Differential gene expression reveals host factors for viral shedding variation in mallards

(*Anas platyrhynchos*) infected with low-pathogenic avian influenza virus

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Abstract

Intraspecific variation in pathogen shedding impacts disease transmission dynamics; therefore, understanding the host factors associated with individual variation in pathogen shedding is key to controlling and preventing outbreaks. In this study, ileum and bursa of Fabricius tissues of mallards (*Anas platyrhynchos*) infected with low-pathogenic avian influenza (LPAIV) were evaluated at various post-infection time points to determine genetic host factors associated with intraspecific variation in viral shedding. By analyzing transcriptome sequencing data (RNA-seq), we found that LPAIV-infected mallards do not exhibit differential gene expression compared to uninfected birds, but that gene expression was associated with viral shedding quantity early in the infection. In both tissues, immune genes were mostly up-regulated in higher shedding birds and had significant positive relationships with viral shedding. In the ileum, host genes involved in viral cell entry were down-regulated in low shedders one day post-infection (DPI), and host genes promoting viral replication were up-regulated in high shedders on two DPI. Our findings indicate that viral shedding is a key factor for gene expression differences in LPAIV-infected mallards, and the genes identified in this study could be important for understanding the molecular mechanisms driving intraspecific variation in pathogen shedding.

Introduction

Heterogeneity in pathogen transmission across individuals can impact the magnitude and duration of a disease outbreak (1,2). Some individuals in a population are super-spreaders; that is, they are disproportionately responsible for secondary transmission cases of a contagious pathogen through increased contact rate and/or higher infectiousness (3). The super-spreader

phenomenon can be traced back to Typhoid Mary in the early 1900s (4), humans infected with human immunodeficiency virus (HIV) in the 1980s (5,6), wildlife spillover events in recent decades (4,7), and more recently, in human outbreaks of novel coronaviruses (8). Super-spreading events are more often attributed to higher contact rates by some infected individuals in a population (2,9,10) rather than to variation in infectiousness; however, for pathogens that can be transmitted without direct contact from infected individuals, it stands to reason that pathogen shedding magnitude and duration may be as important as contact rate in generating super-spreading events. The individuals with the highest pathogen shedding, i.e. the “super-shedders”(11), have been shown to be drivers of super-spreading events as demonstrated in cattle infected with *Escherichia coli* O157 (12) and *Brucella abortus* (13), which are host-pathogen systems with indirect sources of transmission. Despite the apparent importance of individual variation in pathogen shedding to transmission dynamics, we know little about the mechanisms underlying differential pathogen shedding.

Identifying the extrinsic and intrinsic factors that affect an individual host’s pathogen shedding can improve our understanding of mechanisms generating and perpetuating super-spreading events. Extrinsic host factors linked to heterogeneity in pathogen shedding include food availability (14,15), geographical location (16), toxicant exposure (17), and myriad environmental factors that can affect host immune competence through chronically elevated stress hormones (18). Intrinsic factors linked to pathogen shedding are a host’s age (19), sex (20,21), physiology (22–24), and the immune response (24–26). In particular, observing how intraspecific variation in pathogen shedding relates to variation in host biological processes can improve our understanding of host factors involved in pathogen transmission.

The advancement of transcriptomics and gene expression studies have further highlighted the importance of the host immune response and physiology to variation in pathogen shedding. For instance, evaluating the bovine intestinal tract transcriptome revealed that T-cell responses and cholesterol metabolism were associated with super-shedding of *E. coli* O157 (24). Another study found that viral load in rainbow trout (*Oncorhynchus mykiss*) infected with infectious hematopoietic necrosis virus was correlated with type I and type II interferon transcript levels early in the infection (25). Analyzing gene expression simultaneously with pathogen shedding improves our understanding of infectious disease progression and the mechanisms driving transmission dynamics, but due to limited studies in only a few host-pathogen systems, further investigation is warranted.

Gene expression studies have also highlighted the value of examining multiple tissues at various time points post-infection since immune responses are tissue-specific and change over time (27–29). Successful viral infections, for example, are dependent upon cellular entry and the ability to evade the host immune response. The host provides initial protection by activating the innate immune response through pattern recognition receptors (PRR) that activate the transcription of interferons, which leads to the production of antiviral interferon-stimulated genes (ISGs) (30,31). This process provides protection to the host since many ISGs are known to inhibit virus cell entry and replication, degrade viral RNA, regulate cell apoptosis, and have regulatory effects on the interferon pathway (31,32). The host also stimulates immune cell dependent mechanisms (cellular immunity) to eliminate infected cells and activate adaptive immunity consisting of antigen recognition by major histocompatibility complex (MHC) molecules, T-cell activation, and B-cell activation for the production of antibodies later in the infection (33–35). Depending on the type of tissue infected, the innate and adaptive immune

systems respond differently (29), thus it's important to evaluate tissue-specific host gene expression at various time-points post-infection.

In this study, we analyzed the association between gene expression and viral shedding variation in avian influenza virus (AIV) infected mallards (*Anas platyrhynchos*). AIVs are type A influenza viruses that can spillover into domestic swine and poultry, thus AIVs pose a significant health risk to domestic animal and human populations (36). Wild waterfowl are the natural reservoirs for low-pathogenic avian influenza virus (LPAIV), a virus transmitted via the fecal-oral route directly or indirectly through AIV contaminated water sources (37). Previous studies have shown that LPAIV-infected mallards exhibit extreme heterogeneity in pathogen shedding (38), which has been linked to both extrinsic (food availability) (15) and intrinsic (gut physiology) (22) host factors. Yet, these factors alone do not explain all the observed variation in viral shedding. Previous gene expression studies of AIV-infected poultry consistently show that gene expression is up-regulated in AIV-infected birds, many of which are associated with the immune response (28,39–43). To our knowledge, however, a longitudinal transcriptome-wide study is yet to be conducted to analyze the association between gene expression and viral shedding.

We hypothesized that gene expression in the ileum and bursa would be associated with intraspecific viral shedding variation in LPAIV-infected mallards because LPAIV primarily replicates in the epithelial cells of both the intestines and bursa of Fabricius (hereafter referred to as “bursa”) of waterfowl by gaining entry via sialic acid receptors (44–47). Further, the antiviral immune response occurs at the site of replication (31,48,49); therefore, we predicted that gene expression would differ: 1) between infected and uninfected birds, 2) between days post-infection, with innate immunity genes expressed early in the infection and adaptive immunity

genes expressed later in the infection, 3) between bird groups with various viral shedding levels, and 4) that viral shedding magnitude would be correlated with the expression level of genes involved in cell entry and the immune response. We tested our hypotheses by evaluating gene expression at various time points post-infection to detect differential gene expression with respect to infection stage, infection site, and viral shedding in an important reservoir host for AIV, the mallard (50–53). In this study we further our understanding about the genes and pathways associated with individual heterogeneity in pathogen shedding.

Materials and methods

Ethics statement

Eggs were collected under the U.S. Fish and Wildlife Permit (M BI 94270-2) and North Dakota Game and Fish Department License #GNF03639403. All capture, handling, infections, and sampling of mallards was approved by the Michigan State University (MSU) Institutional Animal Care and Use Committee (AUF 12/16-211-00). All euthanasia procedures, including the use of pentobarbital sodium and phenytoin sodium solution (Beuthanasia-D Special, Merck Animal Health, Madison, NJ, USA) and CO₂ inhalation, were in accordance with the Animal Welfare Act and Guidelines to the Use of Wild Birds in Research (54).

Birds and virus

Wild mallards collected *in ovo* were used for this study. Analyzing the gene expression of wild birds is important due to the inherent genetic variation within the population (55), a likely factor driving variation in a species response to viral infection (15). Eggs were collected from hen nests located in uncultivated fields of Towner County, North Dakota, USA (48.44, -99.31) in May -

June 2015, and shipped to MSU where they were incubated (Sportsman 1502 Egg Incubator, GQF Manufacturing Co., Savannah, GA) at 37.5°C with 45-50% humidity until hatched. Full details of egg collection and raising ducks can be found in Dolinski et al. (22).

LPAIV A/northern pintail/California/44221-761/2006 (H5N9) was acquired from the USGS National Wildlife Health Center (NWHC) in Madison, WI (USDA Veterinary Permit 44372). The virus was propagated in embryonated pathogen free eggs (Charles River, Norwich, CT, USA) under biosafety level two laboratory conditions at MSU (56). The propagated virus (stock) was sent to USGS NWHC and determined to have a viral titer of 7.63 log EID₅₀/ml using the Reed-Munch 50% egg infectious dose method (57).

Experimental design

Once all the birds were eight weeks or older, they were moved to individual cages (76x61x41cm) and randomly assigned to one of seven experimental groups using a random-pseudostratified method controlling for age, weight, and nest. Group identification and size are shown in Fig 1, with the group name referring to infection status and the day post-infection (DPI) when the group was sacrificed for tissue sample collection.

Fig 1. Experimental timeline of sample collection days post-infection (DPI). Groups are designated by infection status (I = LPAIV-infected, C = uninfected control), DPI sacrificed and (N) quantity of birds in each group. Ileum and bursa were collected upon sacrifice. Cloacal swabs were collected from all living birds designated with the swab icon.

Mallards (n = 60) were inoculated with 5.63 log EID₅₀/ml of LPAIV H5N9 in Dulbecco's Modified Eagle Medium (DMEM; Gibco® by Life Technologies, Grand Island, NY, USA) and the remaining mallards (n = 10) were sham inoculated with sterile DMEM. Inoculum (1.0 mL) was administered to birds with one drop in each eye and nare, then the rest dispensed in the bird's esophagus.

Cloacal swabs were used for quantification of virus shedding (virus titers), because virus in the cloaca represents transmissible virus and the sample can be repeated over time (58). Cloacal swabs were collected on days shown in Fig 1 using cotton-tipped swabs (Puritan, Guilford, ME), placed in 3.0 mL of brain-heart infusion broth (Sigma-Aldrich, St. Louis, MO, USA), and immediately placed on wet ice and stored at -80°C within three hours after collection.

Birds in each group were euthanized and necropsied at intervals illustrated in Fig 1. Birds sacrificed on one DPI (I1, C1) were euthanized via lethal injection with pentobarbital sodium and phenytoin sodium solution (Beuthanasia-D Special, Merck Animal Health, Madison, NJ, USA), but due to complications with intravenous injections, all other birds were sacrificed by CO₂ inhalation. Birds were stored on beds of ice until necropsies were conducted one to six hours after being euthanized. During necropsies, tissue sections (2 mm³) of ileum and bursa were

collected, and the coelomic cavity was assessed for gross pathology. Each tissue section was placed in 1.0 mL of room temperature RNA stabilizing solution (RNAlater®, Sigma-Aldrich, St. Louis, MO, USA), then removed from the solution after 24 hours and stored at -80°C.

Viral RNA extraction and quantification from cloacal swabs

Cloacal swab samples were thawed at room temperature and viral RNA was extracted from 200 µL of each sample using the MagMAX™-96 AI/ND Viral RNA Isolation Kit (Applied Biosystems® by Thermo Fisher Scientific, Vilnius, Lithuania) with modifications (59). Extracted viral RNA was stored in 500 µL of elution buffer. The virus titer for each cloacal swab sample was quantified by real time reverse transcription-quantitative polymerase chain reaction (qPCR) targeting the matrix protein gene. We used the TaqMan® RNA-to-Ct™ 1- Step Kit (Applied Biosystems® by Thermo Fisher Scientific, Foster City, CA, USA), primers/probe specified by Spackman et. al (60), and 2 µL of sample RNA in three replicates. CT values were used to estimate virus quantity as EID₅₀/mL via standard curve (QuantStudio™ 7 Flex Real-Time PCR Software System v1.3) of stock viral RNA (7.63 log EID₅₀/ml) in a series of 10-fold dilutions. The three replicates per sample were averaged to derive a single estimated virus titer for each sample.

Determining shed level groups

Previous analysis of virus titer data in mallards determined that 99% of cumulative LPAIV was detected during the first five DPI (22), and birds were observed to disproportionately shed LPAIV according to the 20/80 rule (38); therefore, birds in LPAIV-infected groups I1, I2, and I5 were subdivided into three shed level groups (Fig 2). High shedders (H) were the top 20% of birds with the highest cloacal virus titer, moderate shedders (M) were the top half of the lowest 80% of virus titers, and low shedders (L) were the bottom half of the lowest 80% of virus titers

in each group. To detect differential gene expression related to active viral shedding during the innate immune response (48,49), only the virus titer collected on the day the bird was sacrificed was used to categorize shed level groups in early-stage infection (groups I1 and I2). To detect differential gene expression related to the adaptive immune response (35,61), cloacal virus titers on one to five DPI were averaged to create a cumulative virus titer for late-stage infection (group I5).

Fig 2. Cloacal swab virus titer profiles for LPAIV-infected mallards sacrificed on one (I1), two (I2), and five (I5) days post-infection (DPI). Shed status of high (H), moderate (M), or low (L) was assigned to individuals by their last virus titer (I1, I2) or the average virus titer across all DPI (I5). Alternative high (Alt.H) and alternative low (Alt.L) shed status groupings were assigned to individuals in I2 based on cumulative virus titers clustered across one and two DPI. Due to low host RNA quality, *individuals were not included in ileum differential gene expression analyses.

After evaluating virus titer data, we observed that group I2 cumulative virus titer data clustered into two distinct shed level groups (Fig 2); therefore, we also conducted differential gene expression analysis using an alternative two-group shed level classification of high shedders (Alt.H) and low shedders (Alt.L) for group I2.

Tissue mRNA extraction, cDNA library preparation, and sequencing

Total mRNA was extracted from ileum and bursa tissue (15-30 mg) using the Qiagen RNeasy mini kit (QIAGEN®, Hilden, Germany) following the manufacturer's protocol. Total mRNA was stored in 500 µL of RNase free water at -80°C.

RNA quality was determined prior to library preparation using a Qubit® Fluorometer 1.0 (Molecular Probes, Life Technologies, Eugene, OR, USA). Sixty-six of 70 bursa samples had RNA integrity number (RIN) scores >8.0 and one bursa sample had a RIN score of <7.0 (S1 Table). All 70 bursa samples were sequenced at the MSU Research Technology Support Facility. Thirty of the 70 ileum samples had RIN scores >8.0 and 35 had RIN scores <7.0. Library preparation and RNA sequencing was performed at MSU for 22 ileum samples with RIN scores >8.0 in groups I2, I5, and C1. Due to lack of availability at MSU, library preparation and RNA sequencing for an additional 20 ileum samples with RIN scores between 4.9 and 9.4 was conducted at the University of Minnesota (UMN) Genomics Center in St. Paul, MN.

Sequencing libraries were prepared using the NuGEN Ovation® Universal RNA preparation kit (Tecan Genomics, Chesapeake Drive, CA, USA) according to the manufacturer's specifications. Custom probes complementary to mallard rRNA sequences were designed and used for the Insert Dependent Adaptor Cleavage (InDA-C) portion of the protocol. Completed library quality was assessed using Qubit® dsDNA HS (Molecular Probes, Life Technologies, Eugene, OR, USA) and Caliper LabChip® GX DNA HS (Caliper, A PerkinElmer Company,

Hopkinton, MA, USA) assays. Libraries sequenced at MSU were combined into six pools for multiplexed sequencing, including five pools of 16 libraries each, and a sixth pool of 13 libraries. Library pools were quantified using Kapa Biosystems Illumina Library qPCR assay prior to sequencing. Each pool was loaded on one lane of an Illumina HiSeq 2500 High Output flow cell (v4) and sequencing was performed in a 2x150bp paired end format using HiSeq SBS reagents. Libraries at UMN were combined into a single pool and sequenced in one lane of an Illumina NovaSeq S1 2x150-bp run. Base calling was done by Illumina Real Time Analysis (RTA) v1.18.64 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v1.8.4.

RNA-seq read processing

Quality of raw cDNA reads was assessed using FastQC (62) prior to trimming and filtering. Sequence regions with a quality score below 15 and sequences shorter than 40bp were removed using Trimmomatic (63). FastQC was used again to verify quality and trimming.

Sequences were aligned to the most recent mallard reference genome, Ensembl ASM874695v1 (64), using STAR (65,66). Gene-specific read counts for bursa samples were acquired with HT-seq (67) using the STAR output, then RSEM (68) was used to estimate read abundance. Due to low RIN scores of 14 ileum samples (RIN = 4.9 – 7.3) likely caused by time delays between euthanasia and necropsy (69), we used DegNorm (70) to account for effects of degradation in ileum samples. Low RIN scores (RIN <8.0) can negatively impact analyses by misaligning sequences resulting in inaccurate transcript counts (71,72); however, DegNorm adjusts read counts for heterogeneity in mRNA sequence degradation on an individual gene basis while also accounting for sequencing depth (70).

Differential expression analyses

We performed differential gene expression analyses using the R packages “EdgeR” (73,74) and “limma” (75) in R version 4.0.0, using the approach outlined by Law et al. (76). Differential expression analysis was performed separately for each tissue and included gene- and transcript-level analyses for the bursa and only gene-level for the ileum, because isoform counts are not estimated by DegNorm.

Lowly expressed genes and transcripts were filtered by requiring expression of >0.5 counts per million in at least 25% of the birds. A false discovery rate (FDR) corrected alpha value of 0.1 and a log fold count difference (LFC) of 0.5 were required to establish differential expression. These thresholds were established to allow for detection of subtle differential gene expression as previously observed between LPAIV-infected and uninfected ducks (41–43). To account for sex-based differences and variation in sequencing pool, sex and pool were included as covariates in each analysis. Gene names (HGNC symbols) were assigned based on ENSEMBL annotation information that accompanied the reference genome. KEGG pathway analyses were performed using the “kegga” function of “limma”. Pathways with p-values <0.05 were determined as over-represented (enriched) pathways of differentially expressed genes or transcripts (DEG, DET) per analysis.

Preliminary differential expression analysis showed that ileum and bursa samples clustered based on tissue-specific gene expression (Section 1 in S2 Document). Improper tissue-based clustering for two individuals (1 and 72) in group C1 suggested improper sample labeling may have occurred in those cases; therefore, these two individuals were removed from analyses, leaving a total of 68 bursa samples and 40 ileum samples. Additionally, due to an absence of differential gene expression between C1 and C29 (Section 2 in S2 Document), we combined the

two control groups into one group “C” for subsequent analyses. Sample size for each group in each differential expression analysis is provided in Table 1.

Table 1. Group sample size for differential gene expression analyses.

Comparison	Tissue	Group					
		C	I1	I2	I5	I15	I29
Infected vs control	Bursa	8	15	10	15	15	10
	Ileum	5	12	10	13		
Infection by DPI	Bursa		15	10	15	15	10
	Ileum		12	10	13		
		Sample size per viral shedding group High, Moderate, Low					
Shed Level	Bursa		3,6,6	2,4,4	3,6,6		
	Ileum		3,4,5	2,4,4	3,4,6		

To address our hypothesis and predictions, differential gene expression comparisons were conducted separately for ileum and bursa tissues. For our prediction that infection status induces differential gene expression, we compared LPAIV-infected groups on each DPI to the uninfected control group. For our prediction that LPAIV infection induces differential gene expression over time, we also compared each LPAIV-infected group to each other. For our prediction that virus shed level induces differential gene expression, we conducted analyses for early (groups I1 and I2) and late-stage infection (group I5) by comparing high, moderate, and low shed level groups for each DPI. Additionally, an alternative analysis was conducted for group I2 using the Alt.L and Alt.H comparison.

Once analyses were complete, a literature search was conducted for DET/DEGs using gene names and search terms including “immune response,” “influenza virus,” and “viral infection” on google scholar. The search yielded a total of 65 peer-reviewed papers. A total of 37 peer-reviewed journal articles were associated with 36 DEGs involved with the immune response (77–79), and a total of 27 journal articles were associated with 39 DEGS that are host cell factors

of influenza A virus replication (80–83), similar to influenza host factors (orthologs or paralogs), or were involved in the replication of other viruses.

Candidate gene analysis

A candidate gene analysis was conducted to observe linear relationships between gene expression and LPAIV virus titers. We selected candidate genes based on what is broadly known about the host’s immune response to viral infections and more specifically about the avian response to AIV infection (31,39,42,84–87). We also selected genes associated with production of sialic acid receptors (i.e. sialyltransferase genes) (88), since sialic acid receptors bind to influenza A viruses and allow for cell entry (46). Seventy-six search terms were identified based on gene function or name (e.g., interferon, IFIT; Table A in S3 File) for an automated search of the ENSEMBL *Anas platyrhynchos* genome using ‘hgnc_symbol’ and ‘description’ fields. This search identified a list of candidate genes and transcripts that were manually filtered to only include relevant genes. Using only transcripts and genes that showed variability in expression, we used linear mixed models to assess the relationship between gene expression and a bird’s virus titer on the day they were sacrificed. Virus titers were adjusted to $\log_{10}(\text{virus titer} + 1)$ transformed values and gene expression was analyzed as $\log_2$ transformed. Each targeted gene and transcript were assessed separately using automated sub-setting and looping through the dataset. Statistical significance was assessed based on a 0.05 false discovery rate. Sex and DPI were included as fixed effects to account for sex and time-point related differences in gene expression, and sequencing pool was a random effect. P-values and conditional R^2 were reported for each significant model, as well as each LPAIV-infected bird group. The “nlme” R package was used for all linear mixed model analyses (89).

Results and discussion

All LPAIV-infected birds tested positive for LPAIV as demonstrated by at least one cloacal swab, ileum tissue, or bursa tissue sample with a Ct value <40 on qRT-PCR (22,90), whereas all uninfected control birds tested negative. No individuals died prior to scheduled euthanasia or exhibited signs of disease, such as ruffled feathers, lethargy, or gross pathology, consistent with previous findings (91).

RNA sequencing

Library preparations generated ≥ 750 M reads for each lane. Mean quality scores for all libraries were $\geq Q30$. Bursa tissue samples yielded an average of 9,261,147 raw reads (range: 4,141,232 – 14,172,345) and ileum samples yielded an average of 10,881,788 raw reads (range 6,737,825 – 17,963,033). After filtering, we retained an average of 8,984,110 bursa reads (range: 4,062,211 – 13,742,163) and an average of 10,526,592 ileum reads (range: 6,567,571 – 17,444,644) per individual for analyses.

Differential gene expression between LPAIV-infected and uninfected control groups

Differential gene expression was not observed between LPAIV-infected and uninfected control birds at each DPI in the bursa or ileum (Section 3 in S2 Document), despite our lenient DEG screening criteria. In previous studies, differential gene expression was observed between AIV-infected and uninfected poultry (28,41–43,92); yet, in studies that looked at both LPAIV and highly pathogenic AIV (HPAIV), gene expression was greater in HPAIV-infected birds than LPAIV-infected birds (41–43). Unlike other gene expression studies that used captive/hatchery-bred mallards (28,41–43,92), we used wild-bred mallards which have been shown to exhibit greater LPAIV viral shedding variation compared to captive-bred mallards (15). The high

interindividual viral shedding variation of wild-bred LPAIV-infected mallards may therefore have reduced our ability to detect a statistical difference in gene expression between infected and uninfected mallards. Interindividual viral shedding variation was not addressed in any of the previous AIV gene expression studies (28,41–43,92); therefore, it is not possible to state whether the variation in viral shedding reported here differs from other studies; however, previous genetic studies show that there are clear genetic differences between wild- and captive-bred ducks (93), including differences in digestive system morphology (94,95). Given the genetic and viral shedding differences between wild-bred and captive-bred ducks, we recommend that future studies use wild birds to better represent transmission dynamics in nature.

Differential gene expression of LPAIV-infected birds over time

Within LPAIV-infected bird groups only, we observed few DETs and DEGs over the course of the infection. In the bursa, one DET (*HSPA8*) and two DEGs (*FOSB*, *HSPA8*) were down-regulated in group I2 compared to group I29 (Sections 3B and 3C in S2 Document). *HSPA8* and *FOSB* had a similar expression pattern that was lowest on two DPI and gradually increased at each DPI thereafter (Fig 3). *FOSB* codes for a transcription factor subunit for AP-1 which regulates the expression of *IFN-γ* (96). *HSPA8* (aka hsc70) mediates viral production by binding to viral protein M1 and NP transporting these viral proteins from the nucleus to the cytoplasm after replication (97,98). Only *HSPA8* was associated with four enriched KEGG pathways in spliceosome, MAPK signaling, protein processing in the endoplasmic reticulum, and endocytosis functions. Since the highest viral shedding quantities are observed on one, two, and three DPI (22), the expression profile of these two genes suggests that mallards may down-regulate *FOSB* and *HSPA8* in the bursa as a protective mechanism in response to LPAIV infection.

Fig 3. Expression of *FOSB* gene and *HSPA8* gene and transcript (“Gene/Trans”) in the bursa of uninfected control and LPAIV-infected groups. Infected groups I1, I2, I5, I15, and I29 correspond to the DPI birds were sacrificed. Differential expression (FDR <0.1, LFC >0.5) was only observed between I2 and I29. *HSPA8* had identical gene expression at the transcript (trans) and gene levels.

In the ileum, only one gene (*SI100A12*) that codes for a calcium binding protein was differentially expressed and had lower expression in group I1 compared to group I2 (Section 3A in S2 Document). No KEGG pathways were enriched in the ileum.

Differential gene expression between virus shed level groups during early stage infection

We observed differential gene expression between shed level groups early in the infection, with the majority of DEGs observed in the ileum. Between shed level groups in the ileum, we observed 406 DEGs in group I1 (Fig 4, Section 4A in S2 Document) and 186 DEGs in group I2

(Fig 4, Section 5A in S2 Document). In group I1, DEGs were only observed between low and moderate shedders with 97% of the DEGs up-regulated in the moderate shedders. In the ileum of group I2, DEGs were observed between high shedders and low/moderate shedders with 98% of the DEGs up-regulated in high shedders. In the alternate shed level comparison (Alt.L vs Alt.H) we observed 266 DEGs in group I2 (Fig 4, Section 6A in S2 Document) with 54% of the DEGs up-regulated in high shedders.

In the bursa, we did not observe any DETs in group I1 (Section 4C in S2 Document) or group I2 (Section 5C and 6C in S2 Document). While no DEGs were observed between shed level groups in the bursa of group I1 (Section 4B in S2 Document), 17 DEGs were observed in group I2 (Fig 4, Section 5B in S2 Document), 88% of which were up-regulated in high shedders, a pattern consistent with DEGs in the ileum. In the bursa of the Alt.L vs. Alt.H group I2 comparison, we observed only one up-regulated gene in low shedders compared to high shedders (Fig 4, Section 6B in S2 Document). Fold count differences for all DEGs can be viewed in S2 Document.

Fig 4. Number of differentially expressed genes (DEG) between shed level groups (low (L), moderate (M), high (H)) of LPAIV-infected mallards in the ileum and bursa on one (I1) and two (I2) DPI. An additional analysis for I2 divided birds evenly into alternative low and high shedders (Alt.L vs. Alt.H). Arrows reflect the number of DEGs that are up (↑) or down (↓) regulated in the first shed level group listed in each comparison.

KEGG pathway information was only available for DEGs between shed level groups in the ileum. In the ileum of group I1, KEGG pathways could be assigned to 51 out of the 406 DEGs observed which represented more than 50% metabolic pathways (Fig 5, Section 4A in S2 Document). Out of all group I1 KEGG pathways assigned, 11 metabolic and two cellular process pathways were enriched ($p < 0.05$), signifying likely biological significance of these pathways. Metabolic functions are commonly linked to viral infections (99), with lipid metabolism (100) and cellular kinases (101) most associated with influenza infections. In chickens infected with HPAIV H5N1, lipid metabolism was enriched on one DPI (41). It is suggested that lipid metabolism is important during the early stages of influenza infection because viral cell entry relies on membrane trafficking, and the cell membrane is made primarily of phospholipids (41). Interestingly, cholesterol metabolism was recently associated with super-shedding cattle infected with *E.coli* O157 (24), which is also an intestinal pathogen. These findings suggest that these metabolic pathways may be important host factors responsible for intraspecific variation of intestinal pathogens.

Fig 5. KEGG pathways of DEGs in the ileum between shed level group comparisons on one day post-infection (I1). KEGG pathways in darker colors indicate statistically enriched pathways ($p < 0.05$). Abbreviations: biosynthesis (bs), signaling pathway (sp), glycosaminoglycan (ga), glycosphingolipid (gs), phosphate pathway (pp).

unexpected given that the influenza A viral replication cycle utilizes host cell machinery to replicate (102).

Fig 6. KEGG pathways of DEGs in the ileum between shed level group comparisons on two days post-infection (I2). KEGG pathways in darker colors indicate statistically enriched pathways ($p < 0.05$). Abbreviations: signaling pathway (sp), protein processing (pp).

Genes of the immune system were differentially expressed between shed level groups early in the infection in both the ileum and the bursa. In the ileum of groups I1 and I2, genes with interferon pathway, cellular immunity, and apoptosis functions were up-regulated in higher shedding birds for 43 out of the 47 significant comparisons (Fig 8, Table 2). In the bursa of group I2, four interferon-stimulated genes were up-regulated in high shedders compared to low shedders (Table 3). The up-regulation of immune genes in higher shedding birds of I1 and I2 groups supports our prediction that viral shedding is closely associated with innate immunity early in the infection.

Fig 8. Immune genes differentially expressed in the bursa (A) and ileum (B) early in the infection. Individuals and their shed status group are on the x-axis and gene names are on the y-axis. Expression of genes is designated by color corresponding to the row Z-score (distance from the mean). Colors of gene names corresponds to their immune functional category.

Table 2. Log(fold change) differences for immune genes differentially expressed in the ileum between shed level groups.

Immune System Category	Gene	I1	I2			Immune Function
		LvM	MvH	LvH	Alt.L v Alt.H	
Regulator of Interferon Pathway	<i>P2RY4 (P2Y4)</i>	-2.24	ns	ns	ns	ligands of P2RY4 are inhibitors of IFN- α (103)
	<i>MAPK6 (ERK3, PRKM6, MK06)</i>	-1.73	ns	ns	ns	regulator of AP-1 activity, NF κ B activity (104)
	<i>RNF128 (GRAIL, GREUL1, RN128)</i>	-1.67	ns	ns	ns	pos. reg. TBK1/IFN- β (105)
	<i>TXN (ADF, TRX)</i>	-1.64	ns	ns	ns	AP-1/NF κ B Transcriptional Activity (106)
	<i>ATG10 (APG10)</i>	ns	ns	-2.31	-1.97	Autophagy, can activate expression of IFN regulating genes (107)
	<i>MACROH2A1 (MH2AFY)</i>	ns	ns	-2.02	-1.54	interferes with binding of NF κ B (108)
	<i>ZC3H13 (KIAA0853, Xio)</i>	ns	ns	-1.78	-1.49	LMP1-mediated NF κ B activation (109)
	<i>HSPD1 (HSP60, CPN60)</i>	ns	ns	-2.06	-1.75	Interacts with IRF3 to enhance IFN- β induction (110)
	<i>STK4 (MST1, KRS2)</i>	ns	ns	-1.76	ns	neg. reg. TBK1-IRF3 signaling (111,112)
	<i>LRRFIP1 (TRIP)</i>	ns	ns	-1.68	ns	activation of Type I IFN, increases NF κ B activity (77,113,114)
Interferon Stimulated Gene	<i>APOB (APOBEC)</i>	-3.57	ns	ns	2.79	inhibition of HIV/HepB replication – unknown for AIV (115,116)
	<i>PTPRF (LAR)</i>	-1.71	ns	ns	ns	STAT regulation – PTPRK (117,118)

	<i>EPSTI1 (ESIP1)</i>	ns	ns	-5.61	-5.2	expressed in macrophages exposed to IFN γ (49,119)
	<i>IFIT5 (AvIFIT)*</i>	ns	ns	ns	-4.16	broad anti-viral activity (79,120)
	<i>MS4A12 (FLJ20217)</i>	ns	ns	ns	3.5	enhances IFN- β activation (78)
Cellular Immunity	<i>MAL (MAL, VIP17)</i>	-2.91	ns	ns	ns	Membrane signaling in T-cells (121)
	<i>MAL2</i>	-2.52	ns	ns	ns	Paralog of MAL (121)
	<i>MALL (MAL, BENE)</i>	-1.71	ns	ns	ns	Paralog of MAL (121)
	<i>SERPINB8 (PI8, CAP2, PSS5)</i>	-2.54	ns	ns	ns	restricts pro-inflammatory cytokine production (122,123)
	<i>SERPINB1 (PI2, MNEI, ELANH2, LEI)</i>	-1.92	ns	ns	2.4	restricts pro-inflammatory cytokine production (122,123)
	<i>LITAF (SIMPLE, PIG7)</i>	-2.45	ns	ns	ns	stimulator of monocytes and macrophages and regulator of TNF- α (124)
	<i>CCL5 (TCP228, RANTES, SCYA5)</i>	-2.05	ns	ns	ns	Chemokine: Promotes leukocyte proliferation (125)
	<i>IL7R (CD127)</i>	-1.5	ns	ns	ns	Chemokine receptor (125)
	<i>IL22RA2 (IL22BP, CRF2X, ZCYTOR16)</i>	-2.52	ns	ns	ns	IL-22 receptor subunit: defense/repair to epithelial cells post-infections (126)
	<i>PLCD1 (PLCδ1, NDNC3)</i>	-1.57	ns	ns	ns	neg. reg. IL-1 β /Fc γ mediated phagocytosis in macrophages
	<i>CD74 (HLADG, P33)</i>	-1.56	ns	ns	ns	neg. reg. dendritic cell motility (80,127,128)

	<i>ADAM28 (ADA28, ADAM23, MDCL)</i>	-1.3	ns	ns	ns	transendothelial migration of lymphocytes (129)
	<i>PTPRC (CD45, T200, GP180, LCA)</i>	-1.05	ns	ns	ns	required for T-cell activation (117,118,130)
	<i>CSF2RA (GMCSFR, CD116)</i>	ns	ns	-3.31	-2.78	macrophage and T-cell regulation (131)
	<i>XCL1 (SCM1, LPTN, LTN)</i>	ns	-3.45	-2.29	ns	dendritic-cell-mediated cytotoxic immune response (132)
	<i>CD83 (HCD83, BL11, HB15)</i>	ns	ns	-1.88	ns	antigen presentation, MHC class II (133,134)
	<i>SEPTIN11 (SEPT11)</i>	ns	ns	-1.61	-1.43	required for FcγR-mediated phagocytosis (135)
Apoptosis	<i>PERP (KCPI, THW, PIGPC1, KRTCAP1)</i>	-2.59	ns	ns	2.07	p53-dependent apoptosis (136)
	<i>CASP6</i>	-1.45	ns	ns	ns	ZBP1-mediated apoptosis (137)
	<i>NIBAN2 (MINERVA, FAM129B)</i>	-1.19	ns	ns	ns	suppression of apoptosis (138)
	<i>CCAR1 (CARP1, DIS)</i>	ns	ns	-1.53	ns	CD437-dependent apoptosis (139)

(L) low, (M) moderate, and (H) high LPAIV shedders; (I1) one and (I2) two DPI; Alt.LvH, alternative low vs high shed level grouping scheme. (+) up-regulated genes and (-) down-regulated genes in group corresponding to the first shed level group in each comparison; ns, non-significant; gene symbols in parentheses indicate alternative nomenclature.

*Also differentially expressed in bursa.

Table 3. Log(fold change) differences for immune genes differentially expressed in the bursa between shed level groups.

Immune System Category	Gene	I2 - LvH	Immune Function
Interferon Stimulated Gene	<i>IFIT5 (AvIFIT)*</i>	-6.57	Broad anti-viral activity (120)
	<i>IFITM2 (DSPA2c)</i>	-5.55	Inhibition of viral replication by multiple mechanisms (79,140)

	<i>USP18 (UBP18, ISG43)</i>	-3.56	Neg. reg. type I IFN (141)
	<i>IFI35 (IFP35)</i>	-3.36	Neg. reg. RIG-I (142)

(L) low and (H) high LPAIV shedders; (I2) two DPI; (-) down-regulated genes in group corresponding to the first shed level group in each comparison; gene symbols in parentheses indicate alternative nomenclature.

*Also differentially expressed in ileum.

The significance of up-regulated immune genes in higher shedding birds early in the infection could indicate that mallards with higher viral shedding elicit stronger innate immune responses than low viral shedders. ISGs up-regulated in high shedding mallards, such as *IFIT5*, *EPSTI1*, *IFITM2*, *USP18*, and *IFI35*, were also up-regulated in AIV-infected poultry of previous studies when compared to uninfected poultry (41,49,143). These ISGs are specifically important for antiviral defense because they participate in various protective mechanisms that inhibit viral replication and regulate the interferon pathway (Table 2 and Table 3). ISG production is dependent upon a series of biochemical cascades beginning with PRRs recognizing intracellular virus which triggers the transcription of type I and II interferons (31,32,48), thus it is likely that intracellular virus is responsible for the interferon response. Although shed level groups were not characterized using intracellular virus titers, cloacal virus titers are known to be positively correlated with virus titers in ileum and bursa tissue (22); therefore, it is likely that high shedders also have high viral replication in these tissues. We suggest that beyond being activated by a viral infection, the magnitude of the innate immune response is dependent on high viral loads.

Due to the antiviral activity of ISGs, it could be hypothesized that high viral shedders with high ISG expression early in the infection may be able to clear the virus faster than low shedders. If so, then we would predict that high shedding birds early in the infection would have lower virus titers later in the infection. Given that tissue samples for gene expression were

obtained when the bird was sacrificed, we were unable to assess their future viral shedding; however, for the birds in groups I5, I15, and I29, the virus titers early in the infection were positively related to virus shed later in the infection (S4 Table), which is consistent with other experimental infection studies (15). Previous findings also suggest that the up-regulation of interferon genes early in the infection does not lead to lower viral shedding (28,144). Cornelissen et. al (28) did not find associations between interferon pathway genes and AIV clearance, and Helin et. al. (144) found that mallards continued to shed virus after observed up-regulation of interferon genes. For future studies, we recommend evaluating non-lethal samples for gene expression such as whole blood (145) or peripheral blood mononuclear cells (146) to gain further insight into the relationship between the immune response and viral shedding over time in individuals.

Interferon regulating genes associated with the production of ISGs, such as *DDX58*, *MDA-5*, *NFκβ*, *AP-1*, *IRF7*, *IFN-α*, *IFN-β*, and *IFN-λ*, that were previously differentially expressed between AIV-infected and uninfected poultry (28,39,41,43), were not differentially expressed in our study. We did however observe the up-regulation of *P2RY4*, *MAPK6*, *RNF128*, *ATG10*, *MACROH2A1*, *ZC3H13*, *STK4*, and *LRRFIP*, which all participate in regulating the interferon pathway (Table 2); therefore, the expression of genes involved in regulating the production of *DDX58*, *MDA-5*, *NFκβ*, *AP-1*, *IRF7*, *IFN-α*, *IFN-β*, and *IFN-λ* could impact the downstream expression of ISGs. These findings suggest that we may have identified key regulating components of the interferon pathway in LPAIV-infected mallards that are associated with viral shedding variation.

DEGs that are, or are related to, host cell factors (paralogs, orthologs) that promote viral replication were also found between shed level groups early in the infection (Table 4). Thirteen

genes involved in promoting viral cell entry, including two sialyltransferases (*ST3GAL1* and *ST3GAL5*), were down-regulated in group I1 low shedders. These results are expected considering that *ST3GAL1* and *ST3GAL5* are enzymes responsible for the production of SA α 2,3Gal (88), the viral binding receptor preferred by LPAIV to gain cell entry for replication (46). We previously detected a positive relationship between LPAIV shedding and SA α 2,3Gal in ileum enterocytes of mallards (22), indicating that birds with fewer SA α 2,3Gal had lower viral shedding quantities. Additionally, 11 genes that promote viral transcription, translation, and intracellular transport were up-regulated in group I2 high shedders. If viral cell entry is the key factor determining viral shedding on one DPI, it would make sense that genes promoting viral replication would be up-regulated in high shedding birds on two DPI. The observed differential gene expression of host cell factors between LPAIV shed level groups could explain why there is an observed one to two day latent period for AIV detection in poultry (147). Birds with fewer SA α 2,3Gal may take longer to accumulate enough intracellular virus for replication, thus resulting in a delayed immune response. These findings suggest a connection between gene expression of host cell factors and immune genes in the ileum.

Table 4. Log(fold change) differences for host cell factor genes that promote viral replication differentially expressed between shed level groups.

Function	Gene	I1	I2			References
		LvM	MvH	LvH	Alt.Lv Alt.H	
Cell Entry	<i>TMPRSS15 (PRSS7, ENTK)</i>	-3.7	ns	ns	ns	(148)
	<i>TSPAN1 (NET1, TM4C, TM4SF)</i>	-2.66	ns	ns	1.39	(149) ^a
	<i>TSPAN8 (TM4SF3)</i>	-2.56	ns	ns	ns	(149) ^a
	<i>ANXA13 (ISA)</i>	-2.31	ns	ns	ns	(150) ^a
	<i>ANXA2 (ANX2, LPC2D, LIP2, P36)</i>	-1.4	ns	ns	ns	(150,151) ^a

	<i>ST3GAL5 (SIAT9, SPDRS, SATI)</i>	-2.25	ns	ns	ns	(88)
	<i>ST3GAL1 (ST3O, SIATFL, SIAT4A)</i>	-1.62	ns	ns	1.74	(88)
	<i>EPS8 (DFNB102)</i>	-1.5	ns	ns	ns	(152)
	<i>EPS8L2 (DFNB106)</i>	-2.07	ns	ns	ns	(152) ^a
	<i>EPS8L3 (HYPT5)</i>	-1.89	ns	ns	ns	(152) ^a
	<i>MAL, (MAL, VIP17)</i>	-2.91	ns	ns	ns	(153)
	<i>CLTA (LCA)</i>	-1.59	ns	ns	ns	(154) ^a
	<i>PLCD1 (PLCδ1, NDNC3)</i>	-1.57	ns	ns	ns	(127,128) ^a
	<i>ATP6V1D (VATD, VMA8)</i>	ns	ns	-2.23	-1.74	(155) ^a
	<i>ATP6V1G1 (ATP6G, VMA10)</i>	ns	ns	-1.72	-1.4	(155) ^a
Nuclear Import of Viral mRNA/RNP	<i>DNAJC2 (MPHOSPH11, MPP11, ZRF1, ZUO1)</i>	ns	-2.21	-2.49	-2.02	(156) ^a
	<i>DNAJC8 (SPF31, HSPC331)</i>	ns	-2.02	-1.9	ns	(156) ^a
	<i>DNAJC21 (DNAJA5, GS3, JJJ1, BMFS3)</i>	ns	ns	-1.84	ns	(156) ^a
	<i>ST13 (HIP, AAG2, SNC6)</i>	ns	ns	-1.71	ns	(157)
Transcription/ Translation	<i>ROS1 (MCF3)</i>	-2.38	ns	ns	ns	(158,159)
	<i>ATP1A1 (CMT2DD)</i>	-1.83	ns	ns	ns	(160) ^a
	<i>CTSH (ACC4, ACC5, CPSB)</i>	-1.03	ns	ns	ns	(161,162) ^a
	<i>SNRPD1 (SMD1, SNRPD)</i>	ns	ns	-2.62	ns	(83)
	<i>SEC61G (SSS1)</i>	ns	ns	-2.19	ns	(163)
	<i>CDK6 (PLSTIRE, CDKN6, MCPH12)</i>	ns	ns	-2.17	-1.79	(164) ^a
	<i>CCNK (CPR4, IDHDF)</i>	ns	-2.16	-2.15	-1.78	(164) ^a
	<i>CDK11A (CDC2L3, P58GTA, PITSLRE)</i>	ns	-1.96	-1.87	ns	(164) ^a

	<i>CCNI (CYI, CYC1)</i>	ns	ns	2.73	1.96	(164) ^a
	<i>RRP15 (KIAA0507, CGI115)</i>	ns	ns	-1.82	ns	(165) ^a
	<i>PRPF38B (NET1)</i>	ns	ns	-1.77	ns	(166) ^a
	<i>SNRPD3 (SMD3)</i>	ns	ns	-1.67	ns	(83) ^a
	<i>YTHDC1 (KIAA1966, YT521)</i>	ns	ns	-1.59	ns	(167)
	<i>DNAJA1 (HSPF4, HSJ2, NEDD&, DNAJ2)</i>	ns	ns	ns	-1.51	(168)
	<i>HSP90AA1 (HSP90, HSPCA, HSP86, HSP89, LAP2, HSPN, EL52)</i>	ns	ns	ns	-1.36	(169)
Nuclear Export of Viral mRNA/RNP	<i>HSPA8 (HSC70, HSP73, LAPI, NIP71)</i>	ns	ns	ns	-1.25	(97)
	<i>RANBP1 (HTF9A)</i>	ns	ns	ns	-1.19	(170) ^a
Cell Exit	<i>RAB11B (YPT3, NDAGSCW)</i>	-1.92	ns	ns	ns	(171,172)
	<i>HSPA5 (BIP, GRP78)</i>	ns	ns	ns	-1.23	(173,174)
	<i>SEPTIN11 (SEPT11)</i>	ns	ns	-1.61	-1.43	(175) ^a

(L) low, (M) moderate, and (H) high LPAIV shedders; (I1) one and (I2) two DPI; Alt.LvH, alternative low vs high shed level grouping scheme. (+) up-regulated genes and (-) down-regulated genes in group corresponding to the first shed level group in each comparison; ns, non-significant; gene symbols in parentheses indicate alternative nomenclature.

^aReferences an isoform, paralog, or family of genes related to the corresponding DEG

Differential Gene Expression Between Virus Shed Level Groups During Late-Stage Infection

Contrary to our *apriori* predictions, differential expression was not observed between shed level groups during late-stage infection (group I5) at the gene or transcript level in the bursa or ileum (Section 7 in S2 Document). As stated above, genes associated with the adaptive immune response, including T-cell regulation and MHC class II expression were differentially expressed in the ileum early in the infection (Table 2). Our results suggest that immune cell mechanisms

that lead to adaptive immunity, as it may relate to viral shedding, occurs earlier in the infection than we had expected.

Candidate Gene Analysis

We assessed 268 candidate genes (Table B in S3 File), and 433 candidate transcripts (Table C in S3 File) for associations with viral shedding. Of those, 11 genes and seven transcripts in the bursa and thirteen genes in the ileum (transcripts were not assessed in ileum) had significant positive linear relationships with cloacal virus titers (S5 Document). No genes or transcripts had negative linear relationships. All the candidate genes with significant relationships to cloacal virus titers were related to the host immune system (Fig 9). Conversely, none of the sialyltransferase candidate genes assessed were related to cloacal virus titers. Expression levels of 11 out of the 15 genes were significantly associated with virus titers early in the infection (one and two DPI), but not late in the infection (five DPI).

Fig 9. Conditional R^2 values for candidate genes with significant ($p < 0.05$) linear relationships to cloacal swab virus titers. Expression of all genes and transcripts shown are positively related to cloacal virus titers. Candidate genes in the bursa were evaluated at the gene and transcript level. Candidate genes in the ileum were only evaluated at the gene level. Colors of gene names corresponds to their immune gene functional category.

Interferon pathway genes up-regulated in previous LPAIV-infected ducks compared to uninfected ducks, such as PRRs *DDX58* and *DHX58*, and ISGs *IFIT5*, *IFITM2*, *OASL*, *RSAD2*, *MX*, and *PARP12* (40,42,85,87,176), were among the immune genes with positive relationships to viral shedding. Cornelissen et. al. (28) suggests that PRR gene expression is elevated in response to higher viral RNA levels, and Smith et. al. (41) suggests that *IFITM2* is strongly up-regulated in response to HPAIV. Collectively, these studies and our findings further suggest that viral shedding may be affecting the expression of interferon pathway genes.

Variation in viral shedding explained more variation in gene expression in the ileum than the bursa at one DPI. Similarly, our results are consistent with a previous study that found gene expression of PRRs was higher in the ileum compared to the bursa on one DPI (28). We also observed more immune DEGs in the ileum compared to the bursa between shed level groups (Fig 8, Table 2). Given that virus titers in the bursa and ileum are both positively related to cloacal swab virus titers with similar R^2 values of 0.43 and 0.41 respectively (22), less viral replication in the bursa does not explain the closer relationship between viral shedding and immune gene expression in the ileum. Instead, we suggest the relationship between virus titers and gene expression in the ileum is explained by SA α 2,3Gal in the ileum tissue, which we previously found to be positively associated with viral shedding (22). The down-regulation of sialyltransferase genes (*ST3GAL1* and *ST3GAL5*) in the ileum of low shedders in group I1 (Table 3) also supports this hypothesis. With fewer receptors (SA α 2,3Gal) for the virus in the ileum, less virus is bound and replicated; therefore, the low viral shedding may induce a weaker innate immune response. Together, these findings may suggest that the ileum is more involved than the bursa in the relationship between the immune response and viral shedding variation.

Conclusions and future directions

Intraspecific variation in pathogen shedding can alter the magnitude and duration of infectious disease outbreaks; hence, understanding the host intrinsic factors associated with variation in pathogen shedding is key for developing disease management plans. Here, we evaluated the variation in tissue-specific gene expression as it relates to infection status, DPI, and pathogen shedding in LPAIV-infected mallards to obtain a better understanding of the molecular mechanisms underlying intraspecific variation in pathogen shedding. Cloacal viral shedding was measured because it represents transmissible virus and sample collection can be repeated over time (58), thus providing a direct link to questions of transmission dynamics. Gene expression was evaluated in the ileum and bursa because these tissues are sites of high LPAIV replication in mallards (44,45,47). Our findings not only provided evidence that gene expression in wild mallards is more associated with viral shedding than by infection status alone, but also provided insight into the host cell machinery utilized by the virus to successfully replicate. These data improve the current understanding of avian host genes involved in LPAIV-infections and the molecular mechanisms associated with viral shedding magnitude.

Collectively, we have identified specific genes and biological processes in the ileum and bursa of LPAIV-infected mallards that are associated with viral shedding heterogeneity; however, this study alone does not confirm an inherent genetic difference between high viral shedders and low viral shedders. Additionally, while we show a relationship between viral shedding and gene expression, we were unable to determine whether viral shedding influenced gene expression or vice versa, which also makes it difficult to determine an inherent cause and effect. Single nucleotide polymorphisms (SNPs) in relevant genes could be accounting for the differential gene expression observed; however, the gene mapping methods we used to detect gene expression did not account for SNP differences (66). A potential next research step would

be to identify SNPs in observed DEGs to determine if a specific gene SNP is associated with intraspecific viral shedding variation (177). For example, a specific allele identified in cattle has been associated with lower proviral loads of bovine leukemia virus (178). When BLV-infected cattle selected for this specific low proviral allele were introduced to uninfected dairy herds, viral transmission stopped (26). We recommend that the genes identified in our current study, such as interferon pathway genes *DDX58*, *LGP2*, *IFIT5*, *IFITM2*, *IFI35*, *FOSB* (Fig 10), viral replication promoting genes *HSPA8*, *ST3GAL1*, *ST3GAL5*, and other genes described in Tables 2, 3, and 4, be examined further by identifying SNPs, utilizing gene-knockout studies, and conducting site-directed mutagenesis to understand the potential for inherent genetic differences (e.g., gene mutations) and associated molecular mechanisms that may contribute to viral shedding variation.

Fig 10. Intracellular innate immune response to avian influenza. Antiviral interferon stimulated genes (ISGs, purple) are produced through a cascade of intracellular signaling processes (signaling molecules are in red) known as the interferon pathway. Pattern recognition receptors (dark blue) and toll-like receptors (TLRs) stimulate adaptor molecules (green) for the production of transcription factors (orange). Transcription factors relocate to the nucleus to trigger the production of interferons (IFN, light blue). Interferons transport outside the nucleus to initiate the JAK-STAT signaling pathway responsible for the production of the ISGF3 complex, which relocates to the nucleus to stimulate the production of ISGs. ISGs have various antiviral effects targeting viral replication or directly acting on the interferon pathway. Created with BioRender.com.

The data acquired in this study should also be used to determine how the intrinsic host factors associated with viral shedding contribute to pathogen transmission. We know that identifying and removing super-shedders is a method of controlling outbreaks as evidenced by modeling the super-shedders of *E.coli* O157-infected cattle (179). If the individuals with the propensity to be the super-shedders can be determined prior to pathogen exposure, new strategies for outbreak prevention could be realized. This future research depends on applying the knowledge gained from exploratory studies like this one, as well as performing similar studies in multiple host-pathogen systems, to obtain a better understanding of how individual host variation contributes to the complexity of disease transmission dynamics. With more knowledge gained,

the more tools wildlife managers, veterinarians, and public health officials will have to better predict and control future outbreaks.

Supporting information

S1 Table. RNA integrity number (RIN) and sequencing pool for mRNA samples isolated from ileum and bursa tissue.

S2 Document. Complete differential gene expression results for all group comparisons tested in ileum and bursa tissue.

S3 File. Excel spreadsheet with candidate gene search terms (Table A), candidate genes (Table B), and candidate transcripts (Table C).

S4 Table. Correlation matrix with Pearson's R correlation coefficients for cloacal swab virus titers on 1-5 days post infection.

S5 Document. Complete candidate gene analysis results showing positive linear relationships between candidate genes in the ileum and candidate genes/transcripts in the bursa.

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References

1. Lloyd-Smith JO, Schreiber SJ, Kopp PE, Getz WM. Superspreading and the effect of individual variation on disease emergence. *Nature*. 2005;438(7066):355–9.
2. Vanderwaal KL, Ezenwa VO. Heterogeneity in pathogen transmission: mechanisms and methodology. *Funct Ecol*. 2016;30:1606–22.
3. Stein RA. Super-spreaders in infectious diseases. *Int J Infect Dis*. 2011;15(8):510–3.
4. Paull SH, Song S, McClure KM, Sackett LC, Kilpatrick AM, Johnson PTJ. From superspreaders to disease hotspots: Linking transmission across hosts and space. *Front Ecol Environ*. 2012;10(2):75–82.

5. Galvani AP, May RM. Epidemiology: dimensions of superspreading. *Nature*. 2005;438(7066):293–5.
6. Gupta S, Anderson RM, May RM. Networks of sexual contacts: implications for the pattern of spread of HIV. *AIDS*. 1989;3:807–17.
7. Hudson PJ, Perkins SE, Cattadori IM. The Emergence of Wildlife Disease and the Application of Ecology. In: *Infectious Disease Ecology: Effects of Ecosystems on Disease and of Disease on Ecosystems*. Princeton University Press; 2010. p. 347–67.
8. Al-Tawfiq JA, Rodriguez-Morales AJ. Super-spreading events and contribution to transmission of MERS, SARS, and SARS-CoV-2 (COVID-19). *J Hosp Infect*. 2020;105(2):111–2.
9. Mossong J, Hens N, Jit M, Beutels P, Auranen K, Mikolajczyk R, et al. Social contacts and mixing patterns relevant to the spread of infectious diseases. *PLoS Med*. 2008;5(3):0381–91.
10. Godfrey SS. Networks and the ecology of parasite transmission: A framework for wildlife parasitology. *Int J Parasitol Parasites Wildl*. 2013;2(1):235–45.
11. Chase-Topping M, Gally D, Low C, Matthews L, Woolhouse M. Super-shedding and the link between human infection and livestock carriage of *Escherichia coli* O157. *Nat Rev Microbiol*. 2008;6(12):904–12.
12. Matthews L, McKendrick IJ, Ternent H, Gunn GJ, Synge B, Woolhouse MEJ. Super-shedding cattle and the transmission dynamics of *Escherichia coli* O157. *Epidemiol Infect*. 2006;134(01):131–42.
13. Capparelli R, Parlato M, Iannaccone M, Roperto S, Marabelli R, Roperto F, et al. Heterogeneous shedding of *Brucella abortus* in milk and its effect on the control of animal brucellosis. *J Appl Microbiol*. 2009;106(6):2041–7.
14. Tadiri CP, Dargent F, Scott ME. Relative host body condition and food availability influence epidemic dynamics: A *Poecilia reticulata*-*Gyrodactylus turnbulli* host-parasite model. *Parasitology*. 2013;140(3):343–51.
15. Arsnoe DM, Ip HS, Owen JC. Influence of body condition on influenza A virus infection in mallard ducks: Experimental infection data. *PLoS One*. 2011;6(8):1–9.
16. Costa F, Wunder EA, Oliveira D De, Bisht V. Patterns in *Leptospira* Shedding in Norway Rats (*Rattus norvegicus*) from Brazilian Slum Communities at High Risk of Disease Transmission. *PLoS Negl Trop Dis*. 2015;9(6):1–14.
17. Jolly PE, Inusah S, Lu B, Ellis WO, Nyarko A, Phillips TD, et al. Association between high aflatoxin B1 levels and high viral load in HIV-positive people. *World Mycotoxin J*. 2013;6(3):255–61.
18. Jankowski MD, Franson JC, Möstl E, Porter WP, Hofmeister EK. Testing independent and interactive effects of corticosterone and synergized resmethrin on the immune response to West Nile virus in chickens. *Toxicology*. 2010;269:81–8.
19. Costa TP, Brown JD, Howerth EW, Stallknecht DE. The Effect of Age on Avian Influenza Viral Shedding in Mallards (*Anas platyrhynchos*). *Avian Dis*. 2010;54:581–5.
20. Zuk M, McKean KA. Sex differences in parasite infections: Patterns and processes. *Int J Parasitol*. 1996;26(10):1009–24.
21. Siva-Jothy JA, Vale PF. Dissecting genetic and sex-specific sources of host heterogeneity in pathogen shedding and spread. *PLoS Pathog*. 2021;17(1):1–22.
22. Dolinski A, Jankowski M, Fair J, Owen J. The Association Between SAα2,3Gal Occurrence Frequency and Avian Influenza Viral Load in Mallards (*Anas Platyrhynchos*)

- and Blue-Winged Teals (*Spatula discors*). *BMC Vet Res.* 2020;16(430):1–17.
23. Cobbold RN, Hancock DD, Rice DH, Berg J, Stilborn R, Hovde CJ, et al. Rectoanal Junction Colonization of Feedlot Cattle by *Escherichia coli* O157:H7 and Its Association with Supershedders and Excretion Dynamics. *Appl Environ Microbiol.* 2007;73(5):1563–8.
24. Wang O, McAllister TA, Plastow G, Stanford K, Selinger B, Guan LL. Host mechanisms involved in cattle *Escherichia coli* O157 shedding: A fundamental understanding for reducing foodborne pathogen in food animal production. *Sci Rep.* 2017;7(1):1–15.
25. Purcell MK, LaPatra SE, Woodson JC, Kurath G, Winton JR. Early viral replication and induced or constitutive immunity in rainbow trout families with differential resistance to Infectious hematopoietic necrosis virus (IHNV). *Fish Shellfish Immunol.* 2010;28(1):98–105.
26. Juliarena MA, Poli M, Sala L, Ceriani C, Gutierrez S, Dolcini G, et al. Association of BLV infection profiles with alleles of the BoLA-DRB3.2 gene. *Anim Genet.* 2008;39(4):432–8.
27. Deist MS, Gallardo RA, Bunn DA, Dekkers JCM, Zhou H, Lamont SJ. Resistant and susceptible chicken lines show distinctive responses to Newcastle disease virus infection in the lung transcriptome. *BMC Genomics.* 2017;18(1):1–15.
28. Cornelissen JBWJ, Post J, Peeters B, Vervelde L, Rebel MJM. Differential innate responses of chickens and ducks to low-pathogenic avian influenza. *Avian Pathol.* 2012;41(6):519–29.
29. Hu W, Pasare C. Location, location, location: tissue-specific regulation of immune responses. *J Leukoc Biol.* 2013;94(3):409–21.
30. Koyama S, Ishii KJ, Coban C, Akira S. Innate immune response to viral infection. *Cytokine.* 2008;43(3):336–41.
31. Santhakumar D, Rubbenstroth D, Martinez-Sobrido L, Munir M. Avian interferons and their antiviral effectors. *Front Immunol.* 2017;8:1–19.
32. Schoggins JW, Rice CM. Interferon-stimulated genes and their antiviral effector functions. *Curr Opin Virol.* 2011;1(6):519–25.
33. Bonilla FA, Oettgen HC. Adaptive immunity. *J Allergy Clin Immunol.* 2010;125(2):S33–40.
34. Rong E, Wang X, Chen H, Yang C, Hu J, Liu W, et al. Molecular mechanisms for the adaptive switching between the OAS/RNase L and OASL/RIG-I pathways in birds and mammals. *Front Immunol.* 2018;9:1–14.
35. Suarez DL, Schultz-Cherry S. Immunology of avian influenza virus: A review. *Dev Comp Immunol.* 2000;24(2–3):269–83.
36. Webster RG, Bean WJ, Gorman OT, Chambers TM, Kawaoka Y. Evolution and ecology of influenza A viruses. *Microbiol Rev.* 1992;56(1):152–79.
37. Roche B, Lebarbenchon C, Gauthier-Clerc M, Chang C-M, Thomas F, Renaud F, et al. Water-borne transmission drives avian influenza dynamics in wild birds: the case of the 2005–2006 epidemics in the Camargue area. *Infect Genet Evol.* 2009;9(5):800–5.
38. Jankowski MD, Williams CJ, Fair JM, Owen JC. Birds Shed RNA-Viruses According to the Pareto Principle. *PLoS One.* 2013;8(8):e72611.
39. Huang Y, Li Y, Burt DW, Chen H, Zhang Y, Qian W, et al. The duck genome and transcriptome provide insight into an avian influenza virus reservoir species. *Nat Genet.* 2013;45(7):776–83.

40. Ranaware PB, Mishra A, Vijayakumar P, Gandhale PN, Kumar H, Kulkarni DD, et al. Genome wide host gene expression analysis in chicken lungs infected with avian influenza viruses. *PLoS One*. 2016;11(4):1–16.
41. Smith J, Smith N, Yu L, Paton I, Gutowska M, Forrest H, et al. A comparative analysis of host responses to avian influenza infection in ducks and chickens highlights a role for the interferon-induced transmembrane proteins in viral resistance. *BMC Genomics*. 2015;16(1):1–19.
42. Vandervlen HA, Petkau K, Ryan-Jean KEE, Aldridge JR, Webster RG, Magor KE. Avian influenza rapidly induces antiviral genes in duck lung and intestine. *Mol Immunol*. 2012;51(3–4):316–24.
43. Fleming-Canepa X, Aldridge JR, Canniff L, Kobewka M, Jax E, Webster RG, et al. Duck innate immune responses to high and low pathogenicity H5 avian influenza viruses. *Vet Microbiol*. 2019;228:101–11.
44. Costa TP, Chaves AJ, Valle R, Darji A, van Riel D, Kuiken T, et al. Distribution patterns of influenza virus receptors and viral attachment patterns in the respiratory and intestinal tracts of seven avian species. *Vet Res*. 2012;43(28):1–13.
45. Daoust P-Y, Kibenge FSB, Fouchier RAM, Van De Bildt MWG, Van Riel D, Kuiken T. Replication of low pathogenic avian influenza virus in naturally infected mallard ducks (*Anas platyrhynchos*) causes no morphologic lesions. *J Wildl Dis*. 2011;47(2):401–9.
46. Gambaryan A, Yamnikova S, Lvov D, Tuzikov A, Chinarev A, Pazynina G, et al. Receptor specificity of influenza viruses from birds and mammals: new data on involvement of the inner fragments of the carbohydrate chain. *Virology*. 2005;334(2):276–83.
47. Webster RG, Yakhno M, Hinshaw VS, Bean WJ, Copal Murti K. Intestinal influenza: Replication and characterization of influenza viruses in ducks. *Virology*. 1978;84(2):268–78.
48. Campbell LK, Magor KE. Pattern Recognition Receptor Signaling and Innate Responses to Influenza A Viruses in the Mallard Duck, Compared to Humans and Chickens. *Front Cell Infect Microbiol*. 2020;10.
49. Evseev D, Magor KE. Innate immune responses to avian influenza viruses in ducks and chickens. *Vet Sci*. 2019;6(1).
50. Halvorson D, Karunakaran D, Senne D, Kelleher C, Bailey C, Abraham A. Epizootiology of Avian Influenza-Simultaneous Monitoring of Sentinel Ducks and Turkeys in Minnesota. *Avian Dis*. 1983;27(1):77–85.
51. Jimenez-Bluhm P, Di Pillo F, Bahl J, Osorio J, Schultz-Cherry S, Hamilton-West C. Circulation of influenza in backyard productive systems in central Chile and evidence of spillover from wild birds. *Prev Vet Med*. 2018;153:1–6.
52. Li J, Dohna H zu, Anchell NL, Adams SC, Dao NT, Xing Z, et al. Adaptation and transmission of a duck-origin avian influenza virus in poultry species. *Virus Res*. 2010;147(1):40–6.
53. Stallknecht DE, Brown JD. Ecology of Avian Influenza in Wild Birds. In: *Avian Influenza*. 2008. p. 43–58.
54. Fair JM, Paul E, Jones J. Guidelines to the Use of Wild Birds in Research. In: Fair JM, Paul E, Jones J, editors. *The Ornithological Council*. Washington, D.C.; 2010.
55. Svobodová J, Pinkasová H, Hyršl P, Dvořáčková M, Zita L, Kreisinger J. Differences in the growth rate and immune strategies of farmed and wild mallard populations. *PLoS One*.

- 2020;15(8 August):1–19.
56. Woolcock PR. Avian influenza virus isolation and propagation in chicken eggs. *Methods Mol Biol.* 2008;436:35–46.
57. Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. *Am J Hyg.* 1938;27(3):435–69.
58. Henaux V, Samuel MD. Avian influenza shedding patterns in waterfowl: Implications for surveillance, environmental transmission, and disease spread. *J Wildl Dis.* 2011;47(3):566–78.
59. Das A, Spackman E, Pantin-jackwood MJ, Suarez DL. Removal of real-time reverse transcription polymerase chain reaction (RT-PCR) inhibitors associated with cloacal swab samples and tissues for improved diagnosis of Avian influenza virus by RT-PCR. *J Vet Diagnostic Investig.* 2009;21:771–8.
60. Spackman E, Suarez DL. Type a influenza virus detection and quantitation by real-time RT-PCR. *Methods Mol Biol.* 2008;436(M):19–26.
61. Jourdain E, Gunnarsson G, Wahlgren J, Latorre-Margalef N, Br??jer C, Sahlin S, et al. Influenza virus in a natural host, the mallard: Experimental infection data. *PLoS One.* 2010;5(1).
62. Andrews S. FastQC: A quality control tool for high throughput sequence data. Cambridge, UK: Babraham Institute; 2011.
63. Bolger AM, Lohse M, Usadel B. Trimmomatic: A flexible trimmer for Illumina sequence data. *Bioinformatics.* 2014;30(15):2114–20.
64. Mallard assembly and gene annotation [Internet]. 2019 [cited 2020 Nov 24]. Available from: http://uswest.ensembl.org/Anas_platyrhynchos/Info/Annotation
65. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, et al. STAR: Ultrafast universal RNA-seq aligner. *Bioinformatics.* 2013;29(1):15–21.
66. Dobin A, Gingeras TR. Mapping RNA-seq Reads with STAR. *Curr Protoc Bioinforma.* 2015;51(1):11.14.1–11.14.19.
67. Anders S, Pyl PT, Huber W. HTSeq-A Python framework to work with high-throughput sequencing data. *Bioinformatics.* 2015;31(2):166–9.
68. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics.* 2011;12(1):323.
69. Cheviron ZA, Carling MD, Brumfield RT. Effects of postmortem interval and preservation method on rna isolated from field-preserved avian tissues. *Condor.* 2011;113(3):483–9.
70. Xiong B, Yang Y, Fineis FR, Wang JP. DegNorm: Normalization of generalized transcript degradation improves accuracy in RNA-seq analysis. *Genome Biol.* 2019;20(1):1–18.
71. Copois V, Bibeau F, Bascoul-Mollevi C, Salvetat N, Chalbos P, Bareil C, et al. Impact of RNA degradation on gene expression profiles: Assessment of different methods to reliably determine RNA quality. *J Biotechnol.* 2007;127(4):549–59.
72. Gallego Romero I, Pai A, Tung J, Gilad Y. Impact of RNA degradation on measurements of gene expression. *BMC Biol.* 2014;12(42):1–13.
73. Robinson MD, McCarthy DJ, Smyth GK. edgeR: A Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics.* 2010;26(1):139–40.
74. McCarthy DJ, Chen Y, Smyth GK. Differential expression analysis of multifactor RNA-Seq experiments with respect to biological variation. *Nucleic Acids Res.* 2012;1–10.
75. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential

- expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res.* 2015;43(7):e47.
76. Law CW, Alhamdoosh M, Su S, Dong X, Tian L, Smyth GK, et al. RNA-seq analysis is easy as 1-2-3 with limma, Glimma and edgeR. *F1000Research.* 2018;5(1408):1–29.
77. Shim JM, Kim J, Tenson T, Min JY, Kainov DE. Influenza virus infection, interferon response, viral counter-response, and apoptosis. *Viruses.* 2017;9(8):1–12.
78. Zhang X, Yang W, Wang X, Zhang X, Tian H, Deng H, et al. Identification of new type I interferon-stimulated genes and investigation of their involvement in IFN- β activation. *Protein Cell.* 2018;9(9):799–807.
79. Schoggins JW. Interferon-Stimulated Genes: What Do They All Do? *Annu Rev Virol.* 2019;6:567–84.
80. Shaw ML, Stertz S. Role of Host Genes in Influenza Virus Replication. In: *Roles of Host Gene and Non-coding RNA Expression in Virus Infection.* Springer, Cham; 2017. p. 151–89.
81. Peacock TP, Sheppard CM, Staller E, Barclay WS. Host Determinants of Influenza RNA Synthesis. *Annu Rev Virol.* 2019;6:215–33.
82. König R, Stertz S, Zhou Y, Inoue A, Hoffmann HH, Bhattacharyya S, et al. Human host factors required for influenza virus replication. *Nature.* 2010;463(7282):813–7.
83. Dubois J, Terrier O, Rosa-Calatrava M. Influenza viruses and mRNA splicing: Doing more with less. *MBio.* 2014;5(3):1–13.
84. Stambas J, Ye S, Izzard L, Dlugolenski DA, Tripp RA, McCulloch D, et al. ADAMTS5 and its substrate versican play a critical role in influenza virus immunity. *J Immunol.* 2017;198(1):121–3.
85. Cao Y, Huang Y, Xu K, Liu Y, Li X, Xu Y, et al. Differential responses of innate immunity triggered by different subtypes of influenza A viruses in human and avian hosts. *BMC Med Genomics.* 2017;10(4):41–54.
86. Kuchipudi S V, Tellabati M, Sebastian S, Londt BZ, Jansen C, Vervelde L, et al. Highly pathogenic avian influenza virus infection in chickens but not ducks is associated with elevated host immune and pro-inflammatory responses. *Vet Res.* 2014;45(1):1–18.
87. Barber MRW, Aldridge JR, Fleming-Canepa X, Wang YD, Webster RG, Magor KE. Identification of avian RIG-I responsive genes during influenza infection. *Mol Immunol.* 2013;54(1):89–97.
88. Harduin-lepers A. Glycobiology Insights comprehensive Analysis of sialyltransferases in Vertebrate Genomes. *Glycobiol Insights.* 2010;2:29–61.
89. Pinheiro J, Bates D, DebRoy S, Sarkar D. Linear and Nonlinear Mixed Effects Models. R Packag version 3. 2007;1–97.
90. Costa TP, Brown JD, Howerth EW, Stallknecht DE. Variation in viral shedding patterns between different wild bird species infected experimentally with low-pathogenicity avian influenza viruses that originated from wild birds. *Avian Pathol.* 2011;40(2):119–24.
91. Franca M, Stallknecht DE, Poulson R, Brown J, Howerth EW. The Pathogenesis of Low Pathogenic Avian Influenza in Mallards Research Note — The Pathogenesis of Low Pathogenic Avian Influenza in Mallards. *Avian Dis.* 2012;56(4s1):976–80.
92. Maughan MN, Dougherty LS, Preskenis LA, Ladman BS, Gelb Jr J, Spackman E V, et al. Transcriptional analysis of the innate immune response of ducks to different species-of-origin low pathogenic H7 avian influenza viruses. *Virol J.* 2013;10(1):1–11.
93. Söderquist P, Elmberg J, Gunnarsson G, Thulin CG, Champagnon J, Guillemain M, et al.

- Admixture between released and wild game birds: a changing genetic landscape in European mallards (*Anas platyrhynchos*). *Eur J Wildl Res.* 2017;63(6).
94. Champagnon J, Guillemain M, Elmberg J, Massez G, Cavallo F, Gauthier-Clerc M. Low survival after release into the wild: Assessing “the burden of captivity” on Mallard physiology and behaviour. *Eur J Wildl Res.* 2012;58(1):255–67.
95. Moore SJ, Battley PF. Differences in the digestive organ morphology of captive and wild Brown Teal *Anas chlorotis* and implications for releases. *Bird Conserv Int.* 2006;16(3):253–64.
96. Foletta VC, Segal DH, Cohen DR. Transcriptional regulation in the immune system: All roads lead to AP-1. *J Leukoc Biol.* 1998;63(2):139–52.
97. Stricher F, Macri C, Ruff M, Muller S. HSPA8/HSC70 chaperone protein: Structure, function, and chemical targeting. *Autophagy.* 2013;9(12):1937–54.
98. Watanabe K, Fuse T, Asano I, Tsukahara F, Maru Y, Nagata K, et al. Identification of Hsc70 as an influenza virus matrix protein (M1) binding factor involved in the virus life cycle. *FEBS Lett.* 2006;580(24):5785–90.
99. Sanchez EL, Lagunoff M. Viral activation of cellular metabolism. *Virology.* 2015;479–480:609–18.
100. Zhou Y, Pu J, Wu Y. The role of lipid metabolism in influenza A virus infection. *Pathogens.* 2021;10(3):1–11.
101. Meineke R, Rimmelzwaan GF, Elbahesh H. Influenza virus infections and cellular kinases. *Viruses.* 2019;11(2).
102. Watanabe T, Watanabe S, Kawaoka Y. Cellular Networks Involved in the Influenza Virus Life Cycle. *Cell Host Microbe.* 2010;7(6):427–39.
103. Shin A, Toy T, Rothenfusser S, Robson N, Vorac J, Dauer M, et al. P2Y receptor signaling regulates phenotype and IFN- α secretion of human plasmacytoid dendritic cells. *Blood.* 2008;111(6):3062–9.
104. Tripathi S, Pohl MO, Zhou Y, Rodriguez-Frandsen A, Wang G, Stein DA, et al. Meta- and Orthogonal Integration of Influenza “oMICs” Data Defines a Role for UBR4 in Virus Budding. *Cell Host Microbe.* 2015;18(6):723–35.
105. Song G, Liu B, Li Z, Wu H, Wang P, Zhao K, et al. E3 ubiquitin ligase RNF128 promotes innate antiviral immunity through K63-linked ubiquitination of TBK1. *Nat Immunol.* 2016;17(12):1342–51.
106. Hirota K, Matsui M, Iwata S, Nishiyama A, Mori K, Yodoi J. Ap-1 transcriptional activity is regulated by a direct association between thioredoxin and Ref-1. *Proc Natl Acad Sci U S A.* 1997;94(8):3633–8.
107. Zhao Q, Hu ZY, Zhang JP, Jiang JD, Ma YY, Li JR, et al. Dual Roles of Two Isoforms of Autophagy-related Gene ATG10 in HCV-Subgenomic replicon Mediated Autophagy Flux and Innate Immunity. *Sci Rep.* 2017;7(1):1–15.
108. Angelov D, Molla A, Perche PY, Hans F, Côté J, Khochbin S, et al. The histone variant macroH2A interferes with transcription factor binding and SWI/SNF nucleosome remodeling. *Mol Cell.* 2003;11(4):1033–41.
109. Gewurz BE, Towfic F, Mar JC, Shinnars NP, Takasaki K, Zhao B, et al. Genome-wide siRNA screen for mediators of NF- κ B activation. *Proc Natl Acad Sci U S A.* 2012;109(7):2467–72.
110. Lin L, Pan S, Zhao J, Liu C, Wang P, Fu L, et al. HSPD1 interacts with IRF3 to facilitate interferon-beta induction. *PLoS One.* 2014;9(12):1–18.

- 944 111. Jorgensen SE, Al-Mousawi A, Assing K, Hartling U, Grosen D, Fisker N, et al. STK4
945 Deficiency Impairs Innate Immunity and Interferon Production Through Negative
946 Regulation of TBK1-IRF3 Signaling. *J Clin Immunol*. 2020;
- 947 112. Meng F, Zhou R, Wu S, Zhang Q, Jin Q, Zhou Y, et al. Mst1 shuts off cytosolic antiviral
948 defense through IRF3 phosphorylation. *Genes Dev*. 2016;30(9):1086–100.
- 949 113. Bagashev A, Fitzgerald MC, Larosa DF, Rose PP, Cherry S, Johnson AC, et al. Leucine-
950 rich repeat (in Flightless I) interacting protein-1 regulates a rapid type I interferon
951 response. *J Interf Cytokine Res*. 2010;30(11):843–52.
- 952 114. Dai P, Jeong SY, Yu Y, Leng T, Wu W, Xie L, et al. Modulation of TLR Signaling by
953 Multiple MyD88-Interacting Partners Including Leucine-Rich Repeat Fli-I-Interacting
954 Proteins. *J Immunol*. 2009;182(6):3450–60.
- 955 115. Bonvin M, Achermann F, Greeve I, Stroka D, Keogh A, Inderbitzin D, et al. Interferon-
956 inducible expression of APOBEC3 editing enzymes in human hepatocytes and inhibition
957 of hepatitis B virus replication. *Hepatology*. 2006;43(6):1364–74.
- 958 116. Wang FX, Huang J, Zhang H, Ma X, Zhang H. APOBEC3G upregulation by alpha
959 interferon restricts human immunodeficiency virus type 1 infection in human peripheral
960 plasmacytoid dendritic cells. *J Gen Virol*. 2008;89(3):722–30.
- 961 117. Chen YW, Guo T, Shen L, Wong KY, Tao Q, Choi WWL, et al. Receptor-type tyrosine-
962 protein phosphatase κ directly targets STAT3 activation for tumor suppression in nasal
963 NK/T-cell lymphoma. *Blood*. 2015;125(10):1589–600.
- 964 118. Lin R-J, Chang B-L, Yu H-P, Liao C-L, Lin Y-L. Blocking of Interferon-Induced Jak-Stat
965 Signaling by Japanese Encephalitis Virus NS5 through a Protein Tyrosine Phosphatase-
966 Mediated Mechanism. *J Virol*. 2006;80(12):5908–18.
- 967 119. Kim YH, Lee JR, Hahn MJ. Regulation of inflammatory gene expression in macrophages
968 by epithelial-stromal interaction 1 (Epsti1). *Biochem Biophys Res Commun*.
969 2018;496(2):778–83.
- 970 120. Rong E, Hu J, Yang C, Chen H, Wang Z, Liu X, et al. Broad-spectrum antiviral functions
971 of duck interferon-induced protein with tetratricopeptide repeats (AvIFIT). *Dev Comp*
972 *Immunol*. 2018;84:71–81.
- 973 121. Alonso MA, Weissman SM. cDNA cloning and sequence of MAL, a hydrophobic protein
974 associated with human T-cell differentiation. *Proc Natl Acad Sci U S A*.
975 1987;84(7):1997–2001.
- 976 122. Gatto M, Iaccarino L, Ghirardello A, Bassi N, Pontisso P, Punzi L, et al. Serpins,
977 immunity and autoimmunity: Old molecules, new functions. *Clin Rev Allergy Immunol*.
978 2013;45(2):267–80.
- 979 123. Bao J, Pan G, Poncz M, Wei J, Ran M, Zhou Z. Serpin functions in host-pathogen
980 interactions. *PeerJ*. 2018;6(e4557):1–16.
- 981 124. Hong YH, Lillehoj HS, Hyen Lee S, Woon Park D, Lillehoj EP. Molecular cloning and
982 characterization of chicken lipopolysaccharide-induced TNF- α factor (LITAF). *Dev Comp*
983 *Immunol*. 2006;30(10):919–29.
- 984 125. Kaiser P, Staheli P. Avian Cytokines and Chemokines. *Avian Immunol Second Ed*.
985 2013;189–204.
- 986 126. Perusina Lanfranca M, Lin Y, Fang J, Zou W, Frankel T. Biological and pathological
987 activities of interleukin-22. *J Mol Med*. 2016;94(5):523–34.
- 988 127. Zhu L, Ly H, Liang Y. PLC- 1 Signaling Plays a Subtype-Specific Role in Postbinding
989 Cell Entry of Influenza A Virus. *J Virol*. 2014;88(1):417–24.

128. Kudo K, Uchida T, Sawada M, Nakamura Y, Yoneda A, Fukami K. Phospholipase C δ 1 in macrophages negatively regulates TLR4-induced proinflammatory cytokine production and Fc γ receptor-mediated phagocytosis. *Adv Biol Regul.* 2016;61:68–79.
129. McGinn OJ, English WR, Roberts S, Ager A, Newham P, Murphy G. Modulation of integrin α 4 β 1 by ADAM28 promotes lymphocyte adhesion and transendothelial migration. *Cell Biol Int.* 2011;35(10):1043–53.
130. Koretzky GA, Picus J, Schultz T, Weiss A. Tyrosine phosphatase CD45 is required for T-cell antigen receptor and CD2-mediated activation of a protein tyrosine kinase and interleukin 2 production. *Proc Natl Acad Sci U S A.* 1991;88(6):2037–41.
131. Hercus TR, Thomas D, Guthridge MA, Ekert PG, King-Scott J, Parker MW, et al. The granulocyte-macrophage colony-stimulating factor receptor: Linking its structure to cell signaling and its role in disease. *Blood.* 2009;114(7):1289–98.
132. Lei Y, Takahama Y. XCL1 and XCR1 in the immune system. *Microbes Infect.* 2012;14(3):262–7.
133. Hansell C, Zhu XW, Brooks H, Sheppard M, Withanage S, Maskell D, et al. Unique Features and Distribution of the Chicken CD83 + Cell . *J Immunol.* 2007;179(8):5117–25.
134. Kuwano Y, Prazma CM, Yazawa N, Watanabe R, Ishiura N, Kumanogoh A, et al. CD83 influences cell-surface MHC class II expression on B cells and other antigen-presenting cells. *Int Immunol.* 2007;19(8):977–92.
135. Huang Y-W, Yan M, Collins RF, DiCiccio JE, Grinstein S, Trimble WS. Mammalian Septins Are Required for Phagosome Formation. *Mol Biol Cell.* 2008;19:1717–26.
136. Attardi LD, Reczek EE, Cosmas C, Demicco EG, McCurrach ME, Lowe SW, et al. Erratum: PERP, an apoptosis-associated target of p53, is a novel member of the PMP-22/gas3 family (*Genes and Development* (2000) 14 (704-718)). *Genes Dev.* 2000;14(14):1835.
137. Zheng M, Karki R, Vogel P, Kanneganti TD. Caspase-6 Is a Key Regulator of Innate Immunity, Inflammasome Activation, and Host Defense. *Cell.* 2020;181(3):674-687.e13.
138. Chen S, Evan HG, Evans DR. FAM129B/MINERVA, a novel adherens junction-associated protein, suppresses apoptosis in HeLa cells. *J Biol Chem.* 2011;286(12):10201–9.
139. Rishi AK, Zhang L, Boyanapalli M, Wali A, Mohammad RM, Yu Y, et al. Identification and characterization of a cell cycle and apoptosis regulatory protein-1 as a novel mediator of apoptosis signaling by retinoid CD437. *J Biol Chem.* 2003;278(35):33422–35.
140. Brass AL, Huang I-C, Benita Y, John SP, Krishnana MN, Feely EM, et al. The IFITM Proteins Mediate Cellular Resistance to Influenza A H1N1 Virus, West Nile Virus, and Dengue Virus. *2Cell.* 2009;139(7):1243–54.
141. Basters A, Knobloch KP, Fritz G. USP18 - A multifunctional component in the interferon response. *Biosci Rep.* 2018;38(6):1–9.
142. Das A, Dinh P, Panda D, Pattnaik AK. Interferon-Inducible Protein IFI35 Negatively Regulates RIG-I Antiviral Signaling and Supports Vesicular Stomatitis Virus Replication. *J Virol.* 2014;88(6):3103–13.
143. Rohaim MA, Santhakumar D, El Nagggar RF, Iqbal M, Hussein HA, Munir M. Chickens expressing IFIT5 ameliorate clinical outcome and pathology of highly pathogenic avian influenza and velogenic newcastle disease viruses. *Front Immunol.* 2018;9:1–17.
144. Helin AS, Wille M, Atterby C, Järhult JD, Waldenström J, Chapman JR. A rapid and transient innate immune response to avian influenza infection in mallards. *Mol Immunol.*

- 2018;95(February):64–72.
145. Sreekantapuram S, Lehnert T, Prauße MTE, Berndt A, Berens C, Figge MT, et al. Dynamic Interplay of Host and Pathogens in an Avian Whole-Blood Model. *Front Immunol.* 2020;11(March):1–20.
146. Zeng M, Chen S, Wang M, Jia R, Zhu D, Liu M, et al. Molecular identification and comparative transcriptional analysis of myxovirus resistance GTPase (Mx) gene in goose (*Anser cygnoide*) after H9N2 AIV infection. *Comp Immunol Microbiol Infect Dis.* 2016;47:32–40.
147. Van der Goot JA, De Jong MCM, Kochi G, Van Boven M. Comparison of the transmission characteristics of low and high pathogenicity avian influenza A virus (H5N2). *Epidemiol Infect.* 2003;131(2):1003–13.
148. Hayashi H, Kubo Y, Izumida M, Takahashi E, Kido H, Sato K, et al. Enterokinase enhances influenza a virus infection by activating trypsinogen in human cell lines. *Front Cell Infect Microbiol.* 2018;8(MAR):1–14.
149. Florin L, Lang T. Tetraspanin assemblies in virus infection. *Front Immunol.* 2018;9:1–9.
150. Ampomah PB, Kong WT, Zharkova O, Chua SCJH, Samy RP, Lim LHK. Annexins in influenza virus replication and pathogenesis. *Front Pharmacol.* 2018;9(1282):1–9.
151. LeBouder F, Morello E, Rimmelzwaan GF, Bosse F, Péchoux C, Delmas B, et al. Annexin II Incorporated into Influenza Virus Particles Supports Virus Replication by Converting Plasminogen into Plasmin. *J Virol.* 2008;82(14):6820–8.
152. Larson GP, Tran V, Yú S, Cai Y, Higgins CA, Smith DM, et al. EPS8 Facilitates Uncoating of Influenza A Virus. *Cell Rep.* 2019;29(8):2175–2183.e4.
153. Puertollano R, Martín-Belmonte F, Millán J, De Marco MDC, Albar JP, Kremer L, et al. The mal proteolipid is necessary for normal apical transport and accurate sorting of the influenza virus hemagglutinin in Madin-Darby canine kidney cells. *J Cell Biol.* 1999;145(1):141–51.
154. Wang H, Jiang C. Influenza A virus H5N1 entry into host cells is through clathrin-dependent endocytosis. *Sci China, Ser C Life Sci.* 2009;52(5):464–9.
155. Guinea R, Carrasco L. Requirement for vacuolar proton-ATPase activity during entry of influenza virus into cells. *J Virol.* 1995;69(4):2306–12.
156. Qiu XB, Shao YM, Miao S, Wang L. The diversity of the DnaJ/Hsp40 family, the crucial partners for Hsp70 chaperones. *Cell Mol Life Sci.* 2006;63(22):2560–70.
157. Shi Z, Zhang J, Zheng S. What we know about ST13, a co-factor of heat shock protein, or a tumor suppressor? *J Zhejiang Univ Sci B.* 2007;8(3):170–6.
158. Klempner SJ, Ou S-H. ROS1 (ROS proto-oncogene 1, receptor tyrosine kinase). *Atlas Genet Cytogenet Oncol Haematol.* 2017;19(5):337–9.
159. Kumar N, Liang Y, Parslow TG, Liang Y. Receptor Tyrosine Kinase Inhibitors Block Multiple Steps of Influenza A Virus Replication. *J Virol.* 2011;85(6):2818–27.
160. Mi SF, Li Y, Yan JH, Gao GF. Na⁺/K⁺-ATPase β 1 subunit interacts with M2 proteins of influenza A and B viruses and affects the virus replication. *Sci China Life Sci.* 2010;53(9):1098–105.
161. Edinger TO, Pohl MO, Yángüez E, Stertz S. Cathepsin W is required for escape of influenza A virus from late endosomes. *MBio.* 2015;6(3):1–12.
162. Coleman MD, Ha SD, Haeryfar M, Dominic Barr S, Ouk Kim S. Cathepsin B Plays a Key Role in Optimal Production of the Influenza A- Virus. *J Virol Antivir Res.* 2018;07(01):1–16.

163. Heaton NS, Moshkina N, Fenouil R, Gardner TJ, Aguirre S, Shah PS, et al. Targeting Viral Proteostasis Limits Influenza Virus, HIV, and Dengue Virus Infection. *Immunity*. 2016;44(1):46–58.
164. Zhang J, Li G, Ye X. Cyclin T1/CDK9 Interacts with Influenza A Virus Polymerase and Facilitates Its Association with Cellular RNA Polymerase II. *J Virol*. 2010;84(24):12619–27.
165. Su W-C, Hsu S-F, Lee Y-Y, Jeng K-S, Lai MMC. A Nucleolar Protein, Ribosomal RNA Processing 1 Homolog B (RRP1B), Enhances the Recruitment of Cellular mRNA in Influenza Virus Transcription. *J Virol*. 2015;89(22):11245–55.
166. Minakuchi M, Sugiyama K, Kato Y, Naito T, Okuwaki M, Kawaguchi A, et al. Pre-mRNA Processing Factor Prp18 Is a Stimulatory Factor of Influenza Virus RNA Synthesis and Possesses Nucleoprotein Chaperone Activity. *J Virol*. 2017;91(3):1–11.
167. Bayoumi M, Rohaim MA, Munir M. Structural and Virus Regulatory Insights Into Avian N6-Methyladenosine (m6A) Machinery. *Front Cell Dev Biol*. 2020;8(543):1–14.
168. Cao M, Wei C, Zhao L, Wang J, Jia Q, Wang X, et al. DnaJA1/Hsp40 Is Co-Opted by Influenza A Virus To Enhance Its Viral RNA Polymerase Activity. *J Virol*. 2014;88(24):14078–89.
169. Naito T, Momose F, Kawaguchi A, Nagata K. Involvement of Hsp90 in Assembly and Nuclear Import of Influenza Virus RNA Polymerase Subunits. *J Virol*. 2007;81(3):1339–49.
170. Predicala R, Zhou Y. The role of ran-binding protein 3 during influenza A virus replication. *J Gen Virol*. 2013;94:977–84.
171. Bruce EA, Digard P, Stuart AD. The Rab11 Pathway Is Required for Influenza A Virus Budding and Filament Formation. *J Virol*. 2010;84(12):5848–59.
172. Amorim MJ, Bruce EA, Read EKC, Foeglein A, Mahen R, Stuart AD, et al. A Rab11- and Microtubule-Dependent Mechanism for Cytoplasmic Transport of Influenza A Virus Viral RNA. *J Virol*. 2011;85(9):4143–56.
173. Singh I, Doms RW, Wagner KR, Helenius A. Intracellular transport of soluble and membrane-bound glycoproteins: Folding, assembly and secretion of anchor-free influenza hemagglutinin. *EMBO J*. 1990;9(3):631–9.
174. Hogue BG, Nayak DP. Synthesis and processing of the influenza virus neuraminidase, a type II transmembrane glycoprotein. *Virology*. 1992;188(2):510–7.
175. Tokhtaeva E, Capri J, Marcus EA, Whitelegge JP, Khuzakhmetova V, Bukharaeva E, et al. Septin dynamics are essential for exocytosis. *J Biol Chem*. 2015;290(9):5280–8297.
176. Zhang J, Huang Y, Li L, Dong J, Liao M, Sun M. Transcriptome analysis reveals the neuro-immune interactions in duck Tembusu virus-infected brain. *Int J Mol Sci*. 2020;21(7).
177. Zhao Y, Wang K, Wang WL, Yin TT, Dong WQ, Xu CJ. A high-throughput SNP discovery strategy for RNA-seq data. *BMC Genomics*. 2019;20(1):1–10.
178. Juliarena MA, Barrios CN, Ceriani MC, Esteban EN. Hot topic: Bovine leukemia virus (BLV)-infected cows with low proviral load are not a source of infection for BLV-free cattle. *J Dairy Sci*. 2016;99(6):4586–9.
179. Matthews L, Low JC, Gally DL, Pearce MC, Mellor DJ, Heesterbeek J a P, et al. Heterogeneous shedding of Escherichia coli O157 in cattle and its implications for control. *Proc Natl Acad Sci U S A*. 2006;103(3):547–52.

Fig 1

Fig 2

Fig 3

Ileum – I1

Ileum – I2

Bursa – I2

Fig 4

Fig 5

Fig 6

Fig 7

Fig 8

Fig 9

Fig 10