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Transcriptomic responses of Galápagos finches to avian poxvirus infection

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Abstract

Emerging pathogens can have devastating effects on naïve hosts, but disease outcomes often vary among host species. Comparing the cellular response of different hosts to infection can provide insight into mechanisms of host defence. Here, we used RNA-seq to characterize the transcriptomic response of Darwin's finches to avian poxvirus, a disease of concern in the Galápagos Islands. We tested whether gene expression differs between infected and uninfected birds, and whether transcriptomic differences were related either to known antiviral mechanisms and/or the co-option of the host cellular environment by the virus. We compared two species, the medium ground finch (*Geospiza fortis*) and the vegetarian finch (*Platyspiza crassirostris*), to determine whether endemic Galápagos species differ in their response to pox. We found that medium ground finches had a strong transcriptomic response to infection, upregulating genes involved in the innate immune response including interferon production, inflammation, and other immune signalling pathways. In contrast, vegetarian finches had a more limited response, and some changes in this species were consistent with viral manipulation of the host's cellular function and metabolism. Many of the transcriptomic changes mirrored responses documented in model and in vitro studies of poxviruses. Our results thus indicate that many pathways of host defence against poxviruses are conserved among vertebrates and present even in hosts without a long evolutionary history with the virus. At the same time, the differences we observed between closely related species suggests that some endemic species of Galápagos finch could be more susceptible to avian pox than others.

1 | INTRODUCTION

Emerging infectious diseases are a threat to humans and wildlife (Cunningham et al., 2017; Daszak et al., 2000). Novel pathogens have effects across ecological scales: they can cause illness in individuals (Blehert et al., 2009), shifts in the size or distribution of host populations, (van Riper et al., 1986), and may even reshape ecological communities (Holdo et al., 2009; Johnson et al., 2015). However, the magnitude and extent of these effects depends on the interaction between host and pathogen, including how capable the pathogen is at exploiting a novel host, and whether the host can defend itself against attack. Understanding this interaction is crucial for estimating the disease burden of an emerging pathogen and anticipating its effects on a population.

One virus family with worldwide distribution and implications for human and wildlife health are the poxviruses (Poxviridae). Viruses in the genus *Avipoxvirus* infect birds and are distributed worldwide (Williams et al., 2021). Avian pox is transmitted through direct contact between individuals and mechanically by biting arthropods (Zylberberg et al., 2012a). The virus infects epithelial cells

and typically causes cutaneous lesions on the feet, legs, and face (Figure 1), although in more severe cases it can infect mucous membranes of the respiratory tract (Williams et al., 2021). Mild cutaneous infections may not be debilitating; however, large lesions can impede vision, mobility and feeding ability (Parker et al., 2011; Vargas, 1987). The virus causes particularly severe disease in insular bird populations that are naïve to the virus (Williams et al., 2021). For example, pox was well-established in the Hawaiian archipelago by the 19th century (Atkinson & LaPointe, 2009) and was subsequently implicated in the decline and extinction of several Hawaiian honeycreepers (Carduelinae, formerly Drepanidinae; van Riper III et al., 2002). Furthermore, this virus has also recently caused outbreaks of disease in tits (Paridae) in Great Britain (Lawson et al., 2012), Magellanic penguins (*Spheniscus magellanicus*) on the Atlantic coast of Argentina (Kane et al., 2012), and Mediterranean short-toed larks (*Calandrella rufescens*) and Berthelot's pipits (*Anthus berthelotti*) in the Canary Islands (Smits et al., 2005).

Avian pox was introduced to the Galápagos in the late 19th century and has since spread across the archipelago (Lynton-Jenkins et al., 2021; Parker et al., 2011). The prevalence of avian pox fluctuates from year to year, but may be increasing, probably due to the spread of invasive mosquitos and changes to climate and resource availability (Parker et al., 2011; Zylberberg et al., 2012a, 2012b). Pox infects a number of endemic Galápagos passerines including Darwin's finches (Thraupidae) and has recently been reported in the critically endangered waved albatross (*Phoebastria irrorata*; Jiménez-Uzcátegui et al., 2019; Tompkins et al., 2017).

Darwin's finches are an iconic radiation of birds in the Galápagos Islands. They are known for morphological and ecological diversity emerging from a relatively homogenous genetic landscape (Grant & Grant, 2014; Lamichhaney et al., 2015; Sato et al., 1999). Certain loci have been associated with phenotypic differentiation; however, there is strong evidence of genome-wide haplotype sharing and interspecific gene flow, especially within clades (e.g. *Geospiza* ground finches, *Camarhynchus* tree finches) (Chaves et al., 2016; Lamichhaney et al., 2015, 2016). At immune loci, trans-species polymorphism is common (Sato et al., 2011). A study of major histocompatibility complex (MHC) class II genes, a key component of adaptive immunity, found high diversity in MHC haplotypes and that alleles were shared across the Darwin's finch radiation (Sato et al., 2011). This genomic similarity suggests that the various finch species could have similar susceptibility to pox infection.

At the same time, immune phenotypes vary among finch species, indicating that regulatory elements of the immune system may not be conserved. Between 2000 and 2009, pox prevalence and disease severity increased in some populations of ground (*G. fuliginosa*, *G. scandens*), tree (*C. parvulus*), and warbler finches (*Certhidea olivacea*) (Zylberberg et al., 2012a). The increased prevalence of avian pox in these species was correlated with a population-level decrease in acute phase protein levels, a component of the innate immune response (O'Reilly & Eckersall, 2014). These results suggest that a decline in protective immunity could be linked to increased pox susceptibility in some species. In contrast, medium ground finch (*Geospiza fortis*) populations showed signs of reduced disease spread and increased recovery during the same period and had no change in measures of innate immune function (Zylberberg et al., 2012a). Ground finches from populations where pox is common also produce antibodies against pox (Huber et al., 2010). Thus, susceptibility to pox infection may be linked to immune function in Galápagos finches. However, it is unclear how exactly individuals respond to infection, and how much variation there is in this response among individuals and between species.

Understanding the molecular response of hosts to novel pathogens could help explain why disease emerges in some populations and not others, and why some individuals survive while others do not (Liu et al., 2017). The changes in host gene expression following infection are complex and reflect both manipulation of the cellular environment by the pathogen as well as activation of host defence mechanisms (Agudelo-Romero et al., 2008; Videvall et al., 2015, 2020). Laboratory studies of the model poxvirus vaccinia, the virus used to vaccinate against smallpox, have been instrumental in understanding host-viral interactions (Boyle & Traktman, 2009). These studies demonstrate that vertebrate hosts have a complex and robust immune machinery to resist poxviruses. Type I interferons are expressed rapidly in response to infection and activate an antiviral signalling cascade (Seet et al., 2003). This innate immune response also promotes inflammation, recruiting leucocytes to the site of infection (Haga & Bowie, 2005). Cells at the site of infection secrete antiviral and inflammatory cytokines which trigger the adaptive immune response, including the production of lymphocytes and antibodies that clear the pathogen and confer lasting immunity (Haga & Bowie, 2005).

In turn, however, poxviruses have sophisticated mechanisms of immune evasion and host manipulation. Poxviruses dedicate a large proportion of their ~200 genes to encoding immunomodulating proteins (Bidgood & Mercer, 2015). Poxviruses disrupt antiviral defences by inhibiting the apoptotic response, producing proteins that obstruct interferons and other immune signalling pathways, and co-opting host gene expression (Bidgood & Mercer, 2015; Seet et al., 2003; Smith et al., 1997, 2018). In summary, much of the outcome of the pox-host interaction depends on whether the host or virus is more successful at controlling the intracellular environment and cellular metabolism.

Despite the well-described interactions between poxviruses and vertebrate hosts from laboratory experiments, little is known about the cellular and physiological effects of pox infection in wild populations. The fact that avian pox seems to cause particularly severe disease in endemic island host species (Williams et al., 2021) suggests that birds without an evolutionary history with pox could lack effective antiviral defences, and/or might be particularly vulnerable to viral manipulation. Transcriptomics provides a way to characterize how infection changes cellular function in the host, opening a window into the complex interactions between host and virus. RNA-seq can create a complete representation of the transcriptome at the time of sampling and thus identify genes that are expressed

FIGURE 1 Top row: Medium ground finch (left) and vegetarian finch (right). Bottom row: Examples of pox lesions on the feet and nares of a vegetarian finch (left); large lesions on the feet of a medium ground finch (Centre), and significant digit loss in a ground finch previously infected with pox (right).

in tandem as well as new candidate genes and pathways that may not have had previous roles in host-virus interactions (Videvall et al., 2015).

The goal of this study was to investigate the transcriptomic effects of avian pox infection in Darwin's finches. We compared infected and uninfected birds of two species: the medium ground finch and the vegetarian finch (*Platyspiza crassirostris*). These two focal species are common residents of the arid and transitional zones of Santa Cruz Island, and thus are probably exposed to similar environmental stressors, including pathogen exposure. However, they are also from distinct branches of the Darwin's finch tree. The vegetarian finch is monotypic and comprises a lineage sister to a large clade containing the ground finches and tree finches (Lamichhaney et al., 2015). The vegetarian finch is more tolerant to another invasive parasite, *Philornis downsi*, than other species of finches (Heimpel et al., 2017), suggesting that it may respond differently to novel threats. Thus, we compared medium ground finches and vegetarian finches to test whether two species of endemic finches differ in their response to infection.

We used the resulting transcriptomic data set to (1) characterize the transcriptomic response of finches to pox infection, (2) test for differences between species of Darwin's finches in their response, and (3) correlate transcriptomic differences with immune phenotypes based on leucocyte profiles in the peripheral blood. We first predicted that transcriptomic changes would primarily occur in immune genes and pathways. Upregulation of these genes would suggest that finches respond adaptively to the virus, while downregulation would indicate that the virus is successfully interfering with host defences and co-opting the host cellular environment. Next, differences between species in their transcriptomic response to infection would suggest that phylogenetically conserved alleles and/or regulatory elements underlie susceptibility of these birds to pox infection. Finally, we predicted that both innate and adaptive components of the immune system would be involved in the transcriptomic response. We expected upregulation of innate immune and inflammatory pathways to be correlated with the presence of innate immune leucocytes (i.e., heterophils) while upregulation of adaptive immune pathways should be correlated with the presence

of lymphocytes and other leucocytes involved in the adaptive immune system (Davis et al., 2008; Minias, 2019).

2 | MATERIALS AND METHODS

2.1 | Sample collection

Finches were captured by mist-net during January and February of 2019 at the Charles Darwin Research Station on Santa Cruz Island. All procedures were approved by the Cornell IACUC (no. 2015–0065), the Ecuadorian Ministry of the Environment (MAE-DNB-CM-2016-0043) and Galápagos National Park (PC-01-18). We sampled free-living birds and diagnosed infection based on the presence of distinctive cutaneous pox-like lesions (Parker et al., 2011). Although this method is not a definitive diagnosis, it is a common way of identifying avian pox in the Galápagos because there are no other identified etiological agents that cause similar pathologies (Lynton-Jenkins et al., 2021; Parker et al., 2011; Tompkins et al., 2017; Vargas, 1987; Zylberberg et al., 2012b). Birds were categorized as "infected" if they had visible pox-like lesions on the feet, tarsi, or face (Figure 1). Lesions were typically accompanied by swelling in the area and occasionally accompanied by necrosis or recent loss of toes. Finches were scored as "uninfected" if feet and tarsi showed no signs of lesions or swelling and there were no signs of previous pox infection (missing digits). Finches that showed signs of previous pox infection (i.e., healed but missing digits) were categorized as "recovered" and were not included in the genomic study. Hereafter we refer to birds with lesions as "infected" and those without as "uninfected."

Following capture, birds were banded with a uniquely numbered aluminium band, and a blood sample (<75 μl) was taken via brachial venipuncture. Birds were aged as hatch year (i.e., fledglings) or after hatch year (i.e., adults) based on plumage and the presence of a fleshy "gape." Birds were sexed by plumage and the presence of reproductive morphology (cloacal protuberance in males or brood patch in females). Male Darwin's finches gain black plumage with increasing sexual maturity and so can be reliably sexed (Grant & Grant, 2014). However, immature males and nonbreeding females can look similar, and therefore sex assignment was tentative in the field for these individuals. A drop of blood was used to make a blood smear for leucocyte profiling. After the smear dried it was fixed in 100% ethanol for 1 min and then air dried again. The rest of the blood sample was divided into two equal parts: the first part was immediately preserved in RNA-later (Invitrogen). The second part of the sample was preserved on wet ice while in the field. Within 4 h, the sample preserved on wet ice was centrifuged at 2000*g* for 6 min to separate the plasma and erythrocytes. The plasma was frozen at −20°C and the erythrocytes were preserved in Queens lysis buffer at room temperature. Blood samples preserved in RNA-later were lysed at room temperature for 24 h, after which they were centrifuged for 10 min at 2000*g* to compact the blood sample. The supernatant RNA-later was removed with a pipette and the remaining sample was frozen at −20°C. Following the field season, the samples were transported to the Cornell University Museum of Vertebrates where frozen RNA-later and plasma samples were stored at −80°C and lysis buffer samples and blood smears were stored at room temperature.

2.2 | RNA extraction

Total mRNA was extracted from RNA-later preserved blood using Qiagen RNeasy kits (Valencia) following manufacturer's protocol. Cells were disrupted by triturating the 20–30 μl of RNA-later preserved blood with the lysis buffer. An additional DNA digestion step was added during extraction using the Qiagen RNase-Free DNase (Valencia) kit following the manufacturer's instructions. RNA extraction quality was verified first using a NanoDrop spectrophotometer (Thermo Scientific) to determine concentration and chemical purity (A260/230 and A260/280 ratios) and then on a FragmentAnalyser (Agilent) to determine RNA integrity. The RNA quality number (RQN) was >9.0 for all but one sample, which had an RQN of 7.0.

2.3 | Library preparation and sequencing

Forty samples were selected for sequencing: 10 infected and 10 uninfected medium ground finches and 10 infected and 10 uninfected vegetarian finches. Previous transcriptomic studies of birds have used as few as three individuals per treatment (Videvall et al., 2015); 5–6 is typical (Franchini et al., 2017; Videvall et al., 2020; Watson et al., 2017). Since our birds were wild caught and we were comparing both infected versus uninfected as well as two species, we sequenced as many individuals as we could within sample and logistical constraints. We selected only individuals that were adults and preferentially selected male birds. We did not have enough samples to only sequence male birds so ultimately, we sequenced 12 male ground finches and eight female ground finches, as well as 15 male vegetarian finches and five female vegetarian finches. Library preparation took place at the Transcriptional Regulation and Expression (TREx) Facility in the Department of Biomedical Sciences, College of Veterinary Medicine, Cornell University. PolyA+ RNA was isolated from total RNA with the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs). TruSeq-barcoded RNAseq libraries were generated with the NEBNext Ultra II Directional RNA Library Prep Kit (New England Biolabs). Each library was quantified with a Qubit 2.0 (dsDNA HS kit; Thermo Fisher) and the size distribution was determined with a fragment analyser (Agilent) prior to pooling. Libraries were sequenced at Novogene on an Illumina NovaSeq 6000 system.

2.4 | Bioinformatics

Reads were trimmed for low quality and adaptor sequences with TrimGalore version 0.6.0 (https://www.bioinformatics.babraham.

ac.uk/projects/trim_galore/), a wrapper for cutadapt (Martin, 2011) and fastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/). We discarded reads shorter than 50 bp and trimmed ends with a quality score < 20, allowing for a maximum error rate of 0.1. The resulting reads were aligned to the *G. fortis* genome archived in NCBI (GeoFor version 1.0 Li et al., 2012) using STAR version 2.7.0e (Dobin et al., 2013); reads that did not align to the finch genome were filtered out for metatranscriptomic analysis (below). We used the same reference genome for both species because there is no annotated vegetarian finch genome; alignment rates for both species were similar (Results). Downstream analyses were run in R (R Core Team, 2021, version 4.0.4) within R Studio (version 1.4.113). We used DESeq2 version 1.26.0 to model normalized counts, calculate log2 fold change between comparison groups, and identify genes that were significantly differentially expressed after correcting for multiple testing using the Benjamini-Hochberg method (Benjamini & Hochberg, 1995; Love et al., 2014); https://doi.org/doi:10.18129/B9.bioc.DESeq2. Initial characterization of global gene expression profiles using a principal components analysis (PCA) revealed different profiles for male and female individuals (Figure 2). Thus, subsequent gene expression analyses were run on the whole data set ($N = 10$ per treatment per species) as well as just for males (ground finch infected $N = 5$, uninfected $N = 7$; vegetarian finch infected $N = 6$, uninfected $N = 9$). We did not analyse females separately because of the low sample size of females. Analyses of the whole data set excluded sex-linked genes (52 out of 16,001 genes) and genes with fewer than 10 reads (2799 genes).

2.5 | Gene set enrichment analysis

We used gene set enrichment analysis (GSEA) to identify biological processes associated with pox infection. GSEA ranks all genes based on their correlation with a relevant phenotype (e.g., infection status) and then tests whether genes in a particular set of interest (e.g., genes involved with a known biological process such as the immune response) are significantly clustered near the top or bottom of the ranked list (Subramanian et al., 2005). We used the log2 fold change estimates generated by DESeq2 to make comparisons between (1) infected and uninfected finches of each species, (2) between infected vegetarian and ground finches, and (3) between uninfected vegetarian and ground finches. We tested for associations between pox phenotype and the 50 Hallmark Gene Sets in the Molecular Signatures Database (Liberzon et al., 2015) and between pox phenotype and the KEGG Orthology Database (Kanehisa et al., 2016; Kanehisa & Goto, 2000). We used the R package clusterProfiler (version 3.18.1; Yu et al., 2012) to perform GSEA; significant *p*-values were adjusted for multiple comparisons using the FDR method (Benjamini & Hochberg, 1995). For the Hallmark Gene Sets we used the gene sets collection for chicken (*Gallus gallus*) and for KEGG gene lists we used the collection for medium ground finch. For each database these were the most closely related organisms to our study species.

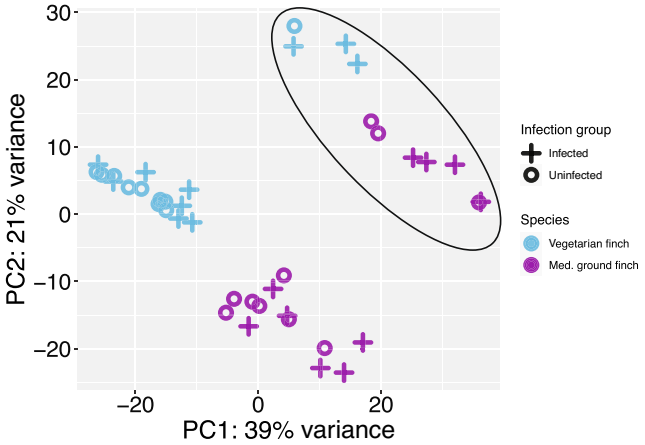

FIGURE 2 PCA of expression profiles for individuals in the study. The cluster of individuals circled in the upper right hand quadrant corresponds to female birds.

2.6 | Metatranscriptomics

We screened the transcriptomic data set for poxvirus reads to verify infection status and quantify viral load. We used Kraken 2 to search for pox reads among all reads that did not align to the ground finch genome (Wood et al., 2019). Kraken 2 uses a k-mer-based approach to classify metagenomic sequences and is highly accurate for classifying viruses (Wood et al., 2019). We used the translated search mode and the standard Kraken 2 database which increases sensitivity when searching for viruses and classified sequences at both a confidence level of 0.00 (default) and 0.05 (slightly more stringent). Because poxviruses are diverse and there are no complete avian pox genomes from the Galápagos available, we filtered results for any sequence that was classified to poxvirus subfamily Chordopoxvirinae (poxviruses of vertebrates).

2.7 | rtPCR and PCR verification of infection

In an additional attempt to confirm infection status, we used PCR and real-time PCR (rtPCR) to amplify poxvirus DNA. We targeted the *Avipoxvirus* 4b core protein gene following methods described in Baek et al. (2020) (for rtPCR) and MacDonald et al. (2019) (for conventional PCR). DNA was extracted from blood samples preserved at room temperature in Queens Lysis Buffer using Qiagen DNeasy kits. Although poxviruses are not typically considered blood-borne pathogens, their DNA is detectable in the blood of human and bird hosts (Baek et al., 2020; Cohen et al., 2007). DNA concentration was quantified on a Qubit 2.0 flourometer (Life Technologies); all samples had a DNA concentration > 5 ng/μl. rtPCR methods followed those described in Baek et al. (2020), with the exception that a standard amount of 25 ng of template DNA was added for all samples. Samples were run in duplicate. Standard PCR methods followed those described in MacDonald et al. (2019); PCR products were then cleaned using a ExoSAP protocol (2 units/μl) (Goldberg

& Mason, 2017) and then Sanger sequenced at the Biotechnology Resource Center at Cornell University.

2.8 | Leucocyte quantification

We characterized immune phenotypes of finches using slide microscopy of blood smears. We profiled leucocytes in the peripheral blood of 39 ground finches (20 infected and 19 uninfected) and 46 vegetarian finches (24 infected and 22 uninfected). Smears were stained in Wright-Geimsa stain for 10 min (or until cells were clearly visible). Smears were examined by one author (C.A.), who was blind to species and pox status, under oil immersion at 1000× magnification until at least 50 leucocytes or 10,000 erythrocytes were counted (median leucocytes = 100; median erythrocytes = 11,153). Leucocytes were identified as lymphocytes, monocytes, eosinophils, or heterophils. We calculated the number of each type of leucocyte per 1000 erythrocytes, as well as the heterophil to lymphocyte ratio, a common indicator of stress in birds (Gross & Siegel, 1983). We confirmed that counts were consistent by initially screening a set of 10 slides twice, in random order, and estimating repeatability using the lmm method in the R package rptR (version 0.9.22). Repeatability for lymphocytes, monocytes, and total leucocytes was >0.80. Basophils, eosinophils, and heterophils were rare or absent from most samples so we did not estimate repeatability for those cell types. We tested for differences in leucocytes per 1000 erythrocytes, lymphocytes per 1000 erythrocytes, monocytes per 1000 erythrocytes, and the heterophil: lymphocyte ratio using generalized linear models with a quasi-Poisson distribution (to account for overdispersion). We originally included sex as a covariate; however, it was not significant in any model and it was removed. We included species and infection status as fixed effects in the final models.

3 | RESULTS

We sequenced a total of 2.9 billion paired end reads with an average of 74.5 million reads per individual. Across all samples the mean percent alignment to the reference genome was 83.0% (range = 74.8%–86.5%). There was no significant difference between species in either the raw number or the percent of reads that aligned to the reference genome (linear model $p > .10$ for both raw number and percent alignment). We obtained expression data for 16,001 genes.

After filtering for coverage, we tested for differential expression of 13,202 genes between various comparison groups: infected versus uninfected finches for each species, infected ground versus infected vegetarian finches, and uninfected ground versus uninfected vegetarian finches. We also repeated the analysis to only include male finches, to eliminate potential confounding effects of sex (Table 1). Between 45 and 1413 genes were significantly differentially expressed between comparison groups (Figure 3, Table 1, Table S1). A total of 80 genes were significantly differentially expressed between infected and uninfected ground finches. Most differentially expressed genes (65/80) were upregulated in infected birds and the mean $\log_2$ fold change between infected and uninfected birds was 1.24 (range = −2.27–4.92; Figure 3, Table S1). A total of 45 genes were significantly differentially expressed between infected and uninfected vegetarian finches. Again, the majority (65/80) of these genes were upregulated in infected birds (mean $\log_2$ fold change = 1.50; range = −2.55–2.78; Figure 3, Table S1). More than 1000 differentially expressed genes were found between infected ground and vegetarian finches, most of which probably reflect interspecific differences in gene expression (Table 1). Similarly, more than 1000 differentially expressed genes were found between uninfected ground and vegetarian finches.

TABLE 1 Numbers of significantly differentially expressed genes (DEG) between comparison groups

	DEG (males and females)	DEG (males only)
Infected ground vs uninfected ground	80	35
Infected vegetarian vs uninfected vegetarian	45	0
Infected ground vs infected vegetarian	1157	1413
Uninfected ground vs uninfected vegetarian	1314	969

Note: Sample size: $N = 10$ individuals per group.
Sample size males only: ground finch infected $N = 5$, uninfected $N = 7$; vegetarian finch infected $N = 6$, uninfected $N = 9$.

We used expression data from all ~13,000 genes to identify biological pathways associated with infection using a GSEA analysis for each of our comparison groups. Between infected and uninfected ground finches, we identified 14 Hallmark sets that were significantly enriched (adjusted $p < .05$; Figure 4a). Among these were several sets associated with innate immune function including *interferon alpha response* and *interferon gamma response*, *IL6 JAK STAT3 signalling*, *complement*, *inflammatory response*, and *allograph rejection*. All pathways were upregulated in infected ground finches. Analysing only male ground finches, we identified eight Hallmark sets, seven of which were included in the larger data set (Figure S1). The only set not identified in the whole data set was *apical surface*, a set related to control of cell polarity in the generation of epithelial cells. The sets with the strongest support between the two analyses were *interferon alpha response*, *interferon gamma response*, *allograph rejection*, and *IL6 JAK STAT3 signalling*. We identified three KEGG pathways that were significantly upregulated in infected ground finches: *toll-like receptor signalling pathway*, *influenza A*, and *phagosome* (Figure 5a). Two different KEGG pathways were significantly enriched between male infected and uninfected ground finches: *phototransduction* and *ribosome* (Figure S2a).

In our other study species, the vegetarian finch, we identified 14 Hallmark sets that were significantly enriched between infected and uninfected individuals (Figure 4b). Overall, the *p*-value support was weaker for Hallmark set enrichment compared to infected

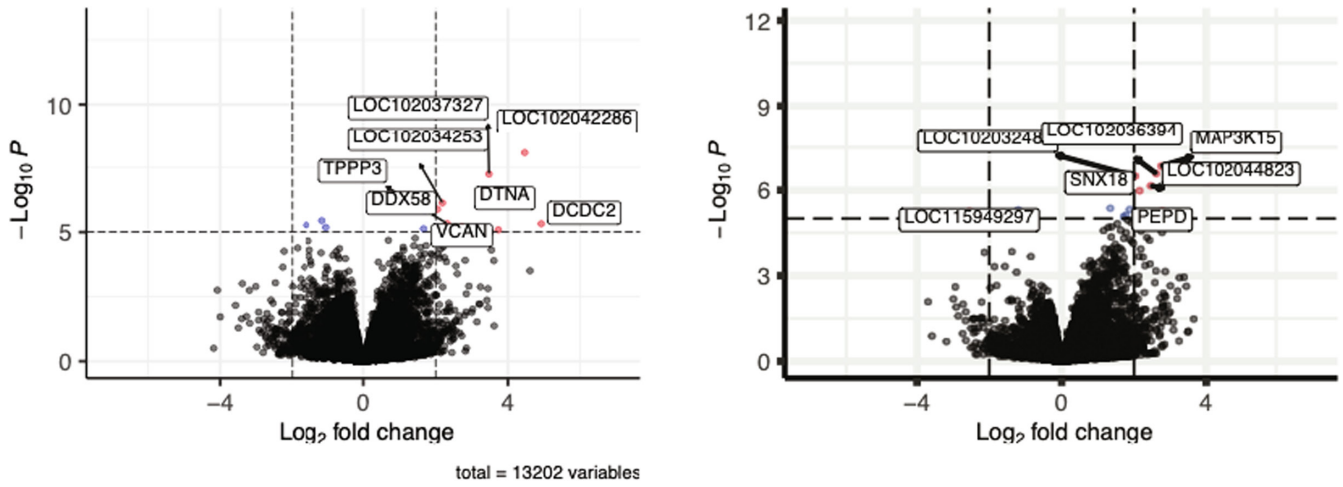

FIGURE 3 Volcano plots of differentially expressed genes in infected versus uninfected ground finches (left) and infected versus uninfected vegetarian finches (right). Each point is a gene. The *x*-axis displays the log2fold change in expression in infected birds compared to uninfected birds. The *y*-axis displays the *p*-value of a Wald test comparing expression of each gene between infected and uninfected groups. Grey points are not significantly differentially expressed. Blue points are genes that are significantly differentially expressed at $p < 1e\text{-}05$. Red points are genes with a log2 fold change >2 that were significantly differentially expressed.

versus uninfected ground finches. Several of the same sets were upregulated in both species in response to infection, including *IL6 JAK STAT3 signalling*, *epithelial mesenchymal transition* (EMT), and *complement*. However, other sets were significantly enriched in just vegetarian finches, including *cholesterol homeostasis* and *mitotic spindle*. One set was significantly downregulated in infected vegetarian finches compared to uninfected vegetarian finches: *oxidative phosphorylation*. Three KEGG pathways were significantly enriched between infected and uninfected vegetarian finches: *cytokine-cytokine receptor interaction*, *phagosome*, which were upregulated in infected finches, and *proteasome*, which was downregulated in infected finches (Figure 5b). Limiting the analysis to just male finches found no significantly enriched Hallmark sets or KEGG pathways.

We then directly compared infected birds of both species to test if vegetarian and ground finches differed in their response to infection. Compared to infected vegetarian finches, infected ground finches upregulated expression of genes significantly associated with 8 Hallmark sets, most notably *interferon response* and *inflammatory response* (Figure 4c). Similar results were found just comparing infected males of each species (Figure S1). Four KEGG pathways were significantly enriched between infected ground and infected vegetarian finches: *cell adhesion molecules*, *toll-like receptor signalling pathway*, *influenza A*, and *neuroactive ligand-receptor interaction* (Figure 5c). Only one KEGG pathway was significantly enriched between infected male ground versus infected male vegetarian finches: *ribosome*, which was downregulated in ground finches (Figure S2b).

We also found significant differences in expression between uninfected finches of each species. Three Hallmark sets were significantly upregulated in ground finches compared to vegetarian finches: *interferon alpha*, *interferon gamma*, and *inflammatory response* (Figure 4d). Of those three, only *inflammatory response* was significantly upregulated comparing uninfected males of each species (Figure S1). One KEGG pathway was significantly enriched in uninfected ground finches compared to uninfected vegetarian finches: *cytokine-cytokine receptor interaction* (Figure 5d). Analysing only male finches identified two significant KEGG pathways: *cytokine-cytokine receptor interaction* and *neuroactive ligand-receptor interaction*, both of which were upregulated in ground finches compared to vegetarian finches (Figure S2c).

Next, we quantified leucocytes in the peripheral blood of ground and vegetarian finches to test if transcriptomic changes were associated with an immune response to infection. Slide microscopy recovered between five and 247 leucocytes per sample (median = 100). The most common types of leucocytes were monocytes and lymphocytes (Table S3); eosinophils and basophils were absent from most samples and so excluded from analysis. There were no significant differences in leucocyte counts between infected and uninfected individuals ($p > .05$ for all cell types). Ground finches had significantly more total leucocytes than vegetarian finches (GLM $p = .04$; Figure 6). On average, ground finches also had more lymphocytes and more monocytes than vegetarian finches; however, the differences were not statistically significant (lymphocytes: GLM $p = .13$, Figure 5b; monocytes: GLM $p = .19$, Figure 6). There was no significant difference in heterophil: lymphocyte ratios between species (GLM $p = .24$, Figure 6).

Finally, we used molecular approaches to attempt to confirm infection and quantify viral load. In our metatranscriptomic analysis, very few sequences were classified as poxviruses. The number of reads assigned to the chordopoxvirinae subfamily in each sample ranged from 44 to 514 under the default confidence criterion. Under a slightly higher stringency criterion of 0.05, the number of reads assigned to chordopoxvirinae ranged between 0 and 17. There were no significant differences in the number of poxvirus reads either between species or between infected/uninfected birds using either the default or more stringent classification standard (Table S2). We additionally attempted to amplify poxvirus DNA using rtPCR and conventional PCR. No sample was positive for poxvirus using the rtPCR screening method. Most samples (33/40)

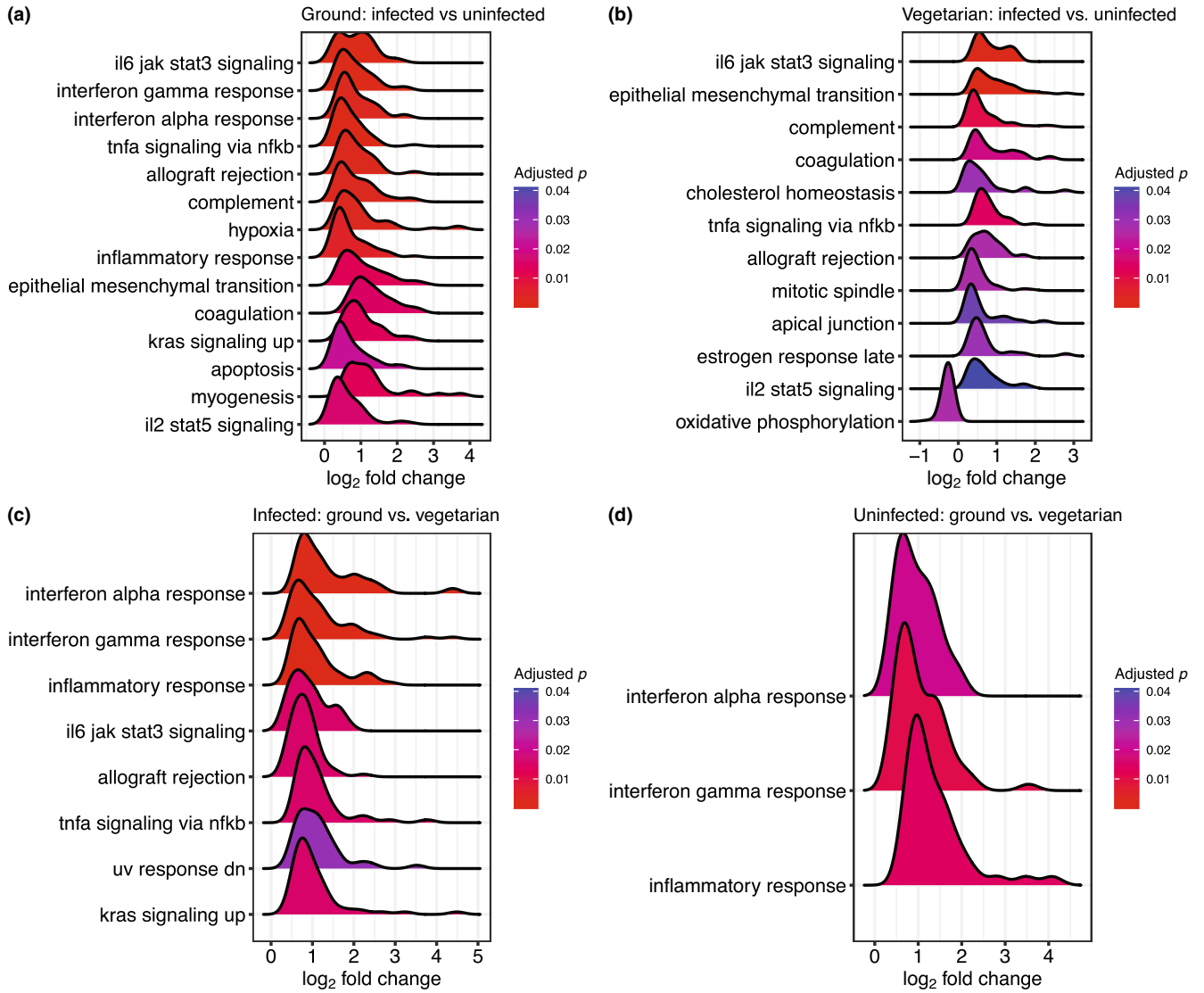

FIGURE 4 Hallmark gene sets significantly enriched between different comparison groups. (a) Infected versus uninfected ground finches, (b) infected versus uninfected vegetarian finches, (c) infected ground versus infected vegetarian, and (d) uninfected ground versus uninfected vegetarian. The *x*-axis displays the frequency distribution of $\log_2$ fold changes of genes in that set. The colour of each set displays the FDR-adjusted *p*-value testing whether expression of genes in that set is significantly associated with infection phenotype.

positively amplified using conventional PCR; however, sequencing results were poor and only two out of 40 sample sequences were a match to poxvirus when searched against the NCBI Genbank nucleotide database. These sequences were a 100% match to avipoxvirus 4b core protein sequences from Galápagos, Hawaiian, and North American birds (Gyuranecz et al., 2013). Both samples were from ground finches classified as infected. Other readable sequences were typically short (<100 bp) and aligned most closely to avian sequences on Genbank.

4 | DISCUSSION

Emerging pathogens can cause disease in naive hosts and are a threat to wildlife populations. Diseases have been particularly devastating in island communities because endemic island species can have limited exposure to parasites and pathogens and few defences against them (Ricklefs, 2010; Williams et al., 2021). However, the severity of disease depends on the interaction between the host and pathogen. Transcriptomics provides one way to characterize this interaction and identify both the costs of infection to the host and the mechanisms of host defence. Here, we document changes in gene expression in two different species of Galápagos finch in response to avian pox virus. Infected birds of both species upregulated gene expression and differentially expressed genes were largely associated with innate immune pathways and processes. However, transcriptomic changes were more pronounced in the ground finch than in the vegetarian finch and some changes in the vegetarian finch are consistent with viral manipulation of the host. Thus, our results suggest that endemic Galápagos finches do detect and respond to infection by an introduced virus, but certain species may be more vulnerable than others to disease.

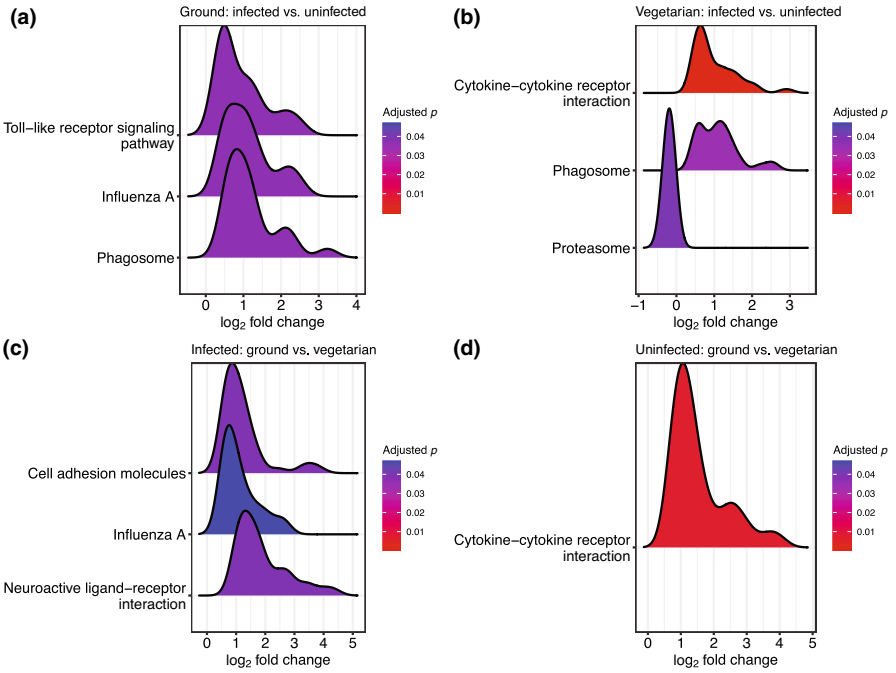

FIGURE 5 KEGG gene pathways significantly enriched between different comparison groups. (a) Infected versus uninfected ground finches, (b) infected versus uninfected vegetarian finches, (c) infected ground versus infected vegetarian, and (d) uninfected ground versus uninfected vegetarian. The *x*-axis displays the frequency distribution of $\log_2$ fold changes of genes in that set. The colour of each set displays the FDR-adjusted *p*-value testing whether expression of genes in that set is significantly associated with infection phenotype.

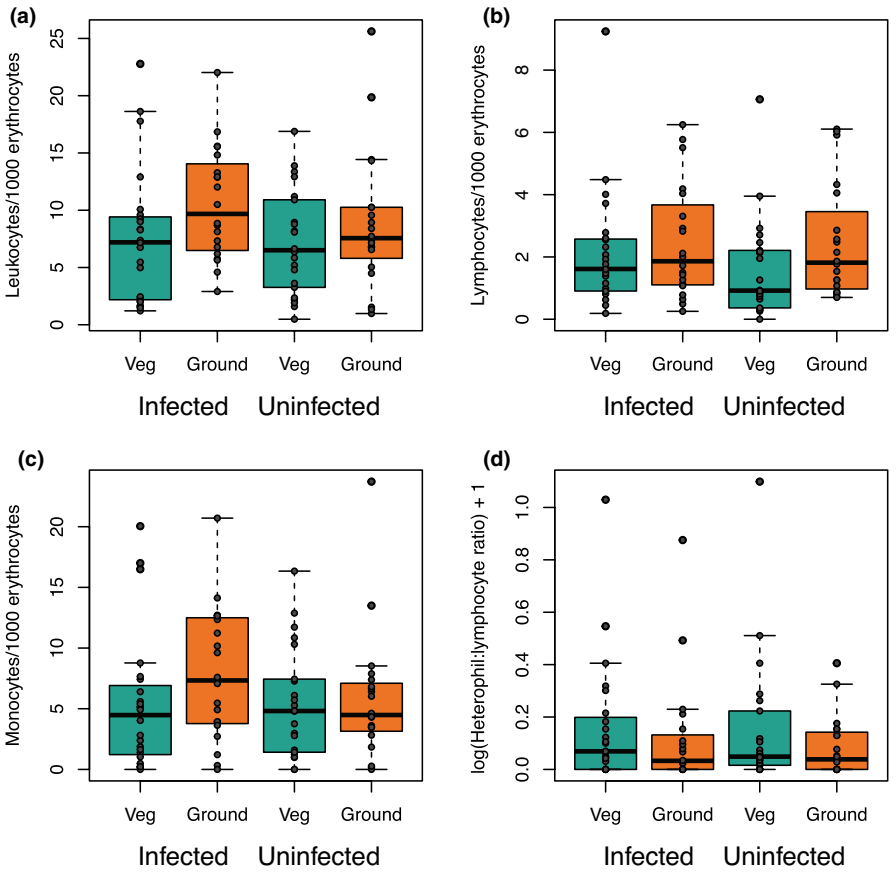

FIGURE 6 Leucocyte counts of infected and uninfected ground and vegetarian finches. (a) Total leucocytes, (b) lymphocytes, (c) monocytes and (d) heterophil: Lymphocyte ratio. (a–c) Counts per 1000 erythrocytes.

4.1 | Upregulation of genes related to host defence against infection

Infected finches of both species upregulated genes involved in immune function. Infected ground finches upregulated interferon and interferon-activated genes (Figure 4a and Figure S1). Interferon is a first line of defence against viral invasion (Guerra et al., 2007; Sen, 2001). Type I interferons (e.g., interferon alpha) are released into the intercellular environment in response to viral infection where they initiate a signalling cascade through the JAK–STAT pathway, which activates a network of hundreds of interferon-stimulated genes (Schneider et al., 2014). The *IL-6*

JAK STAT3 signalling Hallmark set was enriched in both ground and vegetarian finches, suggesting that antiviral signalling is present in both species. Additional KEGG and Hallmark sets associated with type I interferons, *toll-like receptor signalling*, *TNF-α signalling* via *NF-κB*, and *apoptosis*, were upregulated in infected ground finches (Figures 4a and 5a). Toll-like receptors and other viral-sensing proteins detect pathogen infection and trigger the production of interferons (Perdiguero & Esteban, 2009). Tumour necrosis factor (TNF), a cytokine activated by interferon, stimulates the production of nuclear factor kappa B (NF-κB), a transcription factor that is heavily involved in the innate immune response, including by promoting cytokine signalling and apoptosis (Haga & Bowie, 2005). The Hallmark set *interferon gamma* was also enriched in infected ground finches. Interferon gamma is a type II interferon which is promoted by interleukin (IL)-2 and is produced by natural killer cells and T cells during both the innate and adaptive phases of antiviral response (Sen, 2001). Both interferon gamma and apoptosis stop infected cells from producing more virus (Haga & Bowie, 2005; Perdiguero & Esteban, 2009). Thus, most of the Hallmark sets enriched in infected finches—especially ground finches—are involved in the complex innate immune system's response to viral infection.

Transcriptomic responses to infection were also associated with generalized immune responses, indicated by the upregulation of genes in the Hallmark sets *coagulation*, *complement*, *allograft rejection*, and *inflammatory response* and in the KEGG pathways *phagosome* and *cytokine-cytokine receptor interaction* in infected ground and vegetarian finches. Together, these results indicate that Darwin's finches respond to poxvirus infection using known antiviral pathways. The Hallmark and KEGG gene sets that were significantly enriched in infected birds suggest that finches do detect pox infection, respond by producing interferon, a first line antiviral defence, and disseminate the immune response by activating various interferon-stimulated genes.

4.2 | Differences between species in immune response to infection

Although both species upregulated genes involved in immune defence, ground finches had a stronger response to infection than that of vegetarian finches (Figure 4c). Compared to infected vegetarian finches, infected ground finches had higher expression of genes associated with Hallmark immune sets *interferon alpha* and *gamma*, *inflammatory response*, *allograph rejection*, and *IL-6 JAK STAT3 signalling*. Our results suggest that poxvirus may be more successful at manipulating or evading defences of vegetarian finches compared to ground finches. One difference between species was that the *toll-like receptor signalling* KEGG pathway was upregulated in infected ground finches; however, it was not significantly enriched in infected vegetarian finches. Thus, it is possible that vegetarian finches have a more limited ability to detect viral infection compared to ground finches.

Curiously, we found some immune sets were enriched between uninfected vegetarian and uninfected ground finches (Figures 4d and 5d). Compared to uninfected vegetarian finches, uninfected ground finches had higher expression of genes involved with interferon alpha, interferon gamma, and the inflammatory response. We also found that ground finches had significantly higher leucocyte counts than vegetarian finches, regardless of infection status (Figure 6a). These results could indicate that ground finches have higher levels of background immune activation due to biological differences and/or differences in exposure to environmental stressors and pathogens. Previous studies indeed have found variation among other species of Darwin's finches in measures of immune function (Zylberberg et al., 2012a); however, the data presented here are the first to characterize immune defences in vegetarian finches.

4.3 | Potential viral manipulation of the host cellular environment

Other Hallmark and KEGG sets that were significantly enriched in infected birds provide insight into possible effects of infection on host cellular function and metabolism. The Hallmark set *epithelial mesenchymal transition* (EMT) was enriched in both infected ground and infected vegetarian finches compared to uninfected finches (Figure 4a,b). EMT is the process through which epithelial cells shift to a phenotype that is nonpolarized, migratory, and resistant to apoptosis (Kalluri & Weinberg, 2009). This transition is triggered by signalling pathways including inflammation, hypoxia, KRAS and nuclear factor kappa B (NF-κB) (Lin & Wu, 2020), which were also associated with infection (Figure 4a,b). EMT is associated both with tissue regeneration as well as oncogenesis (Gonzalez et al., 2016; Kalluri & Weinberg, 2009). Inflammation triggers EMT, generating fibroblasts, which reconstruct damaged tissues (Kalluri & Weinberg, 2009). Ordinarily, this type of EMT ceases once inflammation reduces (Gonzalez et al., 2016). However, EMT allows dysregulated cells to migrate and invade new tissues, and thus upregulation of EMT is also a hallmark of cancer metastasis (Kalluri & Weinberg, 2009). Several viruses, including herpesviruses, papillomaviruses, and hepatitis viruses promote tumorigenesis by initiating or amplifying EMT (Cyprian et al., 2018; Krump & You, 2018). Poxviruses have not historically been included in this group (Cyprian et al., 2018); however, case studies have speculated that avian poxviruses might indeed have oncogenic properties (Pesaro et al., 2009; Tsai et al., 1997). The upregulation of genes associated with EMT in infected finches thus could reflect both mechanisms of lesion healing as well as potentially dangerous longer-term consequences of viral infection.

The upregulation of genes in the *cholesterol homeostasis* Hallmark set in infected vegetarian finches is another potential sign of host manipulation by the virus. Viruses manipulate host membranes and lipid environment to gain entry to the cell as well as for viral assembly (Deng et al., 2010; Heaton & Randall, 2011). Cholesterol is a particularly important lipid for viruses and a lack of cholesterol can inhibit poxvirus replication (Chung et al., 2005; Deng et al., 2010). Interestingly, the disruption of cholesterol pathways can also

interfere with the interferon-regulated JAK–STAT signalling pathway (Mackenzie et al., 2007) reducing the ability of the host's cells to respond to and signal infection. Vegetarian finches had lower expression of genes in the *IL-6 JAK STAT3 signalling* Hallmark set compared to ground finches (Figure 4c). If the poxvirus effectively manipulates cholesterol homeostasis in vegetarian finches, it may be able to concomitantly interfere with the host immune response. These changes to gene expression point to the complex relationships between immunity and cellular function.

Infection was associated with changes to cellular metabolism, which could also relate to the fight between the host and the virus for control of the cell (Huang et al., 2021). Infected ground finches upregulated genes involved in hypoxia while infected vegetarian finches downregulated genes involved in oxidative phosphorylation. Viruses can manipulate the oxygen environment of the host by activating glycolysis and decreasing oxidative phosphorylation. The shift to anaerobic metabolism supplies energy and biomolecules for viral reproduction (Goodwin et al., 2015; Thyrsted & Holm, 2021). For example, vaccinia virus interferes with prolyl hydroxylase domain 2 (PHD2), an oxygen sensing enzyme, leading to a hypoxic response, which promotes viral replication (Huang et al., 2021; Mazzon et al., 2013). At the same time however, hypoxia can also be part of an immune response by the host to infection. Hypoxia is a common feature of sites of inflammation because of the increased metabolic demand required to synthesize cytokines and anti-microbial enzymes, and support leucocyte activity (Huang et al., 2021; Taylor et al., 2016). Thus, the decreased activity of genes involved in oxidative phosphorylation in vegetarian finches suggests that the poxvirus may be successfully manipulating cellular metabolism in this host to promote replication. In contrast, the upregulation of genes involved in hypoxia in infected ground finches could be related either to immune activation of the host, or evidence of viral manipulation of the cellular environment.

4.4 | Differences in gene expression between sexes

We chose to sequence infected birds with the most visible pox infections regardless of sex, so our data set included both males and females. A PCA of overall gene expression revealed differences between species but also between male and female birds (Figure 1). Thus, we repeated our analysis including just male birds (which comprised most of the samples). The results we found were broadly similar for ground finches (Figures S1 and S2). However, we did not find any significant Hallmark or KEGG sets between infected and uninfected vegetarian finch males (Figure S2). This effect was surprising since only five out of 20 samples were from female birds. These results suggest that responses to pox infection in vegetarian finches are sex-specific and most pronounced in females.

It is possible that pox infection affects males and female finches differently. Male animals are typically more susceptible than females to parasites and pathogens (Zuk & Stoehr, 2010). Moreover, sex-based differences in immune function in birds are stronger during the breeding season than during the nonbreeding period (Valdebenito et al., 2021). Explanations for sex-based differences in include that androgens have an immunosuppressive effect, that females invest more in immune defence, and that males and females differ in their exposure to pathogens (Klein, 2000; Møller et al., 1998).

Evidence that males actually have weaker immune responses than females has been equivocal and dependent on the taxon and immune system aspect under consideration (Kelly et al., 2018; Valdebenito et al., 2021). For instance, recent meta-analyses have found that many common measures of immune function, including the heterophil: lymphocyte ratio, haemagglutination assay, antibody production, and lymphocyte proliferation show no consistent differences between sexes (Kelly et al., 2018; Valdebenito et al., 2021). Overall, adaptive aspects of the immune system do not appear to be strongly sexually dimorphic. In our study we similarly found no differences in lymphocyte counts between sexes.

On the other hand, certain aspects of the innate immune system, including interleukin responses and the TNF-α response to infection, are significantly stronger in females than males (Kelly et al., 2018). In vegetarian finches, transcriptomic responses to infection were dominated by upregulation of innate immune pathways including interlukin-6 and interleukin-2 and TNF-α signalling (Figure 4b). These differences were driven by female birds in our data set and were not significant when analysing only male vegetarian finches. Thus, our results corroborate previous studies and suggest that sex-based differences in gene expression could underlie differences between sexes in innate immune function.

It is unclear why we observed sex-based differences in response to infection in vegetarian finches but not ground finches. Sex differences in immune function are complex and may be specific to species, environment and/or tissue (Valdebenito et al., 2022). It is possible that the breeding periods of vegetarian and ground finches are slightly asynchronous so that the effects of androgens on the vegetarian finch immune system were more pronounced at the point of sample collection. There may also be important differences in the breeding ecology of these species. For instance, if courtship displays and territorial defence in vegetarian finches are more intense than in ground finches, the stress of the breeding season may have had a stronger negative effect on immune function in male vegetarian finches (Hasselquist, 2007). Future work is required to validate these patterns because in this study we did not have the power to directly test for differences in gene expression between males and females.

4.5 | Relationships between gene expression and immune phenotypes

Although we found transcriptomic differences in response to pox, we did not find a significant difference in leucocyte profiles between infected and uninfected birds. Most of the pathways and hallmark sets that were upregulated in infected finches are associated with

the innate immune system. In contrast, apart from heterophils, the leucocytes that we quantified are more typically associated with the adaptive immune system (Davis et al., 2008). We saw few transcriptomic changes associated with adaptive immune activation (e.g., B or T cell signalling, complement) which may explain why we did not see differences in lymphocyte counts between infected and uninfected finches. Although heterophils are part of the innate immune system, they are most associated with antibacterial defences rather than antiviral defences (Harmon, 1998; Minias, 2019).

Our results show the utility of profiling whole mRNA transcriptomes to characterize host responses to infection. Ecoimmunology studies use various assays of immune function to characterize host defence against pathogens or to infer effects of stressors on host health (Davis et al., 2008; Huber et al., 2010; Minias, 2019; Zylberberg et al., 2012a). However, immune assays, including leucocyte profiles, offer only a narrow glimpse into the host's physiology and their interpretation can be complicated (Owen & Clayton, 2007). Whole transcriptome profiling of wild animals provides an agnostic way to characterize the complex and multifaceted response of hosts to viral infection. For instance, we identified effects of pox on not just immune genes and pathways, but also on other aspects of cellular function and metabolism (e.g., cholesterol homeostasis and oxidative phosphorylation in vegetarian finches). These changes show that the effects of viral infection on wild hosts are not just limited to the immune system and reveal other opportunities to study how disease could alter host physiology and health.

4.6 | Study limitations

Darwin's finches are iconic in studies of evolutionary biology and it is vital to understand the threats that emerging diseases pose to these birds. However, research in the Galapagos National Park is highly regulated and experimental manipulations that cause harm to individuals are not permitted. As a result, we had to rely on sampling birds with natural pox infections and could not experimentally infect birds or keep individuals in captivity. Thus, our results are correlative, and there are potential alternative explanations for the patterns we observed. First, we used the presence or absence of lesions as a proxy for pox infection and were unable to confirm infection through molecular methods. Thus, it is possible that some of birds in the “uninfected group” were indeed infected or exposed to pox but did not show symptoms. Second, it is possible that other pathogens in the environment may have contributed to the pathology and/or gene expression patterns we documented.

Diagnosing pox is challenging in wild birds (Baek et al., 2020; Farias et al., 2010). Definitive diagnosis requires isolating live virus and/or using histopathology to confirm the presence of viral inclusion bodies (Bollinger bodies). However, biopsy of lesions from free-living birds presents opportunities for secondary infection (Farias et al., 2010) and histopathology cannot be used to determine if a bird is uninfected. Molecular diagnostics, including PCR screens for pox, do not always agree with histopathology diagnoses or presence of lesions (Baek et al., 2020; Farias et al., 2010; Parker et al., 2011). We had hoped that searching our RNA-seq data set for viral reads would provide an advance in molecular screening for pox, as metatranscriptomic approaches have been useful in studies of other parasites and pathogens (Galen et al., 2020; Shakya et al., 2019). However, we detected few poxvirus reads, either because pox is not transcriptionally active in the blood, or perhaps because viral RNA was not well preserved in RNAlater. Because of these diagnostic challenges, the presence/absence of pox-like lesions is commonly used to diagnose avian pox in the Galápagos, in part because no other etiological agents that would cause similar pathology have been identified (Kleindorfer & Dudaniec, 2006; Lindstrom et al., 2004; Parker et al., 2011; Zylberberg et al., 2012a, 2012b). Nevertheless, a reliable molecular screening method for pox infection would greatly benefit future studies in this system.

Even though we did not detect pox in the blood, we did observe transcriptomic differences in the blood cells between birds with and without pox lesions. These transcriptomic changes might have occurred in leucocytes as they were activated by infection and recruited from general circulation to the site of infection (Chen et al., 2018). It is possible that the transcriptomic effects of pox in infected epithelial cells could be different, and more reflective of viral manipulation of the host's cellular machinery. Still, our data indicate that some effects of poxvirus infection may be observed in noninfected tissues, and that blood can be a useful proxy in cases where wild animals cannot be sampled destructively.

Transcriptomic studies in free-living organisms are important because they can provide novel information about the function of genes that have no analogue in related model organisms (Alvarez et al., 2015). Indeed, several of the genes most differentially expressed in response to pox infection were uncharacterized (Figure 3), meaning we do not have information about their potential function. These genes provide important candidates for further study of host-virus interactions.

4.7 | Implications for Darwin's finch conservation

Eighte of the 29 native species of land birds on the Galápagos Islands are considered threatened by IUCN (Kleindorfer et al., 2022). Emerging disease is a concern in the Galápagos (Causton et al., 2006; Jiménez-Uzcátegui et al., 2019). However, the effects of parasites and pathogens on a population level are difficult to quantify without longitudinal data and the ability to accurately identify disease-caused mortality (Cunningham et al., 2017). Indeed, the role of disease in wildlife declines is probably underestimated due to the cryptic nature of the effects of disease (Cunningham et al., 2017).

It is not clear to what extent disease drives population trends in Galápagos birds. Vegetarian finches are from a monotypic genus in the Galápagos finch radiation (Lamichhaney et al., 2015) and they occur in lower density than most *Geospiza* ground finches (Dvorak

et al., 2012). Population censuses on Santa Cruz during 1997–2010 found that vegetarian finch populations in agricultural zone declined but that medium ground finch populations were stable (Dvorak et al., 2012). A study that took place from 2008 to 2009 found a sharp increase in avian pox prevalence in this same area (Zylberberg et al., 2012b). Neither of our study species is very abundant in the agricultural zone. However, it is possible that avian pox is involved in the differences in population trends between these two species.

Future studies are needed to determine the effects of avian pox on individuals and populations in the Galápagos. Our results suggest that Darwin's finches do detect and respond to pox infection by up-regulating known immune pathways. The presence of this response indicates that Darwin's finches may not be as vulnerable to disease from pox as other endemic island birds, such as the Hawaiian honeycreepers (van Riper III et al., 2002). However, our results add to a growing body of literature showing that Galápagos finch species vary in their vulnerability to novel threats, including other invasive parasites (Cimadom et al., 2014; Heimpel et al., 2017), habitat loss (O'Connor et al., 2010) and introduced predators (Fessl et al., 2010). Still unknown in most cases are the mechanisms that underlie variation in susceptibility to these threats. More work is needed to determine whether avian pox causes a significant disease burden in the vegetarian finch or other species and, if so, why. This information may be useful in effectively allocating resources for conservation and management of Galápagos birds.

AUTHOR CONTRIBUTIONS

Sabrina M. McNew and Birgit Fessl designed the research, Sabrina M. McNew, Janaí Yepez, Diana Carolina Loyola, Kiyoko Gotanda, Catherine Andreadis, and Ashley Saulsberry performed the research, Sabrina M. McNew analysed the data and wrote the study with input from the other authors.

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CONFLICT OF INTEREST

The authors declare no competing interests.

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OPEN RESEARCH BADGES

This article has earned an Open Data Badge for making publicly available the digitally-shareable data necessary to reproduce the reported results. The data is available at [provided https://github.com/smcnew/finch_pox and archived at DOI: 10.5281/zenodo.6984451].

DATA AVAILABILITY STATEMENT

Scripts used for bioinformatics and data analysis are available at https://github.com/smcnew/finch_pox and archived at at DOI: 10.5281/zenodo.6984451. Sequence data are archived in the GenBank SRA database under BioProject PRJNA787639.

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