Quick Stain (Jorgenson Labs, Loveland, CO). Samples were frozen at −20°C until long-term storage was possible at −80°C at San Francisco State University prior to processing by the Avian Parasitology Laboratory.

2.2 DNA extraction and parasite detection

The Promega Wizard SV Genomic DNA Purification System was used to extract DNA from blood samples stored in Queen's Lysis buffer following manufacturer's instructions. Extracted DNA was stored at −20°C while unextracted DNA samples were kept at −80°C for long-term storage. 5' and 3' oligonucleotide primers [forward primer ITS15c (3'-CGATCGAGTGATCCGGTGAATTA-5') and reverse primer ITS13b (5'-GCTGCGTCCTTCATCG TTGTG-3')] were used, per Bostrom et al. (2008), and provided by Elim Biopharm Inc., Hayward, CA to amplify the ITS1 region of *Babesia* DNA. The thermocycler time and temperature profiles used are as follows: an initial activation step at 94.8°C for 1 min was followed by 35 cycles of amplification (94°C for 30 seconds, 62°C for 20 seconds, and 72°C for 30 seconds). The final extension was set at 72°C for 5 minutes, and then cooled to 4°C. Positive controls were identified using thin film microscopy and PCR, and PCR-grade water was used as a negative control. 91 common murre DNA extracts were tested for parasitic infection.

2.3 RNA extraction and sequencing

RNA was extracted from blood samples stored in TRIzol following the protocol in the Qiagen RNeasy Kit with the following modifications. A phenol-chloroform extraction was

performed following the Qiagen RNeasy Lipid Tissue Mini Handbook to separate homogenate into aqueous and organic phases. Prior to extraction, samples were removed from −80°C and thawed to room temperature (approximately 15 minutes at room temperature). 200 mL of chloroform (ThermoScientific Chloroform 99% Extra Dry over Molecular Sieve, Stabilized AcroSeal) was added to the homogenate and shaken vigorously for 15 seconds and incubated on the benchtop at room temperature for 3 minutes. The samples were then centrifuged at 12,000 × g at 4°C for 15 minutes using an OWM Hermle Labnet Z 252 Mk Centrifuge. Standard extraction procedure was then applied until the column wash step. The Qiagen RNase-free DNase Set was added prior to the column wash procedure to ensure purity of the RNA. A final elution of 70 uL of RNase-free water was incubated on the column for 3 minutes and centrifuged at 12,000 g for 1 minute.

Total RNA was assessed for quality control using the Agilent Bioanalyzer Nano RNA chip (Agilent Technologies Inc. Santa Clara, USA) at San Francisco State University's Genomics/Transcriptomics Analysis Core (GTAC). Peaks were assessed by RNA Integrity Number (RIN) values, which ranged from 7.3 to 9.6. A baseline RIN value of 5.0 was set to ensure proper quality. Due to budget restrictions, 3 individuals with the highest RIN value were selected for sequencing as representatives of each condition as biological replicates: *Babesia*-infected, petroleum oil-contaminated, and a control group for a total of 9 samples. Library preparation and RNA sequencing was performed by Novogene Co., LTD (Sacramento, CA) using the NovaSeq PE150 sequencing platform and strategy. Paired-end reads were generated in fastq format and released onto a remote server at SFSU. The estimated average number of reads was 17.6 million reads.

2.4 Gene expression analysis

Files were released and downloaded from Novogene and uploaded on to servers at the California Academy of Sciences in San Francisco, CA, as well as to the galaxy bioinformatics platform (The Galaxy Community at usegalaxy.org). The common murre reference genome and gene annotation files (Accession Number: SAMN12253989) were also uploaded to the galaxy platform. Trimmomatic was used to trim adapter sequences using the default job resource parameters (Bolger et al., 2014) (Galaxy version 0.38.0). FastQC was used to check the quality of the reads using default parameters (Andrews, 2010) (Galaxy version 0.73 + galaxy0). HISAT2 was used to align the reads to the genome sequence using the default parameters, which resulted in binary versions of sequence alignment maps (BAM) (Kim et al., 2015) (Galaxy version 2.2.1 + galaxy1).

FeatureCounts was then used to quantify reads using the default parameters (Liao et al., 2014) (Galaxy version 2.0.1 + galaxy2). Differential gene expression was performed using Limma-voom using the Benjamini and Hochberg (1995) *p*-value adjustment method and the *p*-value adjusted threshold of 0.05 was used as a false-discovery rate control (Law et al., 2014; Liu et al., 2015) (Galaxy version 3.50.1 + galaxy0). The TMM normalization method and robust settings were used to protect against outliers. Results were validated by running the same tests on DeSeq2 using the default job parameters (Love et al., 2014) (Galaxy version 2.11.40.7 + galaxy2.

For this analysis, 9 (Table 1) were selected and divided into three representative groups comprised of 3 birds each: a control group, in which birds were not infected with *Babesia* or contaminated, a *Babesia*-infected group, and an oil-affected group. It was originally planned to include replicate samples from birds that were both infected with *Babesia* and contaminated, however, we did not obtain RNA samples that were of sufficient quality for sequencing and analysis (RIN<6.5).

Two separate analyses were performed using three groups of biological replicates. To test how parasitic infection affects the Common murre, the 3 control group samples were set against the 3 *Babesia* infected samples. The second analysis determined how oil-contamination affected gene expression. The 3 control group samples were tested against the 3 oil-contaminated samples. Expression table data was input into R and volcano plots with labeled genes of interest were created for each test using the ggplot2 and dplyr packages (R Core Team, 2023; Wickham, 2016) (Supplementary Tables S2, S3). R and Microsoft Excel were used to filter through the gene expression data to determine the amount of overlap between the two analyses.

2.5 Gene set enrichment analysis (GSEA)

Statistically significant differentially expressed genes from the limma-voom analysis, were input into the g:Profiler database to convert gene names to ENSEMBL ID's for GSEA (Raudvere et al.,

TABLE 1 Attributes of the 9 common murres selected for the analysis.

IBR ID	Admission Date	Age	Babesia Status	Contamination status
21–1386	7/23/2021	AHY	Negative	Non-Oiled
22–1159	6/20/2022	AHY	Negative	Non-Oiled
21–1233	7/9/2021	HY	Negative	Non-Oiled
21–1416	8/5/2021	AHY	Positive	Non-Oiled
21–1628	10/13/2021	HY	Positive	Non-Oiled
22–1342	7/7/2022	AHY	Positive	Non-Oiled
21–1515	8/20/2021	AHY	Negative	Oiled
22–0246	4/14/2022	AHY	Negative	Oiled
22–0341	4/27/2022	ASY	Negative	Oiled

2019). GSEA was performed in R using the ClusterProfiler package (Yu et al., 2012). Significantly expressed genes, in ENSEMBL format, and their associated fold change values were input into the package with the annotation file for *Gallus gallus* (org.Gg.eg.db) as a reference. While clusterProfiler offers curated gene sets for various organisms, avian annotations, excluding Gallus gallus, are currently unavailable. While using chicken gene sets might not perfectly capture common murre-specific biology, the enriched pathways still provided valuable insights into general stress response mechanisms potentially affected by the chosen stressors. The ontology parameter was set to test for biological function (BP) and set the *p*-value cutoff at 0.05. The Bonferroni-Hotchberg (BH) method for *p*-adjusted value to test for false positives (*q*-value). This method was performed for differentially expressed genes from each analysis. Pathways were identified and annotated using the Disease Ontology Semantic and Enrichment analysis (DOSE) (Yu et al., 2015).

3 Results

3.1 Transcriptome response

667 genes were differentially expressed ($p < 0.05$) in response to *Babesia* infection (Figures 1A, B). 380 (57%) genes were upregulated and 287 (43%) were downregulated (Table 2). Of these genes, 62 were unlabeled and had no known homologues. 39 of these unlabeled genes had a positive change in expression and 23 had a negative change in expression. The top significantly differentially expressed gene was Annexin A2 (*ANXA2*), which had a negative magnitude change in expression by the *Babesia*-infected group compared to the control group ($p < 0.05$).

Oil contamination elicited an even greater response, with 1454 total genes being differentially expressed (Figures 2A, B). Of those genes, 824 (57%) were upregulated and 631 (43%) were downregulated. 142 of these genes were unlabeled and had no known homologues. Of these uncharacterized genes, 72 were upregulated, and 70 were downregulated. The top significantly expressed gene was Interleukin-17 receptor protein E (*IL17RE*), which had a positive magnitude change in expression in the contaminated group. The proportion of significantly upregulated and downregulated genes (57% versus 43%, respectively) were the same in both analyses.

3.2 Similarities in gene expression and GSEA

We found that between the response to infection and the response to contamination, 194 of the same genes were differentially expressed by both groups (Supplementary Figure S1). This accounts for 32% of the genes that were expressed in response to infection and 17% that were expressed in response to contamination. This amount of overlap is much higher than what would be expected at random in response to different conditions ($p < 0.001$, Fisher's Exact Test). Out of the shared 194 genes, 188 were expressed in the same direction by both groups. This accounts for 97% of the shared genes, indicating most of the expression change to both conditions represent a generalized response to stress, also indicating that the stress response to *Babesia* and oil contamination are highly similar. The remaining 6 shared genes had opposite directional changes in expression (Table 3).

Genes differentially expressed in response to *Babesia* infection led to suppression of biological pathways related to immune response (Figure 3). The top two enriched Gene Ontology terms related to biological processes were 'defense response' and 'immune response.' Similarly, terms associated with the immune system were suppressed in response to contamination (Figure 4). "Immune response" and "inflammatory response" were highly significantly suppressed. In each of these instances, there are only two genes significantly driving these patterns. In the infection analysis, "defense response" suppression is driven by Lymphocyte antigen 96 (*LY96*) and Parkinsonism associated deglycase (*PARK7*). *LY96*, as well as Lyphocyte antigen 86 (*LY96*), are also driving the GO "Positive regulation of response to stimuli," and "immune response" is driven down by Pannexin 1 (*PANX1*) and MutL homolog 1 (*MLH1*). In the oil contamination analysis, both GO terms "immune response", and "inflammatory response" are driven by *LY96* and C-C Chemokine receptor type 5 (*CCR5*). Both analyses saw differential expressions of cytokines IL-17 and IL-18, as well as *TNFAIP3*. Contamination saw more DEG involved in cytokine pathways including *SKIL, IL5RA*, and *TNFAIP8*.

Also in the contamination group, ATPase phospholipid transporting 10A (*ATP10A*) and DENN Domain Containing 5B (*DENND5B*) drove "lipid transport." "Lipid localization was also driven by *ATP10A*, as well as Fatty acid binding protein 7 (*FABP7*). "Glycoprotein metabolic process" was suppressed by Sulfatase 1 (*SULF1*) and 2-Phospoxylose Phospatase 1 (*PXYLP1*). Some other DEGs relating to lipid metabolism were *PLA2G2E, ECHDC3, FADS1, FABP4, ACADL, LPIN2, CD36*, and *GATA2*. *PLA2GE* secretes inflammatory lipid mediators (Gubern et al., 2008; Silverstein and Febbraio, 2009; Qin et al., 2018), *ECHDC3* is involved in the biosynthesis of fatty acids (Sharma et al., 2019), *FADS1* encodes a fatty acid desaturase (He et al., 2018), *FABP4* is a gene related to the transport of fatty acids (Furuhashi et al., 2015), *ACADL* aids in fatty acid oxidation (Zhao et al., 2020), *LPIN2* is involved in lipid synthesis (Dwyer et al., 2012).

4 Discussion

In this comparative analysis of gene expression, we investigated the molecular impacts of both oil contamination and infection from an apicomplexan parasite, *Babesia*, in common murres, using three biological replicates for each condition. Despite the small number of biological replicates, the results generated from this analysis are consistent with previous studies exploring similar questions (Videvall et al., 2015; Bonisoli-Alquati et al., 2020). The programs employed in this analysis, DeSeq2 and Limma-voom, while not specifically designed for small sample sizes, utilize various techniques that are designed to handle data with various levels of

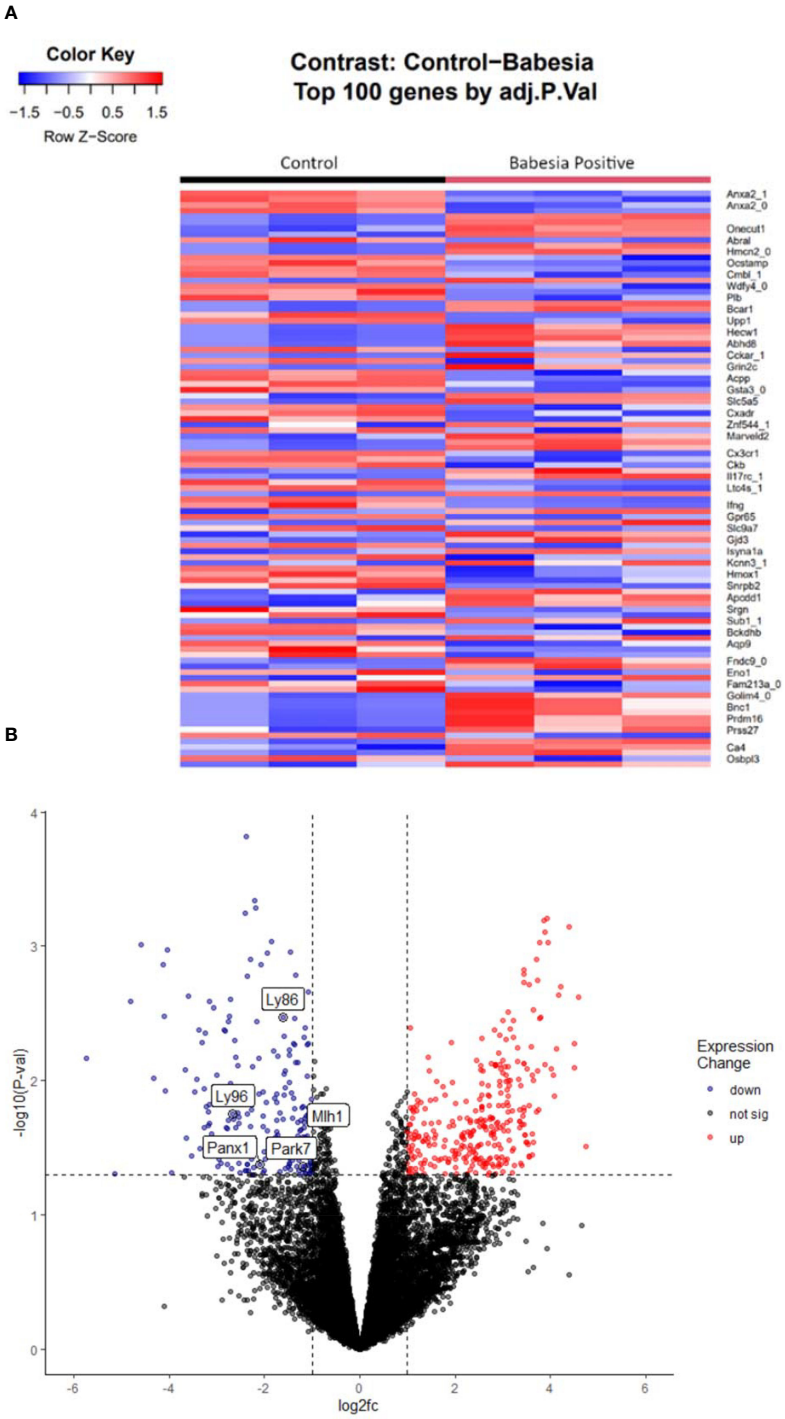

FIGURE 1
Heatmap **(A)** displaying the top 100 differentially expressed genes by adjusted *p*-value (*q*-value). Each column represents a sample, the left being the control group, and the right being the infected group. Each row represents a gene, with the magnitude of change in expression represented by a change in color (red being more upregulated, and blue being more downregulated). The volcano plot **(B)** displays the differentially expressed genes when analyzing the control group versus the *Babesia*-infected group. The log2(fold change) in gene expression is plotted on the x-axis, while the −log10 of the adjusted *p*-value is plotted on the y-axis. Genes that are significantly upregulated ($p < 0.05$ and log2foldchange ≥ 1) are shown in red, while genes that are significantly downregulated ($p < 0.05$ and log2foldchange ≤ -1) are shown in blue. Genes that are not significantly differentially expressed are shown in gray. Labeled genes, *LY96 LY96, MLH1, PANX1*, and *PARK7*, drive down GO terms related to immune response, defense response, and positive regulation of response to stimuli.

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TABLE 2 Number of differentially expressed genes in response to *Babesia* infection and oil contamination in common murres.

DGE Analysis	Differentially Expressed Genes (p<0.05)	Positive change in gene expression (n)	Negative change in gene expression (n)
Control vs. *Babesia*-infected	667	380 (57%)	287 (43%)
Control vs. Oil-contaminated	1454	824 (57%)	630 (43%)

Columns indicate conditions, total number of significantly differentially expressed genes, and the number and ratio of genes that exhibited a change in expression ($p < 0.05$; $-1 \leq$ Log2foldchange ≥ 1).

biological variability (Love et al., 2014). Both programs use shrinkage estimators, such as the Wald statistic or the moderated t-statistic. This reduces the variance of the estimated log2 fold changes, especially for genes with low counts, which in turn helps to differentiate true biological signals from random noise that is more prevalent in datasets with fewer replicates (Love et al., 2014). Both tools also employ normalization procedures that account for technical variability between samples, ensuring that differences in gene expression are more likely due to the biological conditions that are being compared, rather than technical artifacts (Costa-Silva et al., 2017). While there is, of course, room (and a need) for an improved study involving more biological replicates, we believe our results are significant and represent a major first step in uncovering the effects of environmental stress on a severely understudied species.

4.1 Avian host response to *Babesia*

GSEA analysis determined that biological functions relating to the immune response were the top two enriched pathways from this analysis. Further, these two processes were driven to suppression by two genes each; Defense response was driven by *LY96* and *PARK7*, and inflammatory response was driven by *LY96* and *LY96*. While these processes are suppressed, it is likely that the host is not suppressing the entire immune system but dampening certain aspects of the immune response. Lymphocyte antigen 96 (*LY96*) is a transmembrane protein that has been known to play a role in inflammation, immune suppression, and T cell activation, however, the role of this gene in the context of *Babesia* infection and its impact on the immune system is not fully understood (Li et al., 2023). The role of *PARK7* in parasitic infection is also not well understood. The role of this gene is in antioxidative response and maintaining mitochondrial quality control (Wang et al., 2016). It is possible that *LY96* acts as a negative regulator of T cell activation, potentially contributing to the dampened inflammatory and defense responses observed in this analysis. This process is regulatory that eukaryotes possess to suppress overly aggressive immune responses to prevent tissue damage. For example, a study on the role of *LY96* in cancer progression found that suppression, or downregulation of *LY96* can inhibit cancer development (Nie et al., 2022). *PARK7* has been linked to inflammatory processes and may be a part of this suppression process.

While there is currently no information on the avian transcriptome response to *Babesia*, Videvall et al. (2015) analyzed the avian transcriptome response to *Plasmodium ashfordi* (2015), as well as the transcriptome of *P. ashfordi* while infecting an avian host (2017). Although *Plasmodium* and *Babesia* may elicit different overall transcriptome responses, we expect some level of overlap in the host response because both parasites undergo a life cycle stage where they infect, and destroy the red blood cells, leading to an inflammatory response (Clark and Jacobson, 1998; Hunt and Grau, 2003). Videvall et al. (2015) found differential expression of several genes that have also been found to be expressed by mice and humans in response to malaria infection. In the present study, two of these genes, Annexin A2 (*ANXA2*) and Lymphocyte antigen-86 (*LY96*), were downregulated in our *Babesia*-infected group. Both of these genes have also been widely found to be implicated in participating in the inflammatory response, however their role in *Babesia* infection is massively understudied.

ANXA2 has both anti- and pro-inflammatory roles in the innate immune response. *ANXA2* aids in macro autophagy by interacting with Atg16, a protein that is related to autophagy (Dallacasagrande and Hajjar, 2020). In the context of pathogen-caused infection, *ANXA2* plays crucial roles in the mediation of pro-inflammatory cytokines and promotion of anti-inflammatory signals (Dallacasagrande and Hajjar, 2020; Ma et al., 2021). There is also evidence that *ANXA2* may be used to facilitate infection by bacteria such as *Pseudomonas aeruginosa* and *Escheria coli* (Dallacasagrande and Hajjar, 2020). *LY96* is a protein-coding gene, that codes for the protein MD1, and has been shown to be produced by macrophages in the inflammatory response (Su et al., 2014; Thomas et al., 2016). The downregulation of these genes in *Babesia*-infected murres suggests potential mechanisms employed by the parasite to evade the host immune response. For instance, *ANXA2* deficiency might hinder the ability of immune cells to infiltrate *Babesia*-infected tissues, allowing the parasite to establish itself. Similarly, downregulation of *LY96* could suppress T-lymphocyte activation, thereby dampening the adaptive immune response. While these are speculative scenarios, they warrant further investigation to understand *Babesia*'s immunomodulatory strategies in common murres.

The presence of shared genes responding to multiple stresses points toward the possibility of a highly conserved host immune response to both babesiosis and malaria across species. Further research is required to interpret the precise mechanisms and implications of these shared genes in stress and immune responses.

Videvall et al. (2015) also analyzed the transcriptome of *P. ashfordi* after experimentally inoculating Eurasian siskins (*Carduelis spinus*). We compared the results of our *Babesia*-infected analysis to determine if any overlap in gene expression existed that may suggest expression from the *Babesia* parasite itself, rather than from the host. We found only one homologous gene that matched from this study, Subtilisin proteases 1 (*SUB1*; log fold

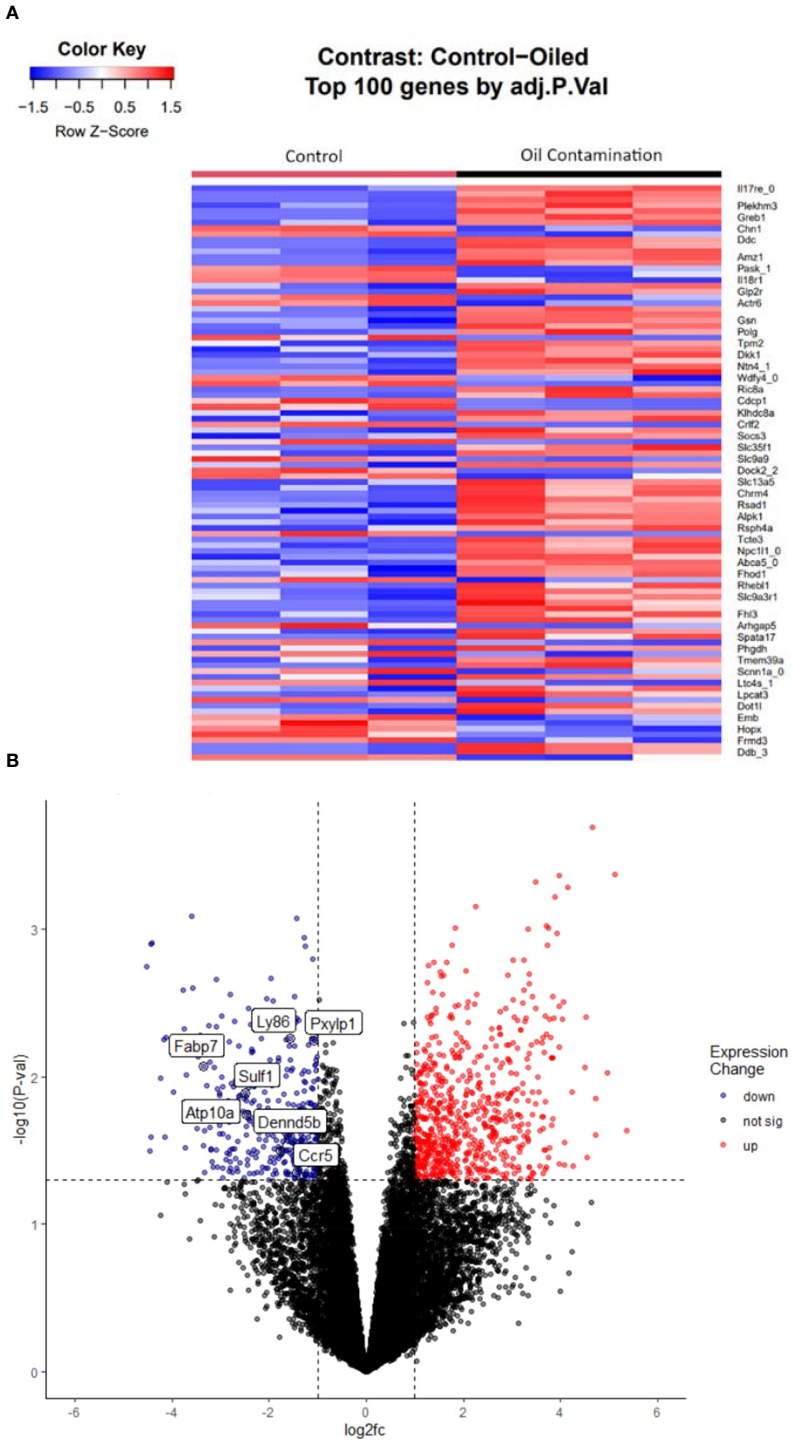

FIGURE 2
Heatmap **(A)** and volcano plot **(B)** displaying the top differentially expressed genes in response to oil contamination. Both figures show a much larger magnitude of differentially expressed genes, as described in Figure 1.

change = 2.9088, *p*-value = 0.00618). This gene encodes proteins with essential roles in the maturation and proteolytic processing of merozoite antigens (Beeson et al., 2016). Although only one gene was found to be similarly differentially expressed, this is not surprising because, despite both *Plasmodium* and *Babesia* being apicomplexan type parasites, it is likely that they have developed distinct needs within erythrocytes, which can lead to differences in gene expression. Many of the genes, however, were found within our dataset, but fell well beneath our significance cutoff. This is most likely because, while comparing data from *Plasmodium* and *Babesia* may provide useful context, it is important to consider the limitations of comparing two different species. While both species

TABLE 3 Summary of the 6 genes that were differentially expressed in both the *Babesia*-infected group and the oil-contaminated group that were expressed in opposite directions.

Gene ID	ENSEMBL	Gene Description	Expression (Babesia)	Expression (Contamination)
CTSL	N/A	N/A	Down	Up
MAPK12	ENSGALG00010001611	Mitogen-activated protein kinase 12	Up	Down
PI4KB	ENSGALG00010028605	Phosphatidylinositol 4-kinase beta	Up	Down
LRRC34	ENSGALG00010016840	Leucine rich repeat containing 34	Up	Down
ABCG2	ENSGALG00010020622	ATP binding cassette subfamily G member 2	Down	Up
ARL4A	ENSGALG00010004029	ADP ribosylation factor-like GTPase 4A	Down	Up

are Apicomplexan type parasites, they belong to two distinctly different Apicomplexan orders that have diverged significantly, *Plasmodium* belongs to the order Haemosporidia, and *Babesia* belongs to Piroplasmida (Martinez-Ocampo, 2018). Furthermore, these two species have established unique life cycles and host-parasite interactions, the most distinct difference being that *Plasmodium* has a replication stage within the host's liver, and *Babesia* does not (Hakimi et al., 2022). Further exploration comparing the similarities and differences between these two genera would be greatly beneficial in the optimization of these types of wildlife genomic studies.

4.2 Avian host response to oil contamination

Functions relating to lipid transport and localization were affected in response to petroleum oil contamination (Figure 4). Lipid transport and localization involves the coordination of various pathways to move insoluble lipolytic products throughout the body (Feingold et al., 2020). This process is essential to organisms because lipids play major roles as the building blocks of cellular membranes, aid in energy storage, act as signaling molecules between and within cells, and are required for proper

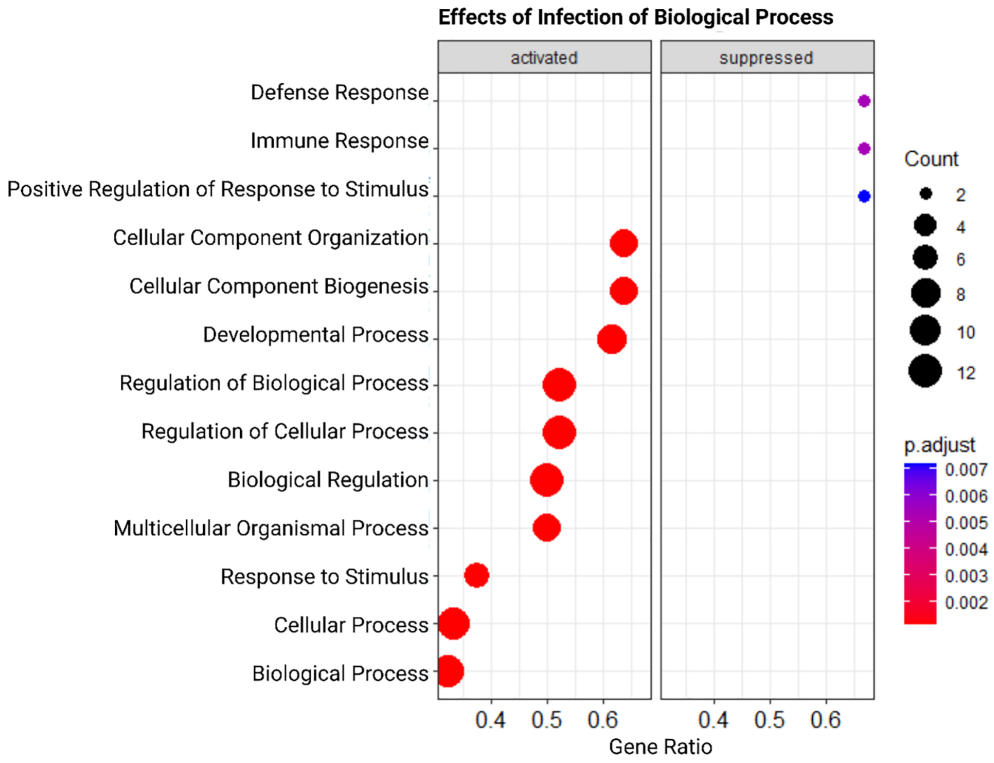

FIGURE 3
Dot-plot representation of Gene Set Enrichment Analysis (GSEA) results highlighting activated and suppressed biological functions in response to *Babesia* infection. Each dot represents a distinct biological function, positioned along the x-axis according to its enrichment score. Activated functions are shown on the left side of the plot, while suppressed functions are displayed on the right side. The color of each dot corresponds to the significance level of the enrichment score, with red dots indicating higher significance. The size of each dot represents the number of genes associated with a biological function. The x-axis represents the gene ratio, or the proportion of genes from the gene set that are present in the analyzed dataset. We can see that terms relating to immune response, inflammatory response, and response to stimulus are all suppressed.

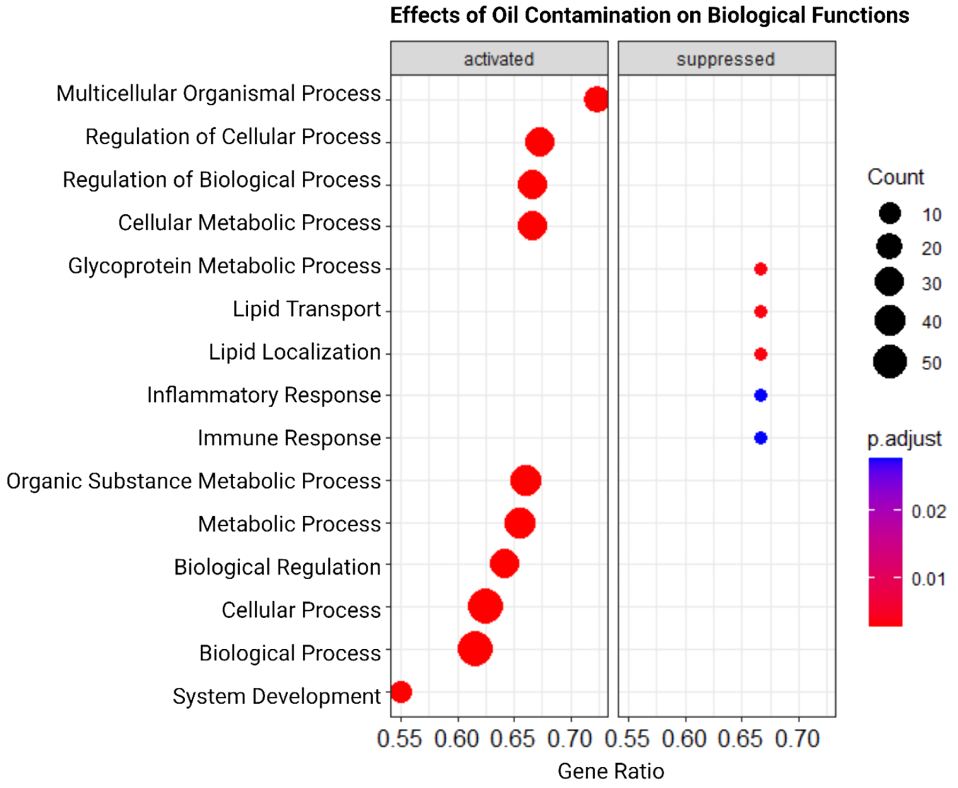

FIGURE 4
Dot-plot representation of Gene Set Enrichment Analysis (GSEA) results highlighting activated and suppressed biological functions in response to exposure to oil contamination, as described in Figure 3.

protein functions. Lipid processes related to metabolism are centralized in the liver, and damage to the liver can affect the lipid amounts circulating throughout the body (Arvind et al., 2000). Further, damage to the kidneys has also been associated with disturbing lipid metabolism (Trevisan et al., 2006). Studies of the effects of petroleum oil on seabirds have reported problems with the kidneys and liver, with damage to these organs being the leading cause of mortality among patients (Briggs et al., 1996). Common murres and Cassin's auklets (*Ptychoramuphus aleuticus*) exposed to petroleum oil in a controlled study exhibited liver problems such as hepatocellular dissociation (Fry and Lowenstine, 1985).

Petroleum oil may also disrupt lipid processes by directly damaging the lipid bilayer of cellular membranes, interfere with proteins that are responsible for transporting lipids throughout the body, and altering lipid environments locally by bioaccumulating in tissues. Studies on Polycyclic Aromatic Hydrocarbons (PAHs) bioaccumulation in marine life are limited, however, a study on the effects of PAHs in gelatinous zooplankton found that bioaccumulation occurred either dermally or through ingestion, and that the lipophilic nature of PAHs are the main cause of accumulation in an organism's lipid content (Almeda et al., 2013). It is highly likely that the Common murres used for this study had significant levels of PAHs bioaccumulation because attempting to preen oiled feathers can likely lead to direct ingestion of PAHs. Dermal absorption of PAHs is also highly likely because contamination disrupts the barrier created by seabirds to protect them from the cold marine environment, leaving their skin exposed and vulnerable to further contamination.

Bonisoli-Alquati et al. (2020) studied the transcriptomes of Seaside sparrows (*A. maritima*) that were exposed, but not physically contaminated, to the Deepwater Horizon oil spill that occurred in the Gulf of Mexico in 2010. This was the only other published transcriptomic analysis of seabirds exposed to petroleum oil to our knowledge, and it was expected that our results would have many similarities, although the birds in that analysis were not physically contaminated by oil. Their results found significant upregulation of *cyp1a2*, a known marker of PAHs in birds and cytochrome p450 subunit member, as well as activation of the PAH-responsive aryl hydrocarbon receptor (AhR) pathway. While our results did not show upregulation of the *cyp1a2* gene, we found significant downregulation of other p450 subunit members: *cyp4b1_1* (log fold change = −2.536, *p*-value = 0.018), *cyp4b1_0* (log fold change = −3.3510, *p*-value = 0.029), and *cyp2r1* (log fold change = −1.0408, *p*-value = 0.0316).

The p450 family of enzymes (CYP), in humans, are membrane-bound proteins involved in drug detoxification and cellular metabolism (Zhao et al., 2021). In humans, the CYP 1 family is involved in drug metabolism, the CYP 2 family is primarily

involved in drug and steroid metabolism, and CYP 4 is involved in fatty acid metabolism (Zhao et al., 2021). In the context of oil exposure, several studies have found expression of *cyp1a* in response to the deepwater horizon oil spill, mediated by AhR (Varanasi, 1989; Whitehead et al., 2012; Brewton et al., 2013; Dubansky et al., 2013; Crowe et al., 2014; Brown-Peterson et al., 2015; Xu et al., 2016, 2017). In a study of double-crested cormorants (*Phalacrocorax auratus*) experimentally dosed with synthetic (DWH Mississippi Canyon 252 oil), *cyp1a* was also found to have elevated expression levels in the liver (Alexander et al., 2017).

In the current study, *cyp1a2* is not expressed, however, AhR is. This gene has been shown to be a regulator of *cyp1a2* (Bonisoli-Alquati et al., 2020). This gene was significantly downregulated in our data, which may have led to the downregulation of *cyp1a2*, as well as other CYP enzymes such as *cyp4b1* and *cyp2r1*. A possible explanation for the downregulation of AhR, as opposed to the activation of this gene, is related to resource allocation by the birds. It is highly likely that the oiled common murres admitted to IBR that were selected for this analysis were in such poor condition, that AhR and CYP enzymes were turned off as a survival mechanism. This method of allocating resources would prioritize essential functions such as maintaining body core temperature and organ function. Further research is needed to understand how the p450 CYP enzymes behave once the birds have had enough supportive care to return to a state of homeostasis.

Bonisoli-Alquati et al. (2020) also detected upregulation of the insulin-like growth factor binding protein (*IGFBP1*) gene, which was assisted by expression of the serine/threonine-protein kinase Pim3 (*PIM3*). Our results show a similar upregulation of the *IGFBP1* gene in the oil contamination group (log fold change = 2.9766, *p*-value = 0.0128). This gene plays a major role in liver regeneration, giving further evidence that birds from our sample group experienced significant liver damage from PAH bioaccumulation. While *PIM3* was not significantly upregulated, *PIM1* was (log fold change = 1.4614, *p*-value = 0.0327), which is highly homologous to *PIM3* (Julson et al., 2022). An investigation of *PIM1* in knockout mice found expression of this gene to be associated with pancreatic, prostate, gastric, and colorectal cancers.

Finally, the Seaside sparrow study found *ELOVL5* and *ELOVL2*, two elongases involved in polyunsaturated fatty acid synthesis, significantly downregulated. These elongases have downstream control in the adipose tissue, and downregulation of these genes may function in preventing lipid peroxidation from oxidative insults. These two elongases were also found in Great tits (*Parus major*) in a study on the effects of urban environments (Watson et al., 2017). While *ELOVL5* and *ELOVL2* were not significantly downregulated, *ELOVL4* was (*elovl4_0* log fold change = −2.9362, *p*-value = 0.020002). The elongase family consists of enzymes involved in the elongation of very-long-chain fatty acids and may have overlapping functions (Wang et al., 2023). It is possible that in Common murres, *ELOV4* is the primary elongase for very-long-chain fatty acid synthesis, and in both Seaside sparrows and Great tits, *ELOVL5* and *ELOVL2* are the primary elongases.

4.3 Similarities in gene expression

As expected, *Babesia* and oil contamination saw DEGs relating to the innate immune system. NF-kB is a transcription factor that regulates expression pro-inflammatory cytokines, chemokines, innate immune cells, and T-cells (Liu et al., 2017). *TNFAIP8*, found to be induced by NF-kB (Niture et al., 2019), and *FABP7*, a promoter of NF-kB-driven inflammatory response (Killoy et al., 2020), were expressed by both groups. Birds that were contaminated with oil saw differential expression of genes associated with the regulation of Interleukin-1 beta (IL-1β), including *CASP4*, which regulates IL-1β synthesis in macrophages (Cheung et al., 2018), and *CASP8*, a gene that plays a role in modulating IL-1β and inflammation (Gurung and Kanneganti, 2015). IL-1β is a pro-inflammatory cytokine that is essential for the host's ability to defend against infection (Lopez-Castejon and Brough, 2011).

Both analyses saw differential expressions of cytokines IL-17 and IL-18, as well as *TNFAIP3*. Contamination saw more DEG involved in cytokine pathways including *SKIL*, *IL5RA*, and *TNFAIP8*. *SKIL* has been shown to play a role in immune escape by upregulating autophagy (Ma et al., 2020). *IL5RA* is an Interleukin 5-receptor subunit that has been implicated in the regulation of white blood cells (Cheong et al., 2005). *TNFAIP3* is a Tumor Necrosis Factor α-Induced Protein that mitigates the response to inflammation (Das et al., 2018). *TNFAIP8* is another Tumor Necrosis Factor α-Induced Protein that helps maintain homeostasis during an immune response (Niture et al., 2019).

4.4 Cellular processes

Both infection and contamination lead to the activation of various cellular processes in common murres (Figures 3, 4). Notably, terms such as "regulation of cellular process," "cellular metabolic process," and "cellular component organization" were found to be among the activated processes in both groups. The activation of cellular response pathways may be a direct consequence of cell invasion by parasites, as shown in previous studies (Sumbria et al., 2021). Additionally, cell stress caused by the presence of these stressors might also contribute to the regulation of cellular processes. Iron plays a crucial role in numerous cellular activities (Rockfield et al., 2018), and its deficiency is a leading cause of anemia (Warner and Kamran, 2023). Specifically, hemolytic anemia, a type of anemia where red blood cells are destroyed, can result from the destruction of red blood cells (Hill and Hill, 2018). *Babesia* directly invades and replicates in the host's red blood cells, ultimately leading to their destruction (Fry and Lowenstine, 1985; Niu et al., 2015; Wu et al., 2017). Similarly, ingestion of petroleum oil has been shown to cause hemolytic anemia in seabirds and other organisms (Leighton et al., 1983; Ostlere et al., 1988). The activation of cellular metabolic process and regulation of cellular process may be associated with the host's defense mechanism. Both parasitic infection and oil contamination can impose significant metabolic burdens on the host, and upregulation of genes associated with this

process may reflect enhanced energy production needs by the increase of immune cell recruitment and tissue repair. It may also be involved in maintaining cellular stability, or homeostasis in response to disruptions or damage caused by inflammation (Chovatiya and Medzhitov, 2014). Therefore, the activation of cellular processes observed in this study might serve as a protective mechanism by the host to mitigate excessive destruction of red blood cells caused by these stressors.

Conversely, there exists the potential for the activation of cellular processes to possess negative consequences in the context of the inflammatory response. The initial increase of metabolic activity in response to cellular invasion is beneficial, however, prolonged increase of metabolic activity can become detrimental (Blanco and Kaplan, 2023). Dysregulation of immune response can lead to tissue damage, inflammation, and autoimmunity (Blanco and Kaplan, 2023). In the context of the current study, while unlikely, it is possible that the activation of cellular processes may be attributed to dysregulation of immune cells and metabolism. Further experimental research is required to elucidate the function of cellular processes in the context of these conditions.

5 Conclusion

The findings of this study highlight the detrimental effects of these environmental stressors on the immune system of common murres. Both apicomplexan infection and oil contamination appear to suppress immune response mechanisms, potentially leaving the birds more susceptible to infections and compromising their overall health. Moreover, this analysis reveals specific impacts of oil contamination on the lipid metabolism of common murres. Genes associated with lipid transport and lipid localization were significantly overrepresented in response to oil contamination. This suggests a disruption in the birds' ability to regulate lipid levels and perform essential functions related to lipid metabolism and glycometabolism. These results also highlight a need for more research in this field, ideally with more biological replicates to increase evidence of the genes observed in this study.

It is important to acknowledge that this study did not include technical replicates. This decision was made due to the author's opinion that the high cost does not justify the added value, given that most DE studies do not include technical replicates. The authors are confident that the results reported are robust despite the lack of technical replicates. It also is important to emphasize that the results generated in this study should be taken with caution, as the sequencing and analysis of the transcriptomes of wildlife is notoriously difficult. One major concern in the present study that should be considered is the method implemented in choosing a control group. The samples collected are all from seabirds admitted into a wildlife hospital after being found beached for an unknown duration of time. Regardless of if the individual displays physical injuries upon admission, there may be underlying physiological or internal ailments that display themselves after intensive supportive care and the bird is returned to homeostasis. However, given the trends found in this study when compared to other studies, the results yielded are significant. Because of the concerns with forming a control group, it should be stated that these results should be regarded as baseline data, emphasizing the need for further experiments. However, this study highlights the potential benefit of using wildlife admitted to rehabilitation centers to study how anthropogenic changes affect their genomic profiles. Longitudinal studies can be implemented to collect blood samples at different time intervals, as opposed to at one single point. This would allow the researcher to track changes in expression before and after oil contamination has been washed off, parasite burden before and after treatment, and overall health over time. Such research could also investigate the effects of oil contamination across different vulnerable species, such as Western grebes (*Aechmophorus occidentalis*), with the purpose of comparing the results. Experimental validation of the results generated from this study would also be useful in informing future projects implementing similar methods.

Overall, this research sheds light on the intricate molecular responses of common murres to two distinct stressors—apicomplexan infection and oil contamination. Understanding the shared and unique pathways affected by these stressors is crucial for developing effective conservation strategies and mitigating the detrimental impacts on the health and survival of these iconic seabirds. Further investigations into the underlying mechanisms and long-term consequences of these gene expression changes will be valuable in enhancing our knowledge of the impacts of environmental stressors on avian populations, and ecosystems.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Ethics statement

The animal study was approved by Oiled Wildlife Care Network/International Bird Rescue IACUC. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

CE: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing. RQ: Data curation, Writing – review & editing. RD:

Resources, Supervision, Validation, Writing – review & editing. SR: Formal analysis, Methodology, Software, Supervision, Validation, Writing – review & editing. RS: Funding acquisition, Resources, Supervision, Validation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcosc.2024.1425484/full#supplementary-material

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