The association between SAα2,3Gal and avian influenza viral load in mallards (Anas Platyrhynchos) and blue-winged teals (Anas discors)

Amanda C Dolinski (dolinsk5@msu.edu)
Michigan State University https://orcid.org/0000-0002-0471-1382
Mark D Jankowski
US Environmental Protection Agency
Jeanne M Fair
Los Alamos National Laboratory
Jen C Owen
Michigan State University

Research article

Keywords: avian, influenza, lectin histochemistry, super-shedder

Posted Date: February 21st, 2020

DOI: https://doi.org/10.21203/rs.2.24168/v1

Version of Record: A version of this preprint was published on November 10th, 2020. See the published version at https://doi.org/10.1186/s12917-020-02642-7.

Abstract

Background Individual heterogeneity in pathogen load can impact disease transmission dynamics; therefore, identifying intrinsic factors responsible for variation in pathogen load is necessary for determining which individuals are prone to be most infectious. Since low pathogenic avian influenza viruses (LPAIV) preferentially bind to alpha-2,3 sialic acid receptors (SAα2,3Gal) in the intestines and bursa of Fabricius in wild ducks (*Anas* spp.), we investigated juvenile mallards (*Anas platyrhyncos*) and blue-winged teals (*Anas discors*) orally inoculated with A/northern pintail/California/44221-761/2006 (H5N9) and the virus titer relationship to percentage of SAα2,3Gal in the intestines and bursa. To evaluate the natural variation of free-ranging duck populations, birds were hatched and raised in captivity from eggs collected from nests of free-ranging birds in North Dakota, USA. Data generated from real time RT-PCR used to quantify virus titers in cloacal swabs, ileum tissue, and bursa of Fabricius tissue and lectin histochemistry used to quantify percentage of SAα2,3Gal was analyzed through a series of multiple linear regression and mixed models.

Results In mallards, we found high individual variation in virus titers significantly related to high variation of SAα2,3Gal in the ileum, with sex being a significant factor. In contrast to mallards, individual variation in teals was minimal and significant relationships between virus titers and SAα2,3Gal were not determined. Collectively, teals had both higher virus titers and a higher percentage of SAα2,3Gal compared to mallards, which may indicate a positive association between viral load and SAα2,3Gal. Statistically significant differences were observed between infected and control birds indicating that LPAIV infection may influence the percentage of SAα2,3Gal, or vice versa, but only in specific tissues.

Conclusions The results of this study provide quantitative evidence that SAα2,3Gal is related to LPAIV titers; thus, SAα2,3Gal should be considered a potential intrinsic factor of variation in LPAIV load.

Background

Wild waterfowl are the natural reservoir for avian influenza viruses (AIV) and a source of infection for domestic poultry (1–3). Highly pathogenic avian influenza virus (HPAIV), which causes devastating impacts to poultry worldwide with some strains fatal to humans, originates from strains of low pathogenic avian influenza virus (LPAIV) circulating in wild ducks (4). LPAIV is transmitted most efficiently via the fecal-oral route (5) and transmitted to poultry via direct contact or contaminated water sources (6); hence, understanding the wild waterfowl host factors responsible for the dissemination of AIV is crucial for improving disease management.

Individual heterogeneity in infectiousness is considered to be a driving force in the development of infectious disease epidemics (7), with high shedding individuals thought to be key in enhancing outbreak intensity (8, 9). Birds infected with RNA viruses, including LPAIV-infected mallards (Anas platyrhynchos), are observed to shed virus with high heterogeneity, where 20% of the birds shed 80% of the total virus shed by all birds (10). While this pattern in infectiousness has been observed and hypothesized to contribute to the dynamics of disease transmission, we know little about what drives this variation.

The intestines and bursa of Fabricius are important sites for LPAIV replication in wild waterfowl (11–13). The majority of LPAIVs circulating in waterfowl preferentially bind to glycans tipped with sialic acid bound to galactose (Gal) in an α-2,3 position (SAα2,3Gal) (14, 15). These receptors found on epithelial cells are located throughout the bird's respiratory tract, intestinal tract (16–18), and bursa of Fabricius (13). In birds, the nucleoprotein antigen for LPAIV has most frequently been detected in the intestines and bursa (12, 13, 19). Additionally, LPAIV-infected birds have more virus isolated from cloacal swab samples than oropharyngeal swabs (20). Therefore, the distribution and abundance of these receptors in avian intestines and bursa are likely to determine the host's susceptibility to infection and the virus's ability to replicate.

Similar to the individual heterogeneity in mallard viral load, individual variation in sialic acid receptors has also been identified. In several avian species, 20% of birds or species expressed 80% of the sialic acid receptors on erythrocytes (21).

Individual variation of SAα2,3Gal expression in mallard intestines has been observed with some individuals reported to have lower expression of SAα2,3Gal in the ileum, cecum, colon, and bursa compared to other individuals (13). Differences in the distribution and intensity of SAα2,3Gal between wild bird species have also been observed, such as red head ducks (Aythya Americana), black swans (Cygnus atratus), and northern pintails (Anas acuta) having limited SAα2,3Gal expression in the duodenum and jejunum compared to other Anseriformes (18). Variation was also found within species, such as mallards, based on the type of lectin used, Maackia amurensis I (MAL I) vs. Maackia amurensis II (MAL II) (18). While the previous literature suggests there is variation in SAα2,3Gal intensity and distribution within and across species, the percentage of SAα2,3Gal in the intestines and bursa has yet to be statistically quantified and related to LPAIV load, a first step in understanding this potential source of AIV variability across individuals and species.

In this study, we address this knowledge gap by investigating the relationship between SAα2,3Gal and LPAIV load in mallards and blue-winged teals (Anas discors, hereafter referred to as “teal”). Both species are important hosts for LPAIV. The mallard is important because of their worldwide distribution, their periodomesticity, and the large diversity of AIV strains isolated from them, including highly pathogenic strains causing high mortality in poultry and people (3, 22, 23). Teals have high infection prevalence (24) and an important role in over-wintering the virus in the southern United States (25, 26).

We hypothesized that higher percentages of SAα2,3Gal in mallard and teals corresponds with higher LPAIV titers. Additionally, we hypothesized that the relationship between virus titers in cloacal swab, ileum tissue, and bursa tissue would all be positively related to each other. Sex-based differences, species-based differences, and comparisons in the percentages of SAα2,3Gal between control and infected birds was also analyzed, where we did not expect to see differences. This research provides a first look into this putative intrinsic factor responsible for LPAIV individual variation in mallards and blue-winged teals.

Results

Viral Infection of Mallards and Teals

All birds inoculated with LPAIV H5N9 (mallard = 60, teal = 44) were infected as demonstrated by positive titers on at least one day between one to five days post infection (DPI) in cloacal swabs, ileum tissue, and/or bursa tissue (Additional File 1). No birds shed virus past 15 DPI, and of the birds that survived to 15 DPI, 99.9 (mallard) and 98.5 (teal) percent of the total virus shed by those birds occurred in the first five DPI. As expected with LPAIV, we observed no clinical signs of disease such as ruffled feathers, lethargy, respiratory distress, or any pathology.

Relationship of Last Cloaca Titer, Ileum Titer, and Bursa Titer

Last Cloaca Titer (last sample taken), Ileum Titer, and Bursa Titer were all significantly positively ($p < 0.05$) related to each other for both mallard and teal (Fig. 1). In mallards, statistically significant positive relationships were observed between Ileum Titer and Last Cloaca Titer for all treatment groups (T1, $R^2 = 0.43$, $p = 0.005$; T2, $R^2 = 0.64$, $p = 0.003$; and T5, $R^2 = 0.66$, $p < 0.001$). Statistically significant positive relationships were observed between Bursa Titer and Last Cloaca Titer for treatment groups T1 ($R^2 = 0.41$, $p = 0.006$) and T5 ($R^2 = 0.63$, $p < 0.001$). Only T5 ($R^2 = 0.68$, $p < 0.001$) had a statistically significant positive relationship between Ileum Titer and Bursa Titer. In teals, the only statistically significant positive relationship for treatment groups was observed for T1 ($R^2 = 0.34$, $p = 0.036$) between Last Cloaca Titer and Bursa Titer.

Species and Sex-based differences in Viral Shedding

Statistically significant differences in viral shedding were found between mallards and teals, but not between males and females within species. Mallards had statistically higher variation than teals in cloacal swab viral titers on one, two, three, and five DPI (Fligner-Killeen $p < 0.05$; Table 1, Fig. 2). For both species, mean cloacal swab virus titers on one, two, and three

DPI were statistically higher than virus titers on four and five DPI ($F_{4,242}$ = 17.61, p < 0.001; Additional File 2). Teals shed statistically more virus than mallards ($F_{1,102}$ = 14.60, p < 0.001) with no interaction between species and DPI ($F_{4,242}$ = 0.91, p = 0.456; Additional File 2). No sex-based differences were observed in cloacal swab virus titers for either species (mallard: $F_{1,58}$ = 0.05, p = 0.818; teal: $F_{1,42}$ = 2.49, p = 0.122) with no statistically significant interaction between sex and DPI (mallard: $F_{4,138}$ = 0.39, p = 0.818; teal: $F_{4,96}$ = 2.43, p = 0.053; Additional File 3).

Table 1
Virus titer descriptive statistics for mallard and blue-winged teal cloacal swabs.

DPI	Species	N	N > DL	N > QL	min +	max	mean	std.dev
					Log10(EID50/mL)	Log10(EID50/mL)	Log10(EID50/mL)	Log10(EID50/mL)
DPI 1	mallard	58	52	38	0.13	6.21	3.26	*1.94
	teal	44	44	41	1.37	6.16	4.06	0.99
DPI 2	mallard	43	38	28	0.38	5.2	3.11	*1.64
	teal	32	32	29	1.78	6.12	3.96	0.97
DPI 3	mallard	35	29	26	0.06	5.72	3.33	*1.90
	teal	32	32	29	0.5	6.15	4.05	0.98
DPI 4	mallard	35	29	19	0.19	5.03	2.38	1.54
	teal	20	19	15	0.98	5.04	3.08	1.16
DPI 5	mallard	35	28	18	0.05	6.43	2.29	*1.75
	teal	20	19	10	0.65	3.99	2.37	1.01

N = total sample size, DL = detection limit of 0.04 Log10(EID_{50}/mL), QL = quantification limit of 2.60 Log10(EID_{50}/mL), min + = minimum N > DL, mean is the geometric mean, and std.dev = one standard deviation. (*) signifies significantly higher titer variation for each DPI between species.

Lectin histochemistry

Both infected and control mallard and teal intestinal and bursa tissues positively stained with MAL I (Additional File 1); however, mallard bursa epithelial cells could not be included in statistical analysis due to autolysis. Overall, lectin scores were not statistically different between LPAIV-infected and control mallards ($F_{1,68}$ = 0.11, p = 0.746); however, there was a statistically significant interaction between infection status and tissue/cell type ($F_{11,693}$ = 4.08, p < 0.001). We found the cecum crypt lectin score in LPAIV-infected mallards to be statistically higher than control mallards (p = 0.046; Fig. 3). Conversely, lectin scores of control mallards' ileum brush border (p = 0.017) and colon brush border (p = 0.015) were statistically significantly higher than infected mallards (Fig. 3). Unlike mallards, LPAIV-infected teals had statistically higher lectin scores than control teals ($F_{1,52}$ = 15.20, p < 0.001), with a statistically significant interaction between infection status and tissue/cell type ($F_{12,611}$ = 8.66, p < 0.001). Post-hoc analysis shows the lectin score in the cecum brush border (p < 0.001) and cecum villi (p < 0.001) was higher in infected birds than control birds (Fig. 3). Higher inter-tissue and inter-individual variation was observed in mallards compared to teals for all tissue/cell types (Fligner-Killeen p < 0.001; Table 2; Fig. 4). LPAIV-infected teals had statistically higher lectin staining than LPAIV-infected mallards ($F_{1,102}$ = 309.92, p < 0.001)

with a statistically significant interaction between species and tissue/cell type ($F_{11,1067} = 9.95$, $p < 0.001$). In mallards, the ileum, cecum, and colon had statistically similar lectin scores for most cell types; however, lectin scores for most cell types in the proximal intestine were significantly lower ($p < 0.05$) than the lectin scores in ileum, cecum, and colon (Fig. 5). In teals, most tissues/cell types had similar lectin scores, except for the cecum brush border and cecum villi, which were statistically significantly lower than all other tissue/cell types (Fig. 5).

In both species, lectin staining was not significantly different between males and females (mallard: $F_{1,58} = 2.243$ $p = 0.141$; teal: $F_{1,42} = 0.24$, $p = 0.626$), and there was no significant interaction between sex and tissue/cell type (mallard, $F_{11,587} = 1.48$, $p = 0.136$; teal, $F_{11,458} = 0.42$, $p = 0.947$; Additional File 4).

Table 2

Lectin histochemistry score descriptive statistics.

Tissue	Cell Type	Species	N	min (%)	max (%)	mean (%)	std.dev (%)
Proximal	crypts	teal	44	44.25	80.00	79.19	5.39
		mallard	60	0	80	10.22	*19.92
	brush border	teal	44	40.25	80.00	74.24	12.12
		mallard	60	0	80	6.91	*16.99
	villi	teal	44	40.55	80.00	74.46	12.12
		mallard	60	0	80	8.82	*16.12
Ileum	crypts	teal	44	80.00	80.00	80.00	0.00
		mallard	54	0	80	14.51	*22.26
	brush border	teal	43	80.00	80.00	80.00	0.00
		mallard	47	0	80	23.36	*27.30
	villi	teal	43	80.00	80.00	80.00	0.00
		mallard	47	0	80	16.87	*20.47
Cecum	crypts	teal	44	38.00	80.00	78.82	6.47
		mallard	58	0	80	18.97	*27.25
	brush border	teal	43	7.00	80.00	62.96	23.49
		mallard	54	0	80	26.24	*33.35
	villi	teal	43	8.00	80.00	59.21	24.95
		mallard	54	0	80	23.12	*30.47
Colon	crypts	teal	44	80.00	80.00	80.00	0.00
		mallard	59	0	80	11.66	*19.36
	brush border	teal	44	76.00	80.00	79.91	0.60
		mallard	58	0	80	23.55	*24.91
	villi	teal	44	76.00	80.00	79.91	0.60
		mallard	58	0	80	16.35	*18.98
Bursa	Epithelial Cells	teal	42	10.00	80.00	68.57	20.61
		mallard	NA	NA	NA	NA	NA

Proximal includes duodenum and jejunum. N = total sample size and std.dev = one standard deviation. (*) signifies significantly higher lectin score variation for each tissue/cell type between species.

Relationship between Lectin Histochemistry Score and Virus Titer – Mallard

For mallard Last Cloaca Titer, initial stepwise variable selection rendered a multiple linear regression (MLR) model which included sex, Proximal PC, Ileum villi, and Ileum brush border (AIC = 11.44, ΔAIC = 1.77; Additional File 5). This reduced

model was tested for co-linearity issues and residual plots were evaluated with no serious problems detected (Additional File 5), so the reduced model was selected as the best fitting model ($R^2 = 0.66$, $p < 0.001$; Table 3). Our results indicated that lectin staining in the ileum villi and being male were positively associated with a higher virus titer, while lectin staining in the ileum brush border was negatively associated with a higher virus titer. Lectin staining in the proximal intestine was not a significant term in the model.

For mallard Ileum Titer, initial stepwise variable selection rendered a model which included sex, ileum villi, and ileum brush border (AIC = 33.57, ΔAIC = 1.32; Additional File 6). This reduced model was tested for co-linearity issues and residual plots were evaluated with no serious problems detected (Additional File 6), thus this model ($R^2 = 0.33$, $p < 0.010$, Table 3) was selected as the best fitting model. Our results indicated that the lectin score of the ileum villi was positively associated with a higher virus titer. LHS in the ileum brush border was negatively associated with a higher virus titer. Sex was not a significant factor in this model.

Table 3
Sex and ileum lectin scores are associated with LPAIV H5N9 virus titers in mallards

y	n	R^2	x	Est. (95% CI) Log10(EID50/mL)	P
Mallard Last Cloaca Titer	25	0.66	Intercept	1.37 (0.14 to 2.60)	0.031
			Sex (Male)	1.66 (0.60 to 2.73)	0.004
			Proximal PC1	0.50 (-0.22 to 1.22)	0.166
			Ileum Villi	2.93 (1.42 to 4.44)	< 0.001
			Ileum Brush Border	-1.96 (-3.12 to -0.80)	0.002
Mallard Ileum Titer	25	0.33	Intercept	2.86 (1.21 to 4.52)	0.002
			Sex (Male)	1.36 (-0.19 to 2.92))	0.083
			Ileum Villi	3.27 (1.18 to 5.36)	0.004
			Ileum Brush Border	-1.93 (-3.73 to -0.14)	0.036

Y = dependent variable, N = number of individual birds in model, X = independent variables in final model, CI = 95% confidence interval, p = p-value. Proximal includes the duodenum and jejunum. PC1 represents the principal component variable for the proximal villi enterocytes, brush border, and crypt enterocytes combined. BCS = Body Condition Score. Group and Sex were treated as factors in each model, and if present in final model, group T1 and females are represented in the intercept.

Relationship between Lectin Histochemistry Score and Virus Titer – Teal

For teal Last Cloaca Titer, initial stepwise variable selection rendered a model which included sex, mass, body condition score (BCS), group, proximal crypts, and bursa (AIC = -8.31, ΔAIC = 0.10; Additional File 7). This reduced model was tested for co-linearity issues and residual plots were evaluated with no serious problems detected, thus this model ($R^2 = 0.61$, $p < 0.010$) was selected as the best fitting model (Additional File 7); however, inconsistent results were observed using the quantification limit validation method (Additional File 8). Due to these inconsistencies, we conclude the Last Cloaca Titer MLR model to be unstable and results unreliable.

For teal Ileum Titer, initial stepwise variable selection rendered a model which included BCS and group (AIC = 32.41, ΔAIC = 1.99; Additional File 9). This reduced model was tested for co-linearity issues and residual plots were evaluated with no serious problems detected, thus this model ($R^2 = 0.44$, $p < 0.001$) was selected as the best fitting model (Additional File 9).

Our results indicated that virus titers were lower on five DPI compared to one and three DPI. BCS was not a significant term in the model.

For teal Bursa Titer, initial stepwise variable selection rendered a model which included mass, BCS, and group (AIC = -1.6, ΔAIC = 1.75; Additional File 10). The reduced model was tested for co-linearity issues with no problems detected. Residual plots were evaluated, and the model did not fit normality assumptions. Mass was removed from the model since it was an insignificant factor, and the residual plots improved; therefore, the model which included BCS and group was accepted as the best fitting model (R^2 = 0.37, p = 0.001; Additional File 10). Our results indicated that virus titer was highest on one DPI, and significantly lower on three and five DPI. BCS was not a significant term in the final model.

Discussion

Mallards and blue-winged teals are important reservoir hosts for avian influenza viruses (3, 24, 25); they are both widely distributed waterfowl species and commonly infected with both LPAIV and HPAIV. Our study documents both within and between-species variation in viral shedding as well as for SAα2,3Gal, the viral receptor for many LPAIVs. In mallards, but not teals, we found viral shedding was related to percentage of SAα2,3Gal. While we expected to see positive linear relationships between virus titers and SAα2,3Gal in all tissues and cell types, the mallard ileum was the most predictive of virus titers, with a positive relationship between virus titers and SAα2,3Gal in ileum villi enterocytes, and a negative relationship between virus titers and SAα2,3Gal in the ileum brush border. Despite the lack of relationship between viral shedding and SAα2,3Gal in teals, we observed significantly higher viral shedding by teals, and a higher percentage of SAα2,3Gal compared to mallards.

As the direction (positive or negative) of the correlation between SAα2,3Gal and virus titer varied across mallard tissue locations, our data highlight the importance of understanding tissue-specific tropism as it relates to cell surface SAα2,3Gal distribution. Within mallards, the positive relationship between virus titer and SAα2,3Gal in the ileum villi enterocytes was expected given that LPAIV replicates in intestinal enterocytes by binding SAα2,3Gal on the surface of the cell for cell entry (27). A reason why ileum villi enterocytes were most correlated with viral titer compared to ileum crypt enterocytes may be that the villi have closer direct contact with digesta and as a result, closer direct contact with virus passing through the gut. For example, previous studies have found LPAIV antigen via immunohistochemistry more consistently in mallard villi enterocytes compared to the crypts (12, 13). Surprisingly, however, the other three intestinal tissue types: proximal, cecum, and colon, were not associated with virus titers in the MLR models.

The lack of a statistically significant relationship between SAα2,3Gal and virus titer in the mallard colon was unexpected, given numerous studies have indicated the colon as a site for high LPAIV replication (11–13, 28). The lack of a statistically significant relationship between colon SAα2,3Gal and viral shedding may be due to the statistical approach we used. The MLR method was designed to identify the tissue or tissues which most contributed to the variation observed in virus titers while eliminating issues from the model that exhibited multicollinearity. Since SAα2,3Gal in the ileum and colon were 63% correlated with each other (Additional File 11), the colon could also have a contributing effect to viral load, but not as strongly as the ileum.

The statistically insignificant relationship between SAα2,3Gal in the mallard proximal intestine and virus titers may be explained by the low percentage of SAα2,3Gal in the proximal intestine compared to the ileum, cecum, and colon. Previous studies have assessed the presence of SAα2,3Gal in villi enterocytes and goblet (mucus producing) cells of the duodenum and jejunum, but with conflicting results. Costa et. al found high expression of SAα2,3Gal in the epithelial cells of the duodenum and jejunum of mallards but not in goblet cells (17), while Franca et. al only found SAα2,3Gal in the goblet cells of the duodenum and jejunum of mallards, but not the enterocytes (13, 18). These differences could be a result of the microscopic imaging techniques used, where Costa et. al used light microscopy and Franca et. al used florescence microscopy. Our analysis purposefully excluded positive staining goblet cells since they produce mucins which also

express SAα2,3Gal and may inhibit cell entry and viral replication (29, 30). Although the consensus is not clear concerning the presence of SAα2,3Gal in the mallard proximal intestine; previous findings indicate that positive viral antigen via immunohistochemistry is more commonly found in the ileum, cecum, and colon when cloacal swab virus titers are high (12, 13), which would indicate that the proximal intestine is not a main site of LPAIV replication. Therefore, we suggest that the lower percentage of SAα2,3Gal in the proximal enterocytes could explain the lack of a relationship to shedding of LPAIV.

Two hypotheses could explain the negative relationship between SAα2,3Gal in the ileum brush border and virus titer. Initially, we expected to see a positive relationship between SAα2,3Gal in the brush border of all intestinal tissues and virus titers since the receptors are located on the surface of the cell and more likely to be exposed to virus (31). However, as a virion attaches to a receptor, the virion along with the receptor becomes engulfed by the cell for replication, therefore removing the receptor from the surface of the cell (32). This idea is also consistent with the differences observed in percentage of SAα2,3Gal between infected and control mallards, where control mallards had higher SAα2,3Gal in the ileum and colon brush border compared to infected birds. Secondly, mucus is also found along the brush border and LPAIV has been found to bind SAα2,3Gal in mucus, which would prohibit the virus from reaching the enterocyte for virus replication (15, 30, 33); thereby reducing the quantity of virus shed. Up-regulation of mucins have also been observed in response to other viruses which bind sialic acid receptors (29), such as human rotavirus infections (34). To investigate the true explanation for the negative relationship between percentage of SAα2,3Gal in the ileum brush border and virus titers, further experimental research is warranted.

The bursa epithelial cells are also considered to be an important site of replication for LPAIV in waterfowl, including mallards (12, 13). However, given autolysis of tissue samples we were unable to analyze the relationship between SAα2,3Gal in the bursa and viral shedding in mallards. In teals, lectin staining was very high in the bursa; however, it was not significantly related to viral shedding. Lack of a significant relationship to viral titer in teals could be attributed to the lack of individual variation in SAα2,3Gal expression in the bursa or to a sporadic correlation between bursa and cloacal swab virus quantity.

The premise of our study was to determine if the percentage of SAα2,3Gal in the intestines and bursa may be associated with cloacal shedding; hence, we predicted the variation of SAα2,3Gal in control and infected birds would not differ. Our data suggest this is not the case. In the cecum, the percentage of SAα2,3Gal was higher in the crypts of infected mallards compared to their conspecific controls. Similarly, in teals the percentage of SAα2,3Gal was higher in the cecum villi and brush border of infected birds. The ceca have a unique role in the functioning of the vertebrate immune system. The cecal tonsils, a major lymphoid tissue in the cecum, enlarges during gut infections due to infiltration of immune cells, which includes macrophages (35). Macrophages express Gal-specific receptors (36), which could explain the higher abundance of SAα2,3Gal in the cecum of infected birds relative to controls. White leghorn chickens have a greater abundance of sialic acid receptors than silky fowl and this corresponded with a higher number of immune cells in the cecum in the leghorns (37). The cecum has a unique response to LPAIV infection compared to other intestinal tissues, which warrants further analysis of SAα2,3Gal in this tissue.

Contrary to differences in SAα2,3Gal expression between LPAIV-infected and control birds in the cecum, control mallards expressed more SAα2,3Gal in the ileum and colon brush border than infected mallards. Franca et. al (13) found that SAα2,3Gal was lower in the cecum, colon, and bursa of infected birds compared to control birds. Their hypothesis indicated that the SAα2,3Gal expression level may decrease after infection because the neuraminidase function of the virus allows cleaving of the receptor releasing virions from the cell (38). When the receptor is cleaved, it is no longer present on the cell surface which would reduce lectin binding. While Franca et. al (13) did not specify whether the decrease in lectin staining was on the surface of the enterocyte, we found mallards to have a higher percentage of SAα2,3Gal only in the brush border. Our results indicate the importance of assessing the specific location of SAα2,3Gal in determining their function in influenza studies.

An interesting finding was the species-specific differences between mallards and teals in variation and viral shedding. The variation observed in mallards in our study is consistent with what has been observed in other experimental infection studies (10, 39). To our knowledge this is the first experimental infection of blue-winged teals with LPAIV; hence we do not have any other study against which to compare our results. We found that teals shed significantly more virus and had a higher percentage of SAα2,3Gal than mallards but expressed less variation in both measurements. Jankowski et al. (21) also found species-based variation in sialic acid receptor expression. In their study, which analyzed the variation of sialic acid receptors expressed by erythrocytes in various avian species, it was found that approximately 20% of the species expressed 80% of the overall sialic acid receptor quantity in all species studied. Although teals were not included in the Jankowski et. al. (21) study, mallards and three other Anas species (A. acuta, A. Americana, and A. crecca) were among the species assessed. Interestingly, mallards had the lowest quantity of sialic acid receptors on erythrocytes compared to the other three Anas species. In our study, the higher shedding of LPAIV and SAα2,3Gal identified in teals is important because teals have the highest LPAIV prevalence rates during winter months in Texas (26, 40), implicating them as important hosts for overwintering LPAIV in the U.S. Our results and the results of Jankowski et. al suggest that species variation is an important factor to consider for sialic acid receptor variation. Since the quantity of virus shed can directly impact transmission dynamics and is an important parameter for predicting disease risk in a population (41), if certain species can be identified as more infectious than other species, species-specific parameters could improve our ability to predict and mitigate disease. Hence, knowing which species are likely to contribute more to environmental contamination is key for determining the transmission risk of LPAIV.

Since both virus titers and the percentage of SAα2,3Gal were significantly higher in teals compared to mallards, it may be hypothesized that the higher teal virus titers were a result of the higher SAα2,3Gal. It has already been shown that teals have higher binding affinity to MAL I lectin than mallards (18). Additionally, different lectins vary in their affinity for SAα2,3Gal with slightly different molecular structure, such that MAL I preferentially binds SAα2,3Gal with a β1-4Glc(NAc) linkage (42), while MAL II preferentially binds SAα2,3Gal with a β1-3Gal(NAc) linkage (43). Different LPAIV strains also vary in binding affinity to SAα2,3Gal with different molecular structures (44). Although, LPAIV H5N9 (Ratite/New York/12716/94) has similar affinity for the receptors targeted by each lectin (43, 44), we did not test the specific receptor affinity of the LPAIV H5N9 (A/northern pintail/California/44221-761/2006) used in this study. If LPAIV H5N9 (A/northern pintail/California/44221-761/2006) does have a higher affinity for SAα2,3Gal with a β1-4Glc(NAc) linkage, which is the preferred binding affinity of MAL I, then further evidence would be provided to explain the higher LPAIV H5N9 virus titers in teals.

Sex was not a significant factor in viral titers nor SAα2,3Gal when examined separately; yet, the MLR analysis did show that when SAα2,3Gal in the ileum villi enterocytes and brush border are held constant, there was a positive relationship between virus titer and SAα2,3Gal in male mallards. We are unsure why this was the case and likely warrants a study with larger samples sizes for both sexes to draw conclusions about the effect of sex on expression of SAα2,3Gal and LPAIV shedding. Few experimental AIV infection studies have investigated sex-based differences in viral shedding (but see Pepin et al. 2012 (45)). Pepin et al. (45) observed significantly higher cloacal shedding of LPAIV in males than females but did not draw a conclusion about the mechanism underlying this difference. There is more evidence for sex-based differences in infectiousness for other host-pathogen systems (46); however, much of the difference is related to a bird's reproductive state and/or hormone levels (47, 48). The birds in our study were only 6–12 weeks old and not reproductively mature; hence, any sex-related differences are unlikely due to variation in hormone production.

The identified positive relationships between viral RNA in cloacal swabs, ileum tissue, and bursa tissue further supports the importance of the ileum and bursa for cloacal shedding of LPAIV. Prior to this study, it was well known that LPAIV replicates in duck intestines and the bursa of Fabricius (11–13). While testing for virus in cloacal swabs is the standard method for determining AIV fecal shedding (49, 50), the direct relationship between tissue replication and virus shed by the cloaca was unknown. Through quantifying viral RNA via RT-qPCR in ileum and bursa tissue, significant positive relationships were found between virus titers in cloacal swabs, ileum tissue, and bursa tissue, indicating the contribution of these tissues to

the cloacal virus shed. The positive relationship between virus titers in the ileum and cloacal swabs provides additional evidence to support our conclusion that ileum SAα2,3Gal was associated with virus titer. Lastly, these positive relationships add validity to collecting cloacal swabs as an indicator of virus titer in the ileum and bursa and perhaps the infection status of the bird as a whole.

Conclusion

Understanding the mechanism underlying variation in infection severity and viral shedding can provide insight into why a few individuals in a population are more infected than others, and perhaps, why some are more infectious than others. LPAIV is a gut-associated pathogen in wild waterfowl; hence, the physiology of the host's gut is an important determinant of within-host-pathogen interaction. The results of this study provide evidence that sialic acid receptors in the gut are associated with viral load. Since sialic acid expression varies both between species (18, 21) and within species (13), this variation has implications for a species' and/or individual bird's contribution to the transmission of avian influenza virus. Furthermore, sialic acid is the cellular receptor for other viruses such as parainfluenza, mumps, corona, noro, rota, and DNA tumor viruses, some of which infect humans, (34) leading to similar questions regarding the effect of sialic acid receptor variation across individuals and species on host-virus interactions. Pathogen receptors are certainly not the only contributing factor to a host's infectiousness. Other intrinsic factors and their relationship to pathogen shedding warrant further investigation. Knowing what underlies variation in pathogen shedding provides further insight into the ecology of disease so we can better predict and minimize disease risk to populations of interest.

Methods

Permits and Protocols

Protocols for animal care and experimental sampling procedures were approved by Michigan State University (MSU) Institutional Animal Care and Use Committee (AUF 12/16-211-00). All euthanasia procedures were in accordance with the Animal Welfare Act and guidelines to the use of wild birds in research (51). Duck eggs were collected with permission from the U.S. Fish and Wildlife Permit (M BI 94270-2) and North Dakota Game and Fish Department License #GNF03639403.

Study Species and Locations

Mallards and teals used for this study were collected as eggs from the nests of wild birds in the southwest corner of Towner County, North Dakota, USA (48.4431853, -99.3156225). In May - June 2015 we collected 90 mallard eggs (1–2 per nest) from a total of 50 nests, with each nest containing an average of eight eggs per clutch. The following summer, May – June 2016 we collected 80 blue-winged teal eggs (1–2 per nest) from a total of 40 nests. Nests were found and eggs collected by dragging a heavy metal-link chain behind two ATVs driving in parallel which initiated hens to fly off their nests (52). Eggs were candled in field to determine age, and any eggs that either had not started incubation or were between 15 and 22 days of incubation were shipped overnight to MSU in East Lansing, Michigan. Each year we made 2–4 shipments of 15 to 40 eggs each over a period of 6 weeks. Unless specified otherwise all procedures were the same for each species/year.

Upon arrival at MSU, eggs were immediately placed into a climate-controlled egg incubator (Sportsman 1502 Egg Incubator, GQF Manufacturing Co., Savannah, GA) housed within a biosafety level two room within the MSU Research Confinement Facility. Eggs were incubated at 37.5 °C with 45–50% humidity and rotated electronically ten times per day. Eggs were candled for viability and age once every three days. As soon as eggs pipped, they were moved into a hatching incubator (Sportsman 1502 Egg Incubator, GQF Manufacturing Co., Savannah, GA) at 37.2 °C with 70–80% humidity. Chicks remained in the hatcher until they were dry, approximately 12–24 hours post hatching. Each bird was then weighed to the nearest 0.1 g, banded with a uniquely numbered plastic leg band, and placed in a brooder (30–35 °C). Birds were kept in brooders for two weeks, then moved to open-room housing where a maximum of 35 birds were housed per room (400sq

feet). Each room maintained a temperature of 23 °C and 45–55% humidity, had two swimming pools (45” diameter, 10” depth), and two dry pools with aspen chip bedding. In both years, birds were maintained on a 13:11hr light:dark photoperiod.

Birds were fed ad libitum Purina® Flock Raiser® Crumbles (Purina, St. Louis, MO, USA) and supplemented with chopped dandelion greens twice per day. Rooms were fully cleaned twice per day. Birds were routinely checked for normal health and weighed every five days. One week prior to inoculation, mallards were separated into individual cages of 20 cages per room. Blue-winged teals were kept in the open room housing separated by experimental group.

Virus

LPAIV A/northern pintail/California/44221-761/2006 (H5N9), originally collected from a Northern Pintail cloacal swab and isolated in specific pathogen free embryonated chicken eggs (ECE), was acquired from the USGS National Wildlife Health Center in Madison, WI (USDA Veterinary Permit 44372). We prepared stock virus propagating the virus in 9 to 11-day old ECE (Charles River, Norwich, CT, USA) (53). The infectious titer of the stock virus of 7.63 log EID_{50}/ml was determined using the 50% egg infectious dose (EID_{50}) and calculated using the Reed & Muench method (54). The viral inoculum was prepared by diluting the stock virus in Dulbecco's Modified Eagle Medium (DMEM) (Gibco® by Life Technologies, Grand Island, NY, USA) to yield a final titer of 5.63 log EID_{50}/ml.

Experimental Design

Mallards were assigned to five treatment (T) and two control (C) groups. Teals were assigned to four T and two C groups. Group assignment was done using pseudo-stratified randomization with birds being stratified by body mass (mallard: range = 640 to 1020 g, mean = 849 g; teal: range = 285 to 473 g, mean = 362 g), age (mallard: range = 60 to 120 days, mean = 87 days; teal: range = 64 to 86 days, mean = 76), and sex (mallard: male = 34, female = 36; teal: male = 26, female = 28). Additionally, individuals from the same nests were assigned to separate groups. Group names refer to the DPI they were sacrificed. In mallards, the groups were T1 (n = 15), T2 (n = 10), T5 (n = 15), T15 (n = 10), T29 (n = 10), C1(n = 5), and C29 (n = 5). In teals, the groups were (n = 12), T3 (n = 12), T5 (n = 12), T14 (n = 8), C1 (n = 5), and C14 (n = 5). The number of birds per group was based on individual viral load variation observed in populations as small as ten individuals (10). Additional birds were placed in groups on DPI of most importance such as high viral shedding (DPI 1–3) and early detection of antibody titer (DPI 5) (55).

All T-group birds (hereafter referred to collectively as “infected”) were inoculated with 1.0 mL of 5.63 log EID50/ml viral inoculum on 0 DPI, diluted in DMEM by placing one drop on each eye and each nare, then dispensing the rest in the esophagus (56, 57). All C-group birds (hereafter referred to collectively as “control”) were sham-inoculated with 1.0 mL of sterile DMEM in a similar fashion. During the inoculation and after inoculation, birds were kept in biosafety level two conditions and personal protective equipment consisted of non-vented, full coverage eye goggles, hair cap, N95 respirator, double gloves, tyvek suit, and plastic booties.

We collected cloacal swabs on all live individuals. Cotton tipped swabs were collected from mallards on 1–5, 8, 11, 13, 15, 17, 19, 22, 24, 26, and 29 DPI, and from teals on 1–7, 9, 11, and 14 DPI (Additional File 12). Swabs were stored in 3.0 mL of brain-heart infusion broth (BHI), transported on ice, and stored in -80 °C until sample processing.

Euthanasia

Mallards, as described by their assigned groups, were sacrificed on 1, 2, 5, 15, and 29 DPI, and teals were sacrificed on 1, 3, 5, and 14 DPI (Additional File 12). Mallards sacrificed on one DPI were euthanized by intravenous lethal injection of pentobarbital sodium and phenytoin sodium solution (Beuthanasia-D Special, Merck Animal Health, Madison, NJ, USA). All other birds were euthanized by carbon dioxide inhalation. Bird carcasses were preserved on ice until necropsy was performed.

Necropsy and Tissue Collection

Mallard necropsy was performed in the same room where birds were kept under biosafety level two conditions mentioned above. Teal necropsies were performed under a biosafety cabinet. Necropsies were performed on mallards within one to six hours of being euthanized, with an average time of approximately four hours post euthanasia. Due to autolysis of tissue samples observed with mallards, we performed necropsies on teals within one hour of being euthanized, with the average time of 22 minutes post euthanasia. We examined birds for any abnormalities and the coelomic cavity for any gross pathology. We also assessed the birds' body condition using a scale of one to five: one being emaciated and five being over-conditioned with presence of fat in intestinal mesentery. Sex was determined by examining the syrinx (58).

We collected 0.5 to 2 cm sections of intestine (duodenum, jejunum, ileum, cecum, colon) and bursa of Fabricius in 10% buffered formalin. The tissues were incubated at room temperature for 24–48 hours to allow time for fixation, then transferred to a histological sectioning cassette in 70% ethanol and embedded in paraffin within 24 hours. We also collected 2 mm sections of ileum and bursa in RNA stabilizing solution (RNAlater®, Sigma-Aldrich, St. Louis, MO, USA) for viral RNA analysis in these tissues.

Viral RNA isolation and RT-PCR

Virus in cloacal swabs, ileum tissue, and bursa tissue was quantified by isolating viral RNA using real time RT-PCR targeting the matrix protein gene (59). Unlike immunohistochemistry which stains for nucleoprotein antigen, real time RT-PCR is quantitative and can detect lower quantities of virus (60). Viral RNA was isolated from cloacal swab material using the MagMAX™-96 AI/ND Viral RNA Isolation Kit (Applied Biosystems® by Thermo Fisher Scientific, Vilnius, Lithuania) with modifications to the manufacturer protocol previously described (61). Viral RNA was extracted with host mRNA from 15–30 mg of ileum and bursa tissue from each bird using the Qiagen RNeasy Mini Kit (QIAGEN®, Hilden, Germany) according to the manufacturer's protocol. For the RT-PCR working solution we used the TaqMan® RNA-to-Ct™ 1-Step Kit (Applied Biosystems® by Thermo Fisher Scientific, Foster City, CA, USA), primer 5'-AGATGAGTCTTCTAACCGTCTCTG (Sigma-Aldrich, St. Louis, MO, USA), probe 5'-[6FAM]TCAGGCCCCCTCAAAGCCGA[BHQ1] (Sigma-Aldrich, St. Louis, MO, USA), and 2∝L of sample RNA for a final well volume of 10∝L. Each sample was processed at least three times on a 384 well plate with a minimum of three negative control wells and three positive control wells. We used LPAIV H5N9 stock virus in a 10-fold dilution on each plate in three replicates to create a reference standard curve. Ct values less than 40 were considered positive for virus. Using QuantStudio™ 6 and 7 Flex Real-Time PCR Software System v1.3, we calculated the standard curve, which was used to estimate virus quantity of each sample by correlating Ct values to 50% egg infectious dose per milliliter (EID_{50}/mL). The reported limit of detection is 0.1 EID_{50} (62); therefore, any samples with undetectable viral RNA were considered negative and assumed to be 0.00 EID_{50}/mL. Virus quantity for each sample was averaged across sample replicates. Failed wells and suspected contaminated wells were removed from final calculations.

The quantification limit of the stock virus 10-fold dilution was approximately 400 EID_{50}; however, 21% of our samples were detected to have positive virus between this threshold and 0.1 EID_{50}. To validate the stability of our statistical analysis, multiple value random imputation (63) was used for any sample with positive virus between 0.1 and 400 EID_{50}, and statistical analysis was repeated. Methods and results of this validation technique are outlined in supplemental material (Additional File 8).

Lectin Histochemistry

We used lectin histochemistry to detect SAα2,3Gal in formalin fixed and paraffin embedded tissues of the intestines and bursa of Fabricius of each bird. Maackia amurensis I (MAL I) agglutinin is a plant lectin which binds specifically to Siaα2-3Galβ1-4Glc(NAc) (42, 43) and has been used in multiple receptor distribution studies in ducks and other influenza hosts (64, 65) to detect SAα2,3Gal. MAL II, which specifically binds Siaα2-3Galβ1–3 (Neu5Acα2–6)GalNAc) (43), is another lectin commonly used in place of, or in conjunction with MAL I (13, 17, 18, 66). Trial protocols were tested to determine the proper

concentration of each lectin needed for proper binding and visual staining of SAα2,3Gal. The trial protocol resulted in a determined concentration for MAL I, but not MAL II; hence MAL I was the only lectin used given that H5 LPAIVs have similar affinity for the receptors targeted by each lectin (43, 44); furthermore, any lack of specificity for sialic acid receptors is shared by both lectins (43).

Paraffin embedded tissue (duodenum, jejunum, ileum, cecum, colon, and bursa of Fabricius) from each bird was sectioned and stained with biotinylated lectin MAL I (Vector Laboratories, Burlingame, CA, USA), using previous described methods (17, 65) with minor modifications. Paraffin embedded tissue sections were deparaffinized and processed with the EnVision FLEX Target Retrieval Solution, Low pH kit wash buffers, blocking agents, and DAB plus chromogen working solution (Agilent, Dako Omnis, Santa Clara, CA, USA). Tissue sections were first treated with 100∝L of 3% Peroxide Block, then Avidin/Biotin blocking agent (Agilent, Dako Omnis, Santa Clara, CA, USA), and protein blocking. The tissue sections were incubated in 100∝L of MAL I for 32 minutes, and then treated for 20 minutes in 100∝L of streptavidin peroxidase (Agilent, Dako Omnis, Santa Clara, CA, USA). The working solution (200 mL) was applied and tissue sections were finally counter stained with 100 mL of hematoxylin (Gill's III, 1:10 dilution) (Astral Diagnostics Incorporated, West Deptford, New Jersey, USA). All tissue sections stained in the same batch were also stained with a known positive control of duck (Anas platyrhynchos domesticus) tissue.

We assessed the percentage of SAα2,3Gal in the proximal intestine (combined duodenum and jejunum), ileum, cecum, colon, and bursa of Fabricius by determining the percentage of lectin stained cells per 5 mm sections of tissue and cell type via a "lectin score." Using brightfield microscopy (400x), we looked specifically at the bursa epithelial cells, and three cell types in each intestinal tissue: the brush border, villi enterocytes, and crypt enterocytes. We scored as many fields of view (FOV) as possible with a maximum of 10 FOVs per cell type in each tissue. Each FOV received a score based on the percentage of cells stained in that FOV. A score of zero indicated that no cells were stained in that field of view. A score of 5 indicated that 1–10% of cells were stained. A score of 35 indicated that 11–60% of cells were stained. A score of 80 indicated that 61–100% of cells were stained. The scores for the FOVs were averaged to obtain a single score for each tissue and cell type, providing 13 separate LHS scores per bird. All samples were scored by the same individual (AD) to eliminate inter-observer error. In some cases, the tissue had become autolyzed and could not be scored, which was more common for the ileum and bursa tissues in mallards possibly due to longer processing times compared to teals.

Since the scoring method used to quantify the percentage of SAα2,3Gal was based off four categories of scores, compared to a quantitative continuous scale, we validated our scoring method with the absolute counts of stained cells for 20 randomly selected birds from mallard groups T1, T2, and T5. For each tissue, a single observer (AD) counted the number of stained cells out of 500 cells for each cell type of the ileum and colon, then took the percentage. With a total of 108 counts for 20 birds, we found high agreement between our scoring method and the absolute counts ($y = -3.15 + 0.88x$; $R^2 = 0.79$, $p < 0.001$).

Statistical Analysis

Statistical software R version 3.4.4 (67) was used for all statistical analyses. P-values of less than 0.05 were considered statistically significant and assumptions of normality were met by $Log_{10}(value + 1)$ transforming all virus titer and lectin histochemistry data. These methods were performed for both mallards and teals unless otherwise indicated. All analyses only included virus titer data collected on one to five DPI, when the majority of virus was shed.

Virus titer in an individual was quantified four ways: (1) cloacal swab titer collected per day between one and five DPI (Cloaca Titer), (2) cloacal swab titer from day of sacrifice (Last Cloaca Titer), (3) titer of virus in ileum tissue on the day of sacrifice (Ileum Titer), and (4) titer of virus in bursa tissue on the day of sacrifice (Bursa Titer). Using the "lm" function and DPI as a factor, we analyzed the relationship between Last Cloaca Titer, Ileum Titer, and Bursa Titer since all three of these variables were collected at the time the bird was sacrificed.

To test for differences in viral titer between species and between sexes within species we used a repeated measure, linear mixed effects model with the “lme” function in the “nlme” R package (68). For each model, Cloaca Titer was the dependent variable. To test species differences, species and DPI, plus their interaction, were set as fixed effects and individual birds were a random effect. To test sex-based differences, mallards and teals were analyzed in two different models with sex and DPI, plus their interaction, set as fixed effects and individual birds were a random effect. Differences in variance were detected using the Fligner-Killeen test (69). When differences in variance were detected, the “weights” argument was used as an adjustment for the model. ANOVA tables were visualized using the “anova.lme” function. We used the post-hoc Tukey’s test to assess inter-variable differences.

Lectin Histochemistry scores were analyzed using a similar approach to virus titer, but with lectin score as the dependent variable. Using the linear mixed model methods described above, we tested for lectin score differences between infected and control birds, setting infection status and tissue/cell-type plus their interaction as fixed effects and individual birds as a random effect. Using data from infected birds only, we also assessed species and sex-based differences in lectin score. Species differences were assessed by setting species and tissue/cell type and their interaction as fixed effects and individual birds as a random effect. Sex-based differences were analyzed in separate models for each species with sex and tissue/cell type, plus their interaction, as fixed effects and individual birds as a random effect.

We also looked at lectin score correlations between cell types within intestinal tissue type using Pearson’s r coefficient. We considered cell types within a tissue type (proximal, ileum, cecum, colon) with a coefficient of 0.8 or higher to indicate a strong correlation. If all three cell types within a tissue were highly correlated, we used the “pccomp” function, a principle component analysis (PCA) method, to reduce the data into one component variable we called “PC tissue type.” Each PC variable accounted for greater than 80% of the variation between the cell types of that particular tissue. PC variables generated from the PCA were used in the multiple linear regression models (MLR) to determine the relationship between virus titer and lectin score.

Virus titer and lectin score relationship was determined by assessing three different MLR models for each species using the Last Cloaca Titer, Ileum Titer, and Bursa Titer as the dependent variable. Independent variables for the Last Cloaca Titer model consisted of the LHS variables, the principal components described above (when appropriate), and five control variables: sex, BCS, treatment group (group), body mass in grams at 55 days after hatch (mass), and inoculation age in days (age). Independent variables for the Ileum Titer MLR model included only the ileum LHS variables and the five control variables. Only the bursa epithelium LHS variable and the five control variables were included in the Bursa Titer MLR model. Due to autolysis of tissue in many mallard bursa samples, the bursa epithelium could not be assessed; therefore, the Bursa Titer MLR was not evaluated in mallards. Also, some additional tissue samples were too autolyzed to assess, which reduced the sample size for mallard MLR models from 40 to 25 birds (T1 = 6; T2 = 8; T5 = 11), and teal MLR models from 36 to 32 birds (T1 = 9, T3 = 11, T = 12).

To determine the best fitting MLR model for each dependent variable, we followed a consistent procedure. Using the “lm” function, global models were tested for dependent variables Last Cloaca Titer, Ileum Titer, and Bursa Titer separately. To select parsimonious model fits to the data, we used the “step” function for stepwise variable selection based on the generalized Akaike’s Information Criterion (AIC) (70). We then used variance inflation factor (VIF) scores, the “vif” function in the “car” package (71), to identify problematic co-linear predictors from the stepwise-chosen models. Independent variables with VIFs > 3.0 were determined problematic and were removed from the model one at a time until all VIFs < 3.0 (72). When two VIFs were > 3.0 and < 1.0 in difference, we tested alternative models. Stepwise variable selection was used for each model to ensure the best fitting model. Residual plots were reviewed. For each of the three dependent variables, the model with both the lowest AIC, highest adjusted R^2, and satisfactory residual patterns (e.g., no linear or nonlinear trend in residuals, little to no heterogeneous variance in residuals, and no suspected outlier observations) was chosen as the best fitting model to the data.

MLR model construction was different for mallards and teals. Mallards had high correlations between cell type in the proximal and cecum intestinal tissues (> 80%; Additional File 11); thus, the proximal and cecum LHS variables were analyzed using PCA to create one PC1 variable for proximal (Proximal PC) and one PC1 variable for cecum (Cecum PC) for the 25 birds included in the mallard MLR model (Additional File 13). In teals, each ileum LHS variable was identical; therefore, PCA could not be performed, and instead, the ileum villi LHS variable alone was used to represent the ileum lectin score in both the Last Cloaca Titer MLR model and the Ileum MLR model.

Abbreviations

AIV
Avian influenza virus
HPAIV
Highly pathogenic avian influenza virus
LPAIV
Low pathogenic avian influenza virus
SAα2,3Gal
alpha-2,3 sialic acid receptor
MAL I
Maackia Amurensis I
MAL II
Maackia Amurensis II
MSU
Michigan State University
ECE
embryonated chicken eggs
EID50
50% egg infection dose
DMEM
Dulbecco's modified eagle medium
DPI
Days post infection
FOV
Fields of view
PCA
Principle component analysis
MLR
Multiple linear regression
BCS
Body condition score
AIC
Akaike's information criteria
VIF
Variance inflation factor

Declarations

Ethics approval and consent to participate

Protocols for animal care and experimental sampling procedures were approved by Michigan State University Institutional Animal Care and Use Committee (AUF 12/16-211-00). All euthanasia procedures were in accordance with the Animal Welfare Act and Guidelines to the Use of Wild Birds in Research. Duck eggs were collected in accordance with a U.S. Fish and Wildlife Permit (M Bl 94270-2) and North Dakota Game and Fish Department License #GNF03639403. Birds used in study were hatched from collected eggs.

Consent for publication

Not Applicable

Availability of Data and Materials

All data analyzed during this study are included in this published article (Additional File 1).

Competing Interests

The authors declare that they have no competing interests. This research does not reflect the official positions and policies of the US EPA. Mention of products/trade names does not constitute recommendation for use by US EPA.

Funding

This study was funded by the National Science Foundation Career Grant 135077 (JCO). The funding body had no role in developing the study design, data collection, analysis and interpretation, decision to publish, or preparation of the manuscript.

Authors' Contributions

JCO, JMF, MDJ together conceptualized the study, and JCO secured funding from NSF. ACD contributed to the development of the final experimental design and methods and was responsible for the care of the birds, collection of the samples, lectin histochemistry scoring, statistical analysis, and writing of the original manuscript with assistance and supervision from JCO. JMF and MDJ reviewed and edited the manuscript, as well as provided technical and statistical expertise.

Acknowledgements

We thank F. Rohwer and M. Buxton from Delta Waterfowl for assistance in egg collection; Dr. S. Fitzgerald and Dr. M. Kuipel at the MSU VDL for assistance in developing the lectin score technique and lectin histochemistry protocols; J. Landgraff from the MSU genomics core for assistance with RT-PCR; A. Dennhardt and D. Pak at MSU CSTAT for guidance with statistical analyses; D. Incorvaia, E. Nelson, M. Gutman, and J. Emerson who were NSF funded undergraduate interns (REU); and E. Hergert, C. Sklarczyk, M. Yang, T. McCord, M. Phillips, O. Lefere, W. Decker, V. Colberg, V. Olszewski, K. Lesko, O. Rose, J. Shemanski, E. Goran, R. Cena, A. Bedore, and L. Carlson who were undergraduate volunteers who assisted with the collection of eggs, care of birds, sample collection, and laboratory sample processing.

References

1. Halvorson D, Karunakaran D, Senne D, Kelleher C, Bailey C, Abraham A, et al. Epizootiology of avian influenza: simultaneous monitoring of sentinel ducks and turkeys in Minnesota. Avian diseases. 1983:77-85.
2. Halvorson DA. Control of Low Pathogenicity Avian Influenza. Avian Influenza: Blackwell Publishing Ltd.; 2009. p. 513-36.
3. Stallknecht D, Shane S. Host range of avian influenza virus in free-living birds. Veterinary research communications. 1988;12(2-3):125-41.
4. Röhm C, Horimoto T, Kawaoka Y, Süss J, Webster RG. Do hemagglutinin genes of highly pathogenic avian influenza viruses constitute unique phylogenetic lineages? Virology. 1995;209(2):664-70.
5. Mamlouk A, Artois M, Ottmann M, Belli P, Lina B, Sabatier P. Shedding and intestinal spread of avian influenza virus H5N2 in dabbling ducks (pre-liminary study). Revue De Medecine Veterinaire. 2011;162(10):454-9.
6. Koch G, Elbers AR. Outdoor ranging of poultry: a major risk factor for the introduction and development of High-Pathogenicity Avian Influenza. NJAS-Wageningen Journal of Life Sciences. 2006;54(2):179-94.
7. Woolhouse ME, Dye C, Etard J-F, Smith T, Charlwood J, Garnett G, et al. Heterogeneities in the transmission of infectious agents: implications for the design of control programs. Proceedings of the National Academy of Sciences. 1997;94(1):338-42.
8. Lloyd-Smith JO, Schreiber SJ, Kopp PE, Getz W. Superspreading and the effect of individual variation on disease emergence. Nature. 2005;438(7066):355-9.
9. Matthews L, McKendrick I, Ternent H, Gunn G, Synge B, Woolhouse M. Super-shedding cattle and the transmission dynamics of Escherichia coli O157. Epidemiology and Infection. 2006;134(01):131-42.
10. Jankowski MD, Williams CJ, Fair JM, Owen JC. Birds Shed RNA-Viruses According to the Pareto Principle. PLoS One. 2013;8(8):e72611.
11. Webster RG, Yakhno M, Hinshaw VS, Bean WJ, Murti KC. Intestinal influenza: replication and characterization of influenza viruses in ducks. Virology. 1978;84(2):268-78.
12. Daoust P-Y, Kibenge FS, Fouchier RA, Van De Bildt MW, Van Riel D, Kuiken T. Replication of low pathogenic avian influenza virus in naturally infected mallard ducks (Anas platyrhynchos) causes no morphologic lesions. Journal of wildlife diseases. 2011;47(2):401-9.
13. Franca M, Stallknecht D, Poulson R, Brown J, Howerth E. The pathogenesis of low pathogenic avian influenza in mallards. Avian diseases. 2012;56(4s1):976-80.
14. 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.
15. Ito T, Suzuki Y, Suzuki T, Takada A, Horimoto T, Wells K, et al. Recognition of N-glycolylneuraminic acid linked to galactose by the α2, 3 linkage is associated with intestinal replication of influenza A virus in ducks. Journal of virology. 2000;74(19):9300-5.
16. Kimble B, Nieto GR, Perez DR. Characterization of influenza virus sialic acid receptors in minor poultry species. Virol J. 2010;7(365):422X-7.
17. Costa T, 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(1):28.
18. Franca M, Stallknecht D, Howerth E. Expression and distribution of sialic acid influenza virus receptors in wild birds. Avian pathology. 2013;42(1):60-71.
19. Wille M, Van Run P, Waldenström J, Kuiken T. Infected or not: are PCR-positive oropharyngeal swabs indicative of low pathogenic influenza A virus infection in the respiratory tract of Mallard Anas platyrhynchos? Veterinary research.

2014;45(1):53.
20. Ellström P, Latorre-Margalef N, Griekspoor P, Waldenström J, Olofsson J, Wahlgren J, et al. Sampling for low-pathogenic avian influenza A virus in wild Mallard ducks: Oropharyngeal versus cloacal swabbing. Vaccine. 2008;26(35):4414-6.
21. Jankowski MD, Glaberman SR, Kimball DB, Taylor-McCabe KJ, Fair JM. Sialic acid on avian erythrocytes. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology. 2019;238:110336.
22. Munster VJ, Wallensten A, Baas C, Rimmelzwaan GF, Schutten M, Olsen B, et al. Mallards and highly pathogenic avian influenza ancestral viruses, northern Europe. Emerging infectious diseases. 2005;11(10):1545.
23. Takekawa JY, Newman SH, Xiao X, Prosser DJ, Spragens KA, Palm EC, et al. Migration of waterfowl in the East Asian flyway and spatial relationship to HPAI H5N1 outbreaks. Avian diseases. 2010;54(s1):466-76.
24. Papp Z, Clark RG, Parmley EJ, Leighton FA, Waldner C, Soos C. The ecology of avian influenza viruses in wild dabbling ducks (Anas spp.) in Canada. PloS one. 2017;12(5):e0176297.
25. Stallknecht D, Shane S, Zwank P, Senne D, Kearney M. Avian influenza viruses from migratory and resident ducks of coastal Louisiana. Avian diseases. 1990:398-405.
26. Ferro PJ, Budke CM, Peterson MJ, Cox D, Roltsch E, Merendino T, et al. Multiyear surveillance for avian influenza virus in waterfowl from wintering grounds, Texas coast, USA. Emerging infectious diseases. 2010;16(8):1224.
27. Cheung TK, Poon LL. Biology of influenza a virus. Annals of the New York Academy of Sciences. 2007;1102(1):1-25.
28. Kida H, Yanagawa R, Matsuoka Y. Duck influenza lacking evidence of disease signs and immune response. Infect Immun. 1980;30(2):547-53.
29. Kim YS, Ho SB. Intestinal goblet cells and mucins in health and disease: recent insights and progress. Curr Gastroenterol Rep. 2010;12(5):319-30.
30. McAuley JL, Corcilius L, Tan HX, Payne RJ, McGuckin MA, Brown LE. The cell surface mucin MUC1 limits the severity of influenza A virus infection. Mucosal Immunology. 2017;10:1581.
31. Kelm S, Schauer R. Sialic acids in molecular and cellular interactions. Int Rev Cytol. 175: Elsevier; 1997. p. 137-240.
32. Samji T. Influenza A: understanding the viral life cycle. The Yale journal of biology and medicine. 2009;82(4):153.
33. Linden S, Sutton P, Karlsson N, Korolik V, McGuckin M. Mucins in the mucosal barrier to infection. Mucosal immunology. 2008;1(3):183-97.
34. Matrosovich M, Herrler G, Klenk HD. Sialic acid receptors of viruses. SialoGlyco Chemistry and Biology II: Springer; 2013. p. 1-28.
35. Davison F, Kaspers B, Schat KA. Avian immunology2008.
36. Munday J, Floyd H, Crocker PR. Sialic acid binding receptors (siglecs) expressed by macrophages. J Leukoc Biol. 1999;66(5):705-11.
37. Han D, Hu Y, Teng K, Deng X. Lower expression of sialic acid receptors in the cecum of silky fowl (Gallus gallus domesticus Brisson) compared to white leghorn. Poultry science. 2016;95(6):1290-5.
38. Byrd-Leotis L, Cummings RD, Steinhauer DA. The interplay between the host receptor and influenza virus hemagglutinin and neuraminidase. International journal of molecular sciences. 2017;18(7):1541.
39. 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):e22633.
40. Hanson BA, Swayne DE, Senne DA, Lobpries DS, Hurst J, Stallknecht DE. Avian influenza viruses and paramyxoviruses in wintering and resident ducks in Texas. Journal of wildlife diseases. 2005;41(3):624-8.
41. Hénaux V, Samuel MD. Avian influenza shedding patterns in waterfowl: implications for surveillance, environmental transmission, and disease spread. Journal of wildlife diseases. 2011;47(3):566-78.

42. Knibbs R, Goldstein I, Ratcliffe RM, Shibuya N. Characterization of the carbohydrate binding specificity of the leukoagglutinating lectin from Maackia amurensis. Comparison with other sialic acid-specific lectins. J Biol Chem. 1991;266(1):83-8.
43. Geisler C, Jarvis DL. Letter to the Glyco-Forum: Effective glycoanalysis with Maackia amurensis lectins requires a clear understanding of their binding specificities. Glycobiology. 2011;21(8):988-93.
44. Gambaryan A, Tuzikov A, Pazynina G, Bovin N, Balish A, Klimov A. Evolution of the receptor binding phenotype of influenza A (H5) viruses. Virology. 2006;344(2):432-8.
45. Pepin KM, VanDalen KK, Mooers N, Ellis J, Sullivan H, Root JJ, et al. Quantification of heterosubtypic immunity between avian influenza subtypes H3N8 and H4N6 in multiple avian host species. The Journal of general virology. 2012;93(Pt 12):2575.
46. Zuk M, McKean KA. Sex differences in parasite infections: patterns and processes. International journal for parasitology. 1996;26(10):1009-24.
47. Zuk M, Johnsen TS. Seasonal changes in the relationship between ornamentation and immune response in red jungle fowl. Proceedings of the Royal Society of London B: Biological Sciences. 1998;265(1406):1631-5.
48. Trager W, McGhee RB. Factors in plasma concerned in natural resistance to an avian malaria parasite (Plasmodium lophurae). J Exp Med. 1950;91(4):365-79.
49. Killian ML. Hemagglutination assay for the avian influenza virus. Methods in molecular biology (Clifton, NJ). 2008;436(Journal Article):47.
50. Ellström P, Latorre-Margalef N, Griekspoor P, Waldenström J, Olofsson J, Wahlgren J, et al. Sampling for low-pathogenic avian influenza A virus in wild Mallard ducks: oropharyngeal versus cloacal swabbing. Vaccine. 2008;35(26):4414-6.
51. Fair JM, Jones J. Guidelines to the use of wild birds in research. Third Edition ed: Ornithological Council; 2010.
52. Higgins KF, Kirsch LM, Ball Jr IJ. A cable-chain device for locating duck nests. The Journal of Wildlife Management. 1969:1009-11.
53. Woolcock PR. Avian influenza virus isolation and propagation in chicken eggs. Avian influenza virus: Springer; 2008. p. 35-46.
54. Reed LJ, Muench H. A simple method of estimating fifty per cent endpoints. Am J Epidemiol. 1938;27(3):493-7.
55. Jourdain E, Gunnarsson G, Wahlgren J, Latorre-Margalef N, Bröjer C, Sahlin S, et al. Influenza virus in a natural host, the mallard: experimental infection data. PLoS One. 2010;5(1):e8935.
56. Cardona CJ, Halvorson DA, Brown JD, Pantin-Jackwood MJ. Conducting influenza virus pathogenesis studies in avian species. Animal Influenza Virus: Springer; 2014. p. 169-83.
57. França M, Poulson R, Brown J, Howerth E, Berghaus R, Carter D, et al. Effect of different routes of inoculation on infectivity and viral shedding of LPAI viruses in mallards. Avian diseases. 2012;56(4s1):981-5.
58. Mohamed R. Sexual Dimorphism in the Anatomical Features of the Syrinx in the White Pekin Ducks (Anas platyrhynchos). International Journal of Agricultural Sciences and Veterinary Medicine. 2017;5(5):78-85.
59. Spackman E, Suarez DL. Type A influenza virus detection and quantitation by real-time RT-PCR. Avian influenza virus: Springer; 2008. p. 19-26.
60. Ward CL, Dempsey MH, Ring CJA, Kempson RE, Zhang L, Gor D, et al. Design and performance testing of quantitative real time PCR assays for influenza A and B viral load measurement. J Clin Virol. 2004;29(3):179-88.
61. 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. Journal of Veterinary Diagnostic Investigation. 2009;21(6):771-8.

62. Spackman E, Senne DA, Myers T, Bulaga LL, Garber LP, Perdue ML, et al. Development of a real-time reverse transcriptase PCR assay for type A influenza virus and the avian H5 and H7 hemagglutinin subtypes. J Clin Microbiol. 2002;40(9):3256-60.
63. Pleil JD. Imputing defensible values for left-censored 'below level of quantitation'(LoQ) biomarker measurements. Journal of breath research. 2016;10(4):045001.
64. Yu JE, Yoon H, Lee HJ, Lee JH, Chang BJ, Song CS, et al. Expression patterns of influenza virus receptors in the respiratory tracts of four species of poultry. Journal of veterinary science. 2011;12(1):7-13.
65. Pillai S, Lee CW. Species and age related differences in the type and distribution of influenza virus receptors in different tissues of chickens, ducks and turkeys. Virol J. 2010;7(5).
66. Kuchipudi SV, Nelli R, White GA, Bain M, Chang KC, Dunham S. Differences in influenza virus receptors in chickens and ducks: implications for interspecies transmission. Journal of molecular and genetic medicine: an international journal of biomedical research. 2009;3(1):143.
67. RCore T. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. URL http://www R-project org. 2016.
68. Pinheiro J, Bates D, DebRoy S, Sarkar D, Team RC. nlme: Linear and nonlinear mixed effects models. R package version. 2012;3(0).
69. Conover WJ, Johnson ME, Johnson MM. A comparative study of tests for homogeneity of variances, with applications to the outer continental shelf bidding data. Technometrics. 1981;23(4):351-61.
70. Hastie TJ, Pregibon D. Generalized linear models. Statistical models in S: Routledge; 2017. p. 195-247.
71. Fox J, Weisberg S. An R companion to applied regression: Sage Publications; 2018.
72. Zuur AF, Ieno EN, Elphick CS. A protocol for data exploration to avoid common statistical problems. Methods in ecology and evolution. 2010;1(1):3-14.

Additional Files

Additional File 1: All analyzed data for LPAIV H5N9 infected and control blue-winged teals and mallards. NA indicates missing data.

Additional File 2: Mean virus titer ± 95% confidence interval for (a) species, (b) days post infection (DPI), and (c) the interaction of species and DPI for mallard and blue-winged teal cloacal swab samples one to five DPI.

Additional File 3: Mean virus titer ± 95% confidence intervals for (a,c) sex, and (b,d) the interaction of sex and days post infection (DPI) for male (M) and female (F) mallard and teal blue-winged teal cloacal swab samples one to five DPI.

Additional File 4: Mean lectin scores ± 95% confidence intervals for intestinal tissues proximal (duodenum and jejunum), ileum, cecum, and colon for LPAIV H5N9 infected male (M) and female (F) mallards and blue-winged teals.

Additional File 5: Residual Plots and AIC table for mallard Last Cloaca Titer multiple linear regression model.

Additional File 6: Residual plots and AIC table for mallard Ileum Titer multiple linear regression model.

Additional File 7: Residual plots and AIC table for blue-winged teal Last Cloaca Titer multiple linear regression model.

Additional File 8: Quantitative limit validation methods and results

Additional File 9: Residual plots and AIC table for blue-winged teal Ileum Titer multiple linear regression model.

Additional File 10: Residual plots and AIC table for blue-winged teal Bursa Titer multiple linear regression model.

Additional File 11: Pearson's r correlation matrix for mallard lectin histochemistry scores.

Additional File 12: Experimental Design

Additional File 13: Mallard PCA results for proximal and cecum lectin scores.

Figures

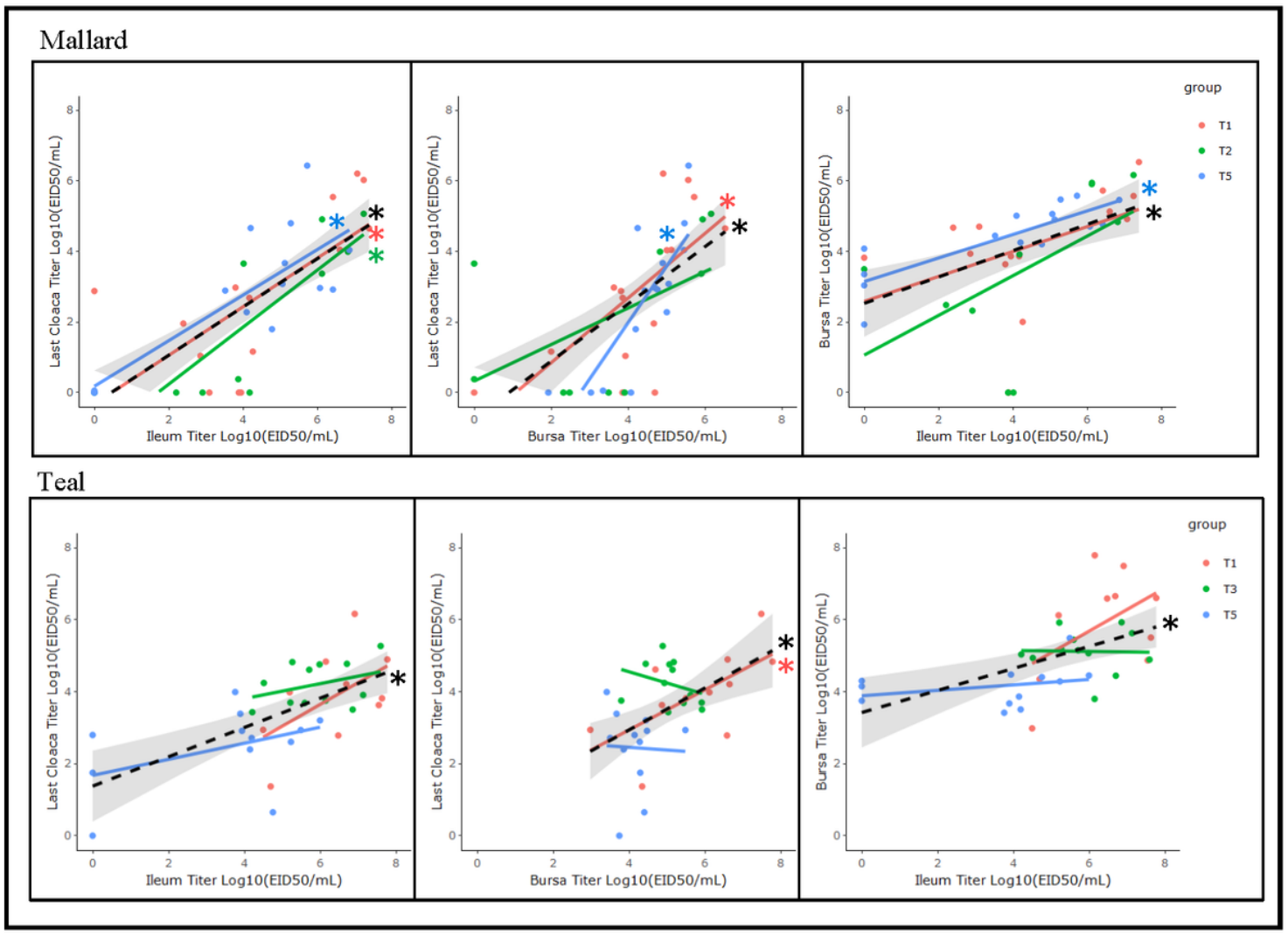

Figure 1

LPAIV H5N9 virus titers in the bursa, ileum, and cloacal swabs are positively related. Black trendline is the linear regression for all birds sacrificed on 1, 2, and 5 days post infection (DPI) with the 95% confidence interval shaded in gray. Colored trendlines represent each treatment group: T1 represents birds sacrificed on 1 DPI, etc. Trendlines indicated with a (*) indicate a statistically significant relationship ($p < 0.05$).

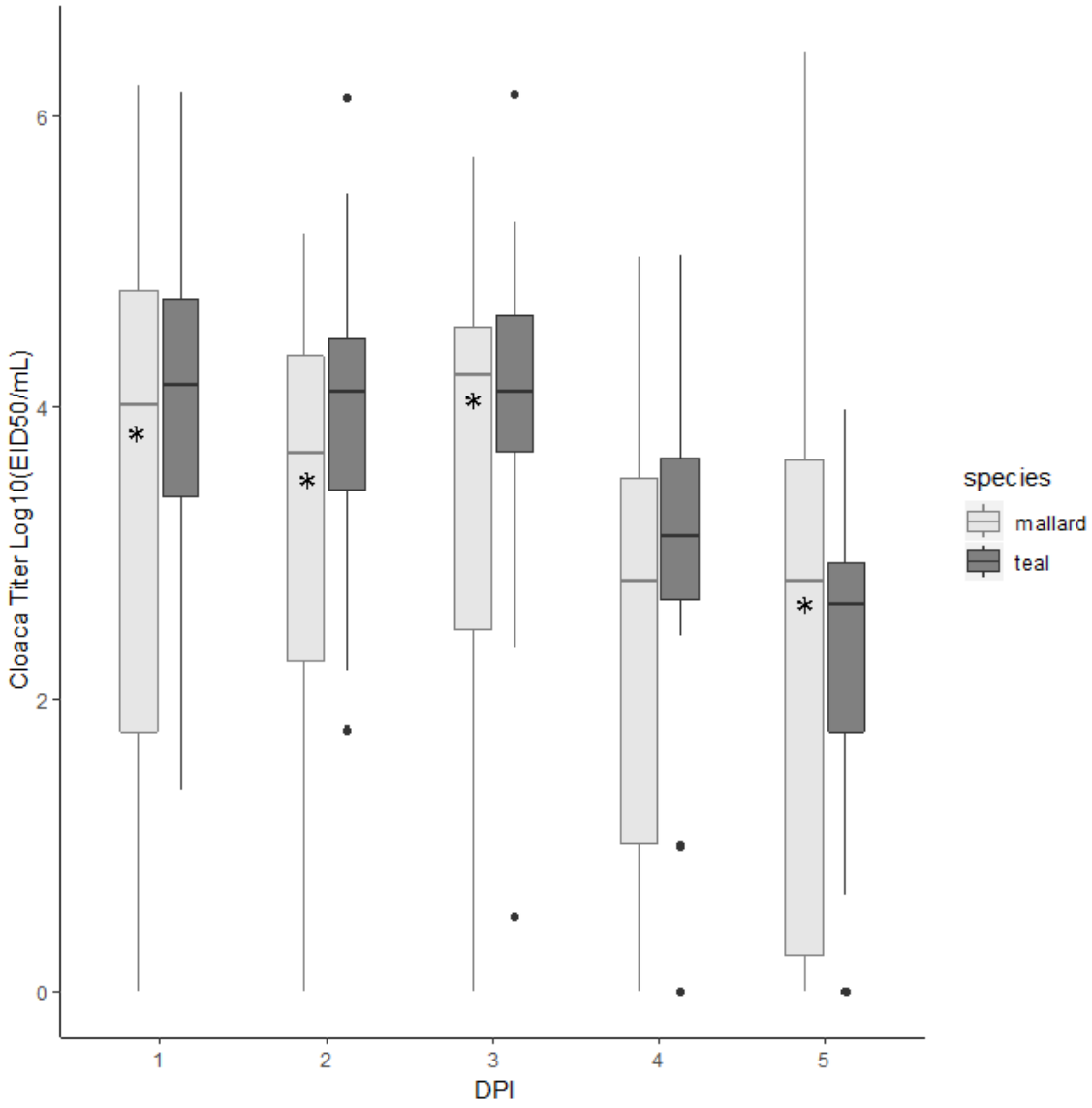

Figure 2

Cloacal swab virus titer boxplots for mallard and blue-winged teals infected with LPAIV H5N9. Horizontal bar within the box is the median value, solid dots indicate values falling above the upper or below the lower quartile + 1.5 times the interquartile distance. (*) indicates statistically higher variation between species for each day post infection (DPI; $p < 0.05$).

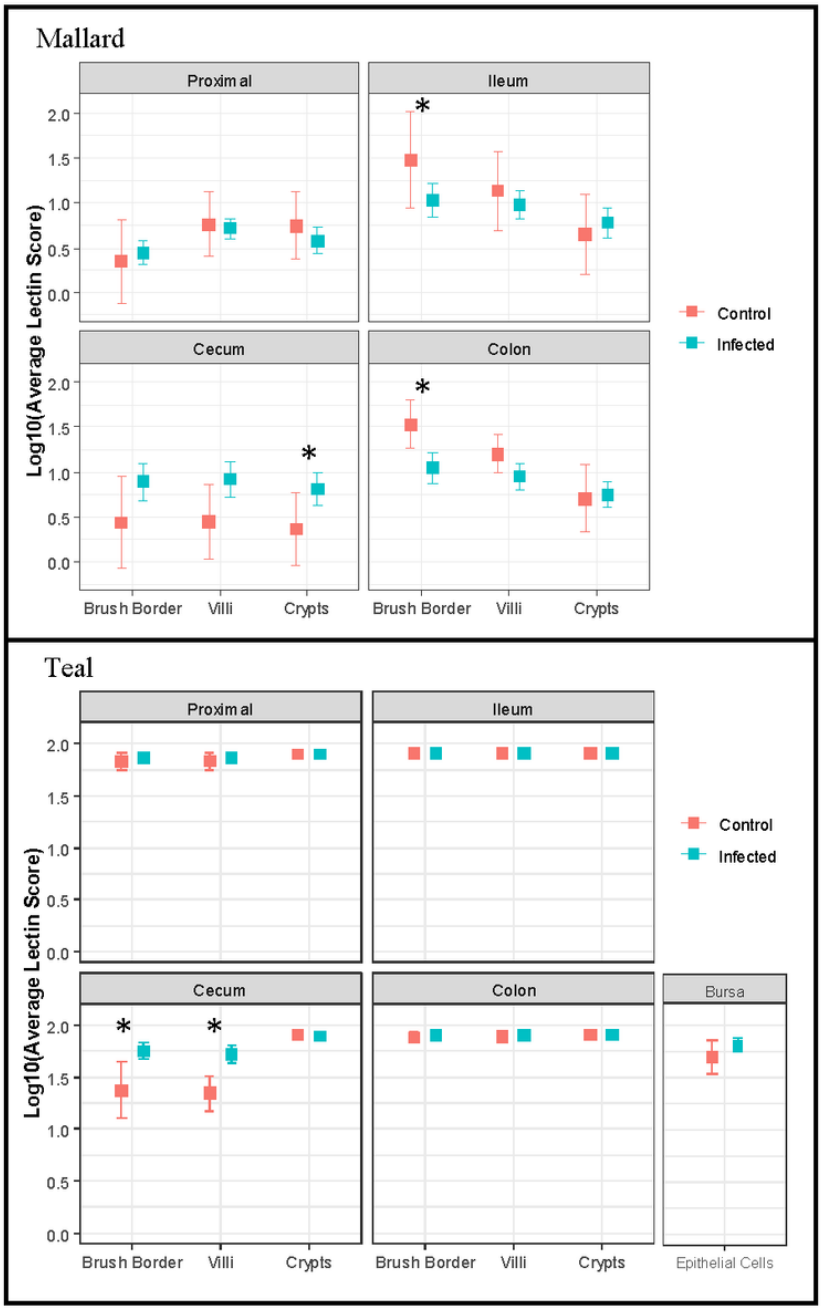

Figure 3

Lectin score differences between control and LPAIV-infected birds. Mean lectin scores + 95% confidence intervals of intestinal tissues proximal (duodenum and jejunum), ileum, cecum, and colon for LPAIV H5N9 infected and control mallards and blue-winged teals. Bursa epithelial cells are included for teals only. (*) indicates tissue/cell type with a statistically significant difference between control and infected birds ($p < 0.05$).

Figure 4

Lectin scores are higher, but less varied in blue-winged teal intestinal tissues compared to mallards. Lectin binding is positive where the brown colored stain is visible. The individual bird ID, tissue, and lectin score (villi enterocyte/epithelial cells) are given for each histological photograph. Scores were determined by averaging the scores for each field of view evaluated at 400x. Each field of view was given the following score: 0, no cells stained; 5, 1-10% of cells were stained; 35,

11-60% of cells stained; and 80, 61-100% of cells stained. Segments (a) and (b) show the range of lectin scores between sections of intestinal tissue in one individual (proximal represents duodenum or jejunum). Segments (c) and (d) show the range of lectin between individuals for the ileum tissue specifically. Segments (e) and (f) show lectin scores in the bursa of Fabricius. All photos were taken at 200x brightfield microscopy.

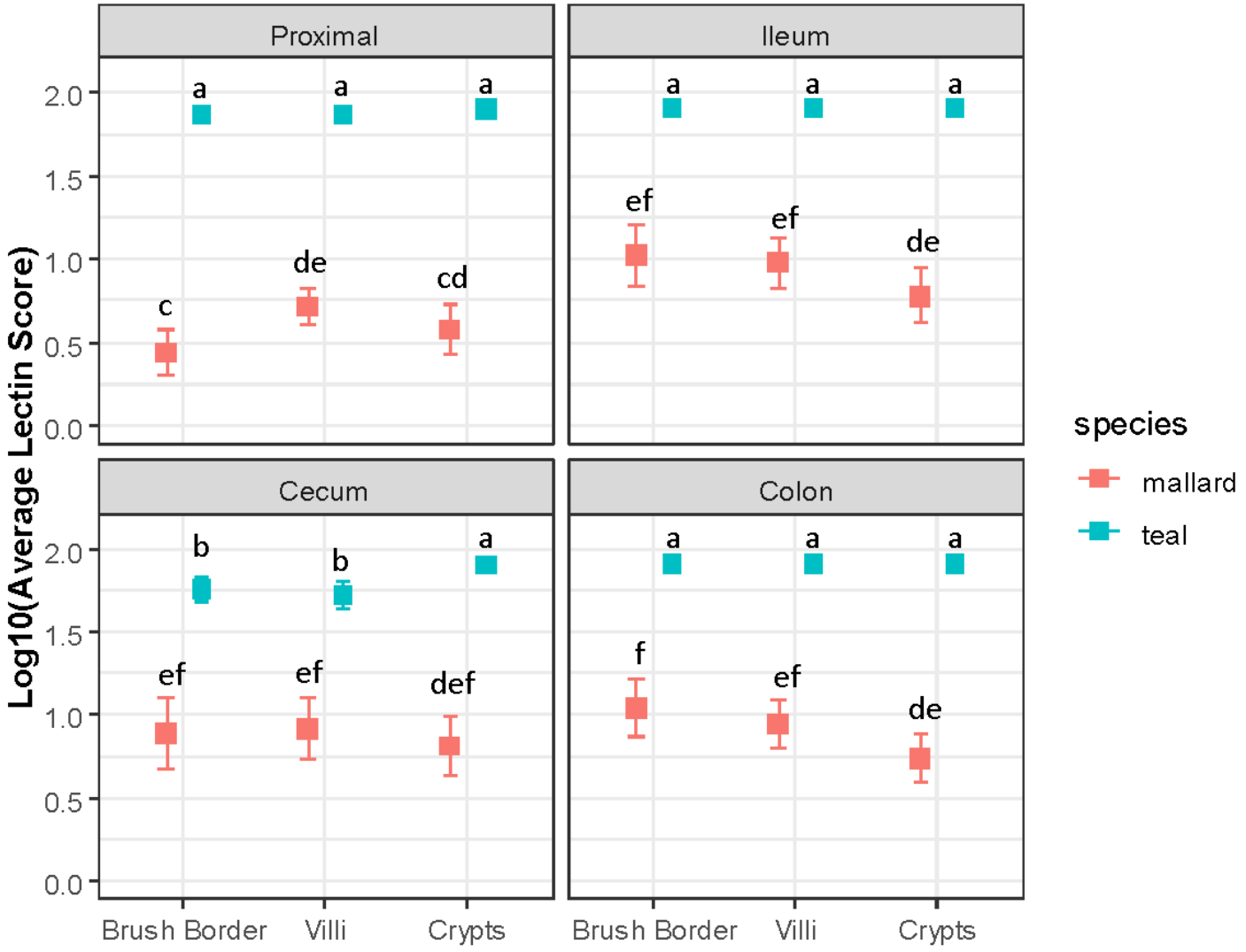

Figure 5

Teals had higher lectin scores than mallards, but with less variation. Mean lectin scores + 95% confidence intervals for intestinal tissues proximal (duodenum and jejunum), ileum, cecum, and colon for LPAIV H5N9 infected mallards and blue-winged teals. Across all panels, points with different letters are considered significantly different ($p < 0.05$).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalFile10.pdf
- AdditionalFile12.pdf
- AddtionalFile9.pdf
- AdditionalFile8.docx

- AdditionalFile5.pdf
- AdditionalFile6.pdf
- ARRIVEGuidelinesChecklist.docx
- AdditionalFile2.pdf
- AdditionalFile3.pdf
- AdditionalFile1.xlsx
- AdditionalFile11.csv
- AdditionalFile4.pdf
- AdditionalFile13.csv
- AdditionalFile7.pdf