Industry-wide surveillance of Marek's disease virus on commercial poultry farms: underlying potential for virulence evolution and vaccine escape

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Abstract

Marek's disease virus is a herpesvirus of chickens that costs the worldwide poultry industry over 1 billion USD annually. Severity of disease has increased over the last half century due to evolution of the virus, a trajectory accompanied by reduced efficacy of two generations of Marek's disease vaccines. Whether continued evolution will erode the efficacy of current vaccines is an open question. We conducted a three-year surveillance study to assess the prevalence of Marek's disease virus on commercial poultry farms, determine the effect of

various factors on virus prevalence, and document virus dynamics on broiler chicken houses over short (weeks) and long (years) timescales. We extracted DNA from dust samples collected from commercial chicken and egg production facilities in Pennsylvania, USA. Quantitative polymerase chain reaction (qPCR) was used to assess wild-type virus detectability and concentration. Using data from 1018 dust samples with Bayesian generalized linear mixed effects models, we determined the factors that correlated with virus incidence. Maximum likelihood and autocorrelation function estimation on 3727 dust samples were used to document and characterize virus concentrations within houses over time. Overall, wild-type virus was detectable at least once on 36 of 104 farms at rates that varied substantially between farms. Virus was detected in 1 of 3 broiler-breeder operations (companies), 4 of 5 broiler operations, and 3 of 5 egg layer operations. Marek's disease virus detectability differed by production type, bird age, day of the year, operation (company), farm, house, flock, and sample. Operation (company) was the most important factor, accounting for between 12% and 63.4% of the variation in virus detectability. Within individual houses, virus concentration often dropped below detectable levels and reemerged later. The data presented here characterize Marek's disease virus dynamics, a prerequisite in determining whether current vaccine protection will be eroded by future virus evolution.

Keywords: *Marek's disease virus; surveillance; epidemiology; virulence evolution; vaccine escape*

Introduction

Marek's disease, caused by Marek's disease virus (*Gallid herpesvirus II*), was first described over a century ago as a relatively mild polyneuritis condition in chickens. Over time the disease has increased in severity due to evolution of the virus (Osterrieder et al., 2006; Witter, 1997). Hyper virulent strains of the virus, termed "very virulent plus" strains, can be collected today that are capable of killing all unvaccinated chickens within ten days of exposure (Read et al., 2015). Since the development of the first vaccine against this disease, mass vaccination has been a key feature in sustaining industrial-scale poultry production (Davison and Nair, 2004). These vaccines are described as "leaky", because they protect vaccinated hosts from developing symptoms of disease, but they nonetheless allow for infection and onward transmission of the virus (Islam et al., 2008; Ralapanawe et al., 2016; Witter et al., 1971). This feature of Marek's disease vaccination may have played a key role in the pathogen's evolutionary increase in virulence (Read et al., 2015). Moreover, the increase in virulence has been accompanied by a reduction in vaccine efficacy (Witter, 1997). Two generations of Marek's disease vaccines have been undermined by virus evolution, and this evolutionary trajectory has been well documented (Witter, 1997). Marek's disease virus might therefore be the single best system for studying the effects of mass vaccination on evolutionary reductions in vaccine efficacy. Whether the efficacy of existing vaccine control strategies will decline in the future is an open question (Nair, 2005), whose answer partially depends on the ecology of the virus. This is because evolutionary outcomes can vary greatly depending on ecological details, which in this case depend on where in the industry the

evolution is occurring (Atkins et al., 2013). Nevertheless, very little is known about the distribution of the virus across the industry. Here we surveilled virus across the industry by sampling dust (the infectious vehicle) from commercial chicken facilities located throughout Pennsylvania from 2012 to 2015. We used these data to ask where Marek's disease virus is found, how its prevalence differs across the industry, and how its concentration changes within flocks over time.

Early efforts to quantify Marek's disease virus prevalence in the field used serological data to determine that infection was extremely prevalent (Biggs et al., 1972; Chubb and Churchill, 1968; Ianconescu and Samberg, 1971; Witter et al., 1971). Clinical disease and production losses coupled with these observations motivated near-universal vaccination in commercial poultry farming in the United States and many other nations. More recently, virus prevalence has been inferred from disease data (Dunn and Gimeno, 2013; Kennedy et al., 2015; Purchase, 1985; Witter, 1996), but the reliability of these methods are limited by biases inherent in disease data generally (King et al., 2008) and these data in particular (Kennedy et al., 2015). The development of quantitative polymerase chain reaction (qPCR) protocols specific for Marek's disease virus have made it possible to detect and quantify virus collected from field settings (Baigent et al., 2016; Gimeno et al., 2014; Walkden-Brown et al., 2013a). Of the three studies that have used qPCR methods to study field samples, two were performed in unvaccinated (Wajid et al., 2013) or mostly unvaccinated (Walkden-Brown et al., 2013b) chickens, and the third found extremely low prevalence of Marek's disease virus while looking in only a single sector of poultry farming (Ralapanawe et al.,

2015). These results might therefore be of limited value with regard to understanding the evolution of Marek's disease virus in the field. Here we performed quantitative polymerase chain reaction (qPCR) on samples collected from chicken farms throughout Pennsylvania, USA, to directly examine Marek's disease virus dynamics on commercial poultry farms. The farms used in this study encompass much of the diversity of industrial-scale commercial chicken-meat and egg production.

When quantifying virus dynamics, it is important to appreciate that commercial poultry farming is highly structured (fig. 1). At its highest level, industrialized commercial chicken production is broadly divided into egg laying birds, broiler birds, and layer-breeder or broiler-breeder birds. This distinction is important because the natural history, genetics, and management practices can often different dramatically between these production types. Further structure exists within these production types, because a single company, hereafter referred to as an "operation", will often target particular sectors of the poultry market (e.g. kosher, organic, live bird market, cage-free eggs, etc.) requiring different management practices which can potentially impact virus dynamics. Biosecurity practices, equipment, and feed mills are also often shared within operations, and so some operations may better control virus dynamics than others. Within an operation there is an additional layer of structure because birds are reared on moderately small premises (farms) that are managed by a single person or a small number of people. These people, often referred to as "growers", are in part responsible for monitoring and maintaining bird health, and thus their actions could in turn affect virus dynamics. Within single farms, there are usually multiple houses.

Within these houses, there are successive flocks of birds. Finally, within houses at any point in time there may be variation between individual samples if the virus is not perfectly mixed throughout the house. Our goal was to quantify the relative importance of these factors on the variation we observed in the prevalence of Marek's disease virus. This is a critical first step in evaluating risk factors both for disease outbreaks, and for virus evolution that might undermine current vaccine strategies and lead to increased pathogen virulence.

Methods

Background and sample collection

Marek's disease virus is a herpesvirus (Calnek et al., 1970) that is transmitted through inhalation of virus-contaminated dust (Colwell and Schmittle, 1968). Once inside a host, the virus goes through an incubation period of one to three weeks, after which new virus particles are produced and shed from feather follicle epithelial cells (Baigent et al., 2005; Islam and Walkden-Brown, 2007). The shedding of this infectious virus co-occurs with the shedding of epithelial cells, and so the virus can be found in "chicken dust" (Carrozza et al., 1973), a by-product of chicken farming made up of sloughed off epithelial cells, feathers, fecal material, chicken feed, and bedding material (Collins and Algers, 1986). Once shedding is initiated, it is believed to occur for the rest of the chicken's life, although studies quantifying virus shed have been limited to relatively young birds. We collected samples of chicken dust from farms, and measured virus concentrations to gain insight into the virus's dynamics in

the field.

We visited and collected dust from as many different farms as possible to gain insights into whether and where the virus was detectable. Logistical constraints including those imposed by biosecurity concerns, industry participation, total availability of farms, and time-varying presence of chicken cohorts resulted in a sampling schedule best described formally as haphazard rather than random. After finding farms where virus was repeatedly detected, we began collecting from these farms at approximately weekly intervals to quantify changes over time (hereafter referred to as the "longitudinal data"). All samples collected during this study are being stored indefinitely at -80 °C.

All samples used in this study were collected by the authors. To mitigate the spread of pathogens between poultry farms, only farms from a single operation were visited in a single day. We wore hairnets, beard covers, plastic boots, and coverall suits while in poultry houses, and these materials were changed between each farm to minimize opportunities for virus spread. Plastic 1.5 ml Eppendorf tubes were filled with dust by mechanically "scooping" dust with the collection tube, or "brushing" dust with glove-covered fingers. To prevent cross contamination of dust samples, gloves were changed between each sample. In Appendix A.1, we showed that the dust that collected on fan covers, or "louvers", showed less spatial variation in virus concentration than dust collected from ledge-like surfaces. We therefore collected between two and six dust samples from fan louvers in each house each visit. In total, we visited 104 unique commercial combinations of farm and operation (three farms changed operations during surveillance). These combinations were comprised

of 29 broiler-breeder facilities, 52 broiler (meat-chicken) facilities, and 23 egg-laying facilities (no egg-breeder facilities were included). This included a total of 13 operations, 101 unique farms, 226 houses, 469 unique flocks, 4848 total dust samples, and 9498 total qPCR runs. To generate the subset of data that we used to quantify Marek's disease prevalence we excluded the longitudinal data, and the 103 samples for which bird age was unavailable. This left us with 13 operations, 90 farms, 192 houses, 297 flocks, 1018 samples, and 1915 qPCR runs (hereafter referred to as the "surveillance data").

On two farms, in addition to dust collection we collected data on airborne virus concentration and host infection status. Airborne virus concentration was assessed by securing a total of six 1.5 ml Eppendorf tubes to the arms, hips, and legs of the authors during routine dust collection. Tubes were oriented horizontally with tops pointing to the front of the collector's body. The tubes were opened upon entering the house, and closed upon leaving. This period lasted approximately fifteen to twenty minutes. These data are hereafter referred to as the "air tube data." Feathers were also collected from individual birds following a procedure approved by the Institutional Animal Care and Use Committee of The Pennsylvania State University (IACUC Protocol#: 46599). Two feathers were pulled from the breast of each target bird. The pulpy distal end of each feather was clipped and placed into individual Eppenorf tubes. Scissors used to clip feathers were sterilized between birds using 70% isopropyl alcohol wipes. Ten total birds were sampled from each house during each visit (hereafter referred to as the "feather tip data"). Target birds for feather collection were chosen such that they were spatially distributed throughout the house. Individual birds

were selected at the discretion of the collector with a goal of random selection. To account for the possibility of airborne virus contamination, we also had two control tubes, one that was left open during the collection of a single feather from a single bird, and one that was left open during the collection of feathers from all ten birds.

qPCR

All samples were brought back to the lab and stored at 4 °C prior to processing. For dust samples, at the earliest convenience, typically within one week, we weighed out duplicate 2 mg samples of dust on a Mettler Toledo balance (Cat # 97035-620). Virus DNA was extracted from dust samples using the Qiagen DNeasy Blood & Tissue kit (Cat # 69506). The following changes were made to the standard "Animal Tissue" protocol. In step 1, we used 2 ± 0.2 mg of chicken dust, and we increased the amount of Buffer ATL to 380 μl. In step 2, we incubated our samples overnight at 56 °C with constant shaking at 1,100 RPM on a VWR symphony Incubating Microplate Shaker (Cat # 12620-930). In step 3, we doubled the volume of ethanol and Buffer AL to 400 μl each. Between steps 3 and 4, we centrifuged samples at 17,000 RCF to pellet undigested dust to avoid clogging the spin column in step 4. Lastly, in step 4, we bound the DNA to the spin column twice using half of our supernatant each time. This last step was necessary because of the increased sample volume that was generated from our modifications to steps 1 and 3. Air tube and feather tip samples were processed identically standard "Animal Tissue" protocol, with the exception of the added centrifugation between steps 3 and 4. Unlike the dust samples, the entire template was used

in initial DNA extraction, and so these samples were processed in singlicate.

Three types of live vaccine virus strains are used on Pennsylvania farms to control Marek's disease. These strains are related but not identical to wild-type virus. Once vaccinated, a bird can shed these vaccine strains (Baigent et al., 2005; Islam and Walkden-Brown, 2007), and so we used the primer-probe combination of Baigent et al. (2016) that is capable of quantifying wild-type virus in the presence of each of the vaccine strains. This assay targets a portion of the *pp38* gene, which is present only in Marek's disease virus serotype 1. Two of these vaccine strains (HVT and SB-1) are different serotypes; their genomes do not contain a *pp38* gene, and therefore they do not amplify in the qPCR assay. The third vaccine strain (Rispens) is a serotype 1 virus, as are wild-type strains, and all have a *pp38* gene. The qPCR was designed to target a region of the *pp38* gene that contains a single nucleotide polymorphism that differs between Rispens virus and all sequenced wild-type strains. The Rispens virus did amplify minimally in the qPCR, resulting in minor fluorescence. We were nonetheless able to accurately determine the presence and quantity of wild-type virus when it was present at a ratio of as low as 1 copy per 10 Rispens virus copies (Appendix A.2). Below this ratio, the wild-type virus was often still detected, but the estimate of its quantity became biased. In appendix A.3 we show that this interference was unlikely to affect our conclusions.

All qPCR assays were run on a 7500 Fast Real-Time PCR System machine (Cat #4351107). The pp38-FP and pp38-RP primers and the pp38-Vir(1) probe from Baigent et al. (2016) were used at concentrations of 300 nM, 300 nM, and 100 nM respectively. We used the

PerfeCTa qPCR FastMix, UNG, Low ROX reaction mix (Cat #95078), and we followed the standard protocol with the exception of adding bovine serum albumin (BSA) from Sigma Aldrich (Cat #B4287) to a final concentration of 1 μg/μl. The final reaction volume contained 21 μl of master mix and 4 μl of DNA template. Our cycling conditions included an initial denaturation at 95 °C for 20 seconds, followed by 40 two-step cycles of denaturation at 95 °C for 3 seconds and annealing/extension at 60 °C for 30 seconds. All assays were carried out using 'ABI Sequence Detection Software' version 1.4. We used the prMd5pp38-1 plasmid provided by John Dunn at the Avian Disease and Oncology Lab as a standard. For all qPCR runs, a quantification curve was generated using five serial tenfold dilutions of the standard in duplicate, and C_T numbers were translated into DNA copy numbers using this quantification curve. As a positive control, each run contained a well of Marek's disease virus DNA extracted from an *in vivo* infection. As negative controls, each run contained a well with distilled water, and a well with a high concentration of Rispens virus DNA. As previously mentioned, Rispens virus DNA can result in minor fluorescence, and so this Rispens-virus-positive well was used to set the critical threshold of our qPCR. In practice, this meant that the critical threshold was set to just above the fluorescence value of this well at cycle 40. The standard deviation between technical replicates of a dust sample was estimated to be 0.319 ± 0.006 $\log_{10}$ units of virus per mg dust, which is approximately equivalent to a 2.09 ± 0.03 fold standard deviation in virus concentration (Appendix A.4). This error estimate included multiple sources of variation, including heterogeneity within a dust sample, in the DNA extraction efficiency, and in the qPCR amplification and quantification.

The standard deviation between biological replicates was 0.556 ± 0.022 $\log_{10}$ units, which is approximately equivalent to a 3.60 ± 0.18 fold standard deviation (Appendix A.4). Our tests to confirm the accuracy of our qPCR approach are summarized in Appendix A.5.

Statistical analysis

Analysis of the surveillance data

To study the variation in the presence and absence of Marek's disease virus across chicken dust samples it was useful to treat the results of our qPCR tests on dust samples as binomial data. Feather tip data were treated similarly (Appendix A.6). Our qPCR data was interpreted as binomial data with those qPCR runs that had at some point crossed the qPCR florescence threshold treated as positive outcomes, and those that had not treated as negative outcomes. This method was similar in effect to running a traditional PCR and checking for the amplification of a target using gel electrophoresis. In practice, our limit of detection was approximately 100 template DNA copies per mg of dust (Appendix A.5), which is close to the concentration of virus that would be expected if about 20 to 50 chickens were infected per flock of 30,000 chickens and virus was randomly mixed throughout the dust (Appendix A.7).

To account for the lack of balance in our sampling scheme, we analyzed our data using generalized linear mixed effects models (Gelman and Hill, 2009). For this analysis, we excluded the data that were collected from five farms at regular intervals (i.e. the longitudinal data), because we were concerned that these farms would have outsized importance on factors

such as seasonal patterns and flock to flock variation. We additionally excluded samples where bird age was unavailable (103 samples). Depth of coverage on the remaining farms ranged from 1 to 90 dust samples, with a median of 6 (fig. 2). Our models had random effects for "Operation", "Farm", "House", "Flock", and "Sample" to account for these levels of clustering in the data. We were also interested in whether virus dynamics differed between broiler, broiler breeder, and layer farms, and so we included a fixed effect of "Production type". We allowed for seasonal variation in virus dynamics by including fixed effects on the sine and cosine of the collection date, transformed such that one year corresponds to a period of 2π. This method fit a sigmoidal curve with a flexible amplitude and offset that was constrained to have a cycle period of one year. Lastly, we included an effect of cohort age by using a spline function on bird age. This approach allowed us to fit a non-linear effect of age while nonetheless taking advantage of the computational benefits of generalized linear models (Härdle, 1990; Huang and Stone, 2003). This spline contained knots at cohort ages of 21, 42, 100, and 315 days. These ages were selected for their biological and empirical relevance: virus concentration in shed dust first peaks around 21 days post infection (Islam and Walkden-Brown, 2007; Read et al., 2015), commercial broiler cohorts in our data were typically processed at around 42 days of age, the longer-lived broilers in our data were typically processed at around 100 days, and the halfway point for our oldest flock sampled was 315 days. In practice, the spline was generated using the "bs" function in the R package "splines". We generated five candidate models consisting of the full model that contains all of the factors listed above, the three models that lacked exactly one of these fixed effects,

and one model that lacked the random effect of "Sample". We did not explore the effect of removing the higher level random effects. Instead, we explored the importance of each random effect by examining the magnitude of its estimated effect.

We analyzed the generalized linear mixed effects models using Bayesian methods. There are several advantages to this approach, including readily interpretable confidence intervals on all model parameters (Hadfield, 2010), the ability to incorporate many random effect levels (Bolker et al., 2009), and the ability to perform model parameter estimation in the presence of quasi-complete separation (Gelman et al., 2008). This analysis was done in the R statistical computing language (R Development Core Team, 2014), using the function 'MCMCglmm' (Hadfield, 2010) with family set to "categorical", and "slice" sampling. A caveat of this approach was that every parameter in the model including both fixed and random effects required the specification of a prior distribution.

Following Hadfield (2010), prior distributions for fixed effects were univariate normal distributions, and for random effects were inverse Wishart distributions. For each fixed effect we used mean 0, and standard deviation 7. For each random effect, we used scale 5 and degrees of freedom 3. These prior distributions were set for practical, rather than biological reasons, because less informative priors resulted in models that failed to converge in a reasonable number of MCMC iterations. However, we explored the effects of using different parameters in the prior distributions and found that the results were qualitatively similar for other priors that were able to achieve convergence (Appendix A.8). Each model was run for 4.1×10^6 iterations with a burn in of 1×10^5 steps, and a thinning interval of

2×10^3. This resulted in 2000 parameter samples for each model run. This process was repeated to generate a total of three chains for each model.

Posterior convergence was tested in three steps, following the method used by Kennedy et al. (2014). First, we visually examined trace plots for each model parameter to explore whether the chains appeared to be mixing. Second, we tested for stationarity of the posterior distribution using the stationarity test proposed by Heidelberger and Welch (1983). Third, we tested whether the between chain variances for parameter estimates were similar to the within chain variances by using the test proposed by Gelman and Rubin (1992). Upon seeing no obvious problems with convergence, we concluded that the chains had likely converged.

The models were compared using the Deviance Information Criterion (DIC). DIC is a tool, in many ways similar to the Akaike Information Criterion (AIC), that is useful for comparing the relative goodness of fit of various models (Spiegelhalter et al., 2002). To foster model comparison, we presented ΔDIC scores, which are the differences in DIC between the best model and each alternative model. Like AIC, there is no precise threshold for significance of ΔDIC scores, but (Bolker, 2008) argued that it is on the same scale as AIC. We therefore followed the suggested rule of thumb for AIC (Burnham and Anderson, 2002) that ΔDIC scores less than 2 suggest substantial support for a model, scores between 3 and 7 indicate considerably less support, and scores greater than 10 suggest that a model is very unlikely. If the difference in structure between the model with little support and the best fitting model, for example, were to include an effect of factor "x" that the poorer fitting focal model lacked, the interpretation of a large ΔDIC would be that "x" is important to explaining the data.

We also explored the importance of model factors using fraction of variance explained (R^2) where the calculation of R^2 was modified for use with generalized linear mixed models (Nakagawa and Schielzeth, 2013). We presented marginal R^2 and conditional R^2 values that describe the fraction of variance on the latent scale of the data that can be attributable to fixed and fixed plus random effects, respectively. We then extended this method to explore the contribution to R^2 that can be attributed to each single factor in the model. Credible intervals for all estimates came from the posterior distributions of the fitted models.

We explored the statistical significance of differences between production types by performing pairwise comparisons on the estimated effect sizes of production type. In practice, this was done by asking what fraction of samples from the posterior estimated a larger effect size for production type level 1 than for production type level 2 or the reverse. This value was multiplied by two to account for it being a two-tailed hypothesis test. These tests were performed for all three pairwise comparisons between broiler-breeders, broilers, and layers.

Previous work has shown that Marek's disease associated condemnation rates historically varied across broad geographic area such as between states (Kennedy et al., 2015). We explored whether there was evidence of clustering in virus detection across the finer spatial scales found in our surveillance data. We did this by calculating distances and correlations in effect sizes between each pairwise farm location. We then used the "lm" function in "R" to generate two models. The first was an intercept only model that functioned as a null model. The second was an intercept plus distance effect model, where distance was transformed by adding one and then taking the $\log_{10}$. The importance of distance was assessed by performing

a likelihood ratio test.

Analysis of the longitudinal data

To study the variation in Marek's disease virus dynamics within a focal chicken house over time, we used the quantitative values returned by qPCR analysis, rather than the presence-absence used for the surveillance data, because the quantitative data are more sensitive to changes in virus concentration. We assumed lognormal error in these quantities, because variation in qPCR data tends to occur on a log scale. In our analyses, we therefore transformed the virus-copy-number-per-mg-of-dust data by adding one and $\log_{10}$ transforming that value. We explored the suitability of this lognormal assumption for our data in Appendix A.4. Additionally, many of our samples yielded virus concentrations below our limit of detection. We thus performed our analyses while treating these data in two different ways, first as a value of zero virus copies representing virus absence, and second as a value of our limit of detection representing virus presence at an undetectable level. Our limit of detection was generally better than 100 virus copies per mg of dust (Appendix A.5), and so in practice, we used this quantity as our value in the latter case. For this analysis, all samples that had detectable virus below this quantity were treated identically to negative samples.

We sampled from five broiler farms at approximately weekly intervals. One of our main goals was to quantify how virus concentrations changed over the duration of a cohort, and across different cohorts, and so we began by simply plotting the data. A similar plot was generated for the air tube data. We then explored a cohort age effect by fitting smoothing

splines to the raw data from each farm where the data are sorted by cohort age. Each spline was fit in "R" using the function "smooth.spline". We used the option "nknots=4" for this function because this was the smallest number of knots that did not return an error. Very similar conclusions were obtained using any number of knots from four to nine. We explored seasonality in these data by subtracting cohort age effects from the raw data and plotting the residual virus concentration. We assessed the degree of correlation between houses within farms using the "cor" function in "R". We also examined autocorrelations within houses using the "acf" function for data within each house.

Results

Surveillance data

Samples used in our model comparisons were collected from multiple operations that reared broiler-breeder, broiler, and egg laying chickens. Samples were collected throughout the year from flocks of varying sizes, ages, and sexes in Pennsylvania (fig. 2). Among all samples collected (combining surveillance and longitudinal data), wild-type Marek's disease virus was detected at least once on 36 of the 104 farms (fig. 3). Virus was detected in 1 of 3 broiler-breeder operations, 4 of 5 broiler operations, and 3 of 5 egg layer operations. The fraction of samples in which virus was detectable varied substantially among farms with detectable virus, and less so between houses within a farm (fig. 3). Summary plots of virus prevalence as a function of production type, bird age, date of sample collection, and bird sex

Table 1: DIC table for models considered. "Mean deviance" is the average deviance of the posterior. Note that the "Full model" is in bold to highlight that it was the best model according to DIC.

Model name	Mean Deviance	Number of parameters	DIC	ΔDIC
Full model	**336.9**	**17**	**494.5**	**0**
No production type	339.7	15	497.1	2.5
No bird age	345.8	15	503.7	9.1
No collection date	341.2	10	499.1	4.6
No sample	450.1	16	575.3	80.7

can be found in Appendix A.9. Note, however, that a visual inspection of patterns in these data could be misleading because of potential confounding with other covarying factors. We therefore used statistical models to further explore the effects of these factors on the data.

Our analysis of the virus prevalence data using DIC scores revealed that our best model was our most complicated model, which included effects of production type, bird age, collection date, and variation between dust samples (Table 1). Comparing our most complicated model to the other models through ΔDIC using the rule of thumb suggested by Burnham and Anderson (2002), we found moderate support for an effect of production type, reasonable support for an effect of collection date, relatively strong support for an effect of bird age, and overwhelming support for variation between dust samples. Taken together these results suggest that, to varying degrees, each of these factors had detectable effects on the prevalence of Marek's disease virus on farms.

We further explored the importance of these effects by examining the fraction of variance in our data explained by each model factor for our best model (fig. 4). This showed that the fraction of variance attributable to production type was highly uncertain, with the 95% credible interval ranging from 1.5% to 38.4%. This large degree of uncertainty likely resulted

from partial confounding between production-type effects and operation effects, and it may explain why support for an effect of production type was only moderate. Indeed, exploring the variance explained by these two factors combined, we found that they accounted for between 26.7% and 74.4% of the variance. This confounding likely occurred because there were relatively few operations in our study area.

Fig. 5 shows the effect sizes of production type, bird age, and collection date observed in the full model. Virus prevalence was higher on broiler farms than on layer farms ($p = 0.023$), but there was no statistically significant difference between breeder and broiler ($p = 0.268$), or breeder and layer farms ($p = 0.150$). During the first few weeks of a bird cohort the probability of detecting virus decreased, and then as birds continued to age this probability began to increase. Note that after cohorts reached about 100 days, the median effect was close to neutral and the confidence intervals on the effect size was fairly large (fig. 5 middle panel). This uncertainty was likely because we have relatively few data from older cohorts. We additionally saw a seasonal pattern in Marek's disease virus prevalence with a fairly wide credible interval. Our probability of detecting virus was lowest in the winter months and highest in the summer months (fig. 5 bottom panel).

Additionally, we found that the estimated effect that "Farm" had on virus detection tended to be positively correlated for nearby farms, and this correlation decayed with distance between farms ($\chi^2 = 28.5, d.f. = 1, p < 0.001$). However, the effect size was relatively small, with a maximum correlation of 0.029 ± 0.004 for nearby farms and a decay of 0.014 ± 0.003 for every 10-fold increase in distance. Moreover, this correlation with distance might have

been a statistical artifact resulting from geographic clustering of farms belonging to the same operation: no significant correlations by distance were detected between farms within single operations. We therefore would suggest caution when interpreting the effect of nearby farms on Marek's disease virus dynamics.

Longitudinal data

The longitudinal data from five broiler farms revealed several patterns. These data visually confirmed the conclusion from the surveillance data that virus densities varied substantially between farms, and between flocks, but varied less between houses located on the same farm (figs. 6 and 7). This similarity between houses was also seen as a correlation of virus quantities between houses within farms (average correlations between houses within each of the five farms were 0.215, 0.320, 0.738, 0.763, and 0.918). The data also confirmed the observation that virus densities tended to decrease during the early phase of a cohort, and tended to increase during the later phase of a cohort (Appendix A.11). This created "U" shaped curves in virus concentration within cohorts (figs. 6 and 7). The initial decrease might be explained either by a dilution of virus in dust early in cohorts, when birds were shedding virus-free dust into holdover dust from the previous cohort, or by degradation of virus DNA early in flocks. The subsequent increase could then be explained by the hyper-concentration of virus in dust later in cohorts, when birds were shedding dust that was highly contaminated with virus. This pattern is not explained by differences in sample humidity or qPCR inhibition (Appendix A.13). Consistent with the surveillance data in which seasonal

effects were small, we were unable to find any consistent seasonal effect on Marek's disease virus dynamics in these data.

Three additional patterns were also detectable in the longitudinal data. First, virus concentrations often dropped to below detectable levels, and returned to detectable levels at a later time point (figs. 6 and 7). Second, there was an autocorrelation in virus concentration within single houses over time. This effect was seen as an autocorrelation between samples collected seven days apart ($\text{Acf}(7)_{avg} = 0.579$, $\text{Acf}(7)_{min} = 0.226$, $\text{Acf}(7)_{max} = 0.967$), although this correlation was also observed over longer periods (Appendix A.12). Third, during farm down time, when birds were absent from houses, there were many cases where virus concentration did not change (figs. 6 and 7). Patterns consistent with the first two of these observations were also seen in the air tube and feather tip data (fig. 8).

Discussion

The historical evolution of Marek's disease virus towards reduced vaccine efficacy and increased virulence was both economically important and academically interesting. Determining whether this evolutionary trajectory will continue depends critically on the factors driving evolution, and thus depends on the distribution of Marek's disease virus in the industry. Here, we surveyed commercial chicken farms in Pennsylvania to generate the first industry-wide dataset exploring the modern distribution of this virus. We found that the virus was at detectably high densities in only a third of farms, that bird age, collection date, and production type affected the probability that we detected virus, and that the

vast majority of variation in the data was not attributable to those factors, but instead was attributable to differences between the companies, farms, houses, flocks and samples. Longitudinal sampling on five focal broiler farms revealed substantial autocorrelation in virus density within houses over time, and demonstrated that virus concentrations often dropped to undetectable levels on farms but reappeared in future flocks. Taken together, these results imply that selective pressures on the virus may be highly heterogeneous across the industry and over time.

Previous studies on the evolution of Marek's disease virus in the poultry industry have focused entirely on endemic virus persistence in broiler chicken houses (Atkins et al., 2013; Read et al., 2015; Rozins and Day, prep). Our data, however, reveal that the virus can be found in each of the sectors of chicken farming, including broiler, layer, and breeder chicken facilities. The vastly different host genetics, rearing duration, host densities, vaccination strategies, and biosecurity measures of these different sectors imply the potential for vastly different evolutionary outcomes depending on where evolutionary forces are most strongly acting.

Conventional wisdom is that Marek's disease virus is sufficiently pervasive that it should be considered ubiquitous (Dunn, 2013; Office International des- Epizooties, 2010; Purchase, 1976). This idea came from observations that the virus is highly stable in the environment (Jurajda and Klimes, 1970), that problems with Marek's disease can occur quickly and without warning when there are issues with vaccine administration, and that vaccination does not preclude infection with and transmission of the virus (Islam and Walkden-Brown,

2007; Purchase and Okazaki, 1971; Ralapanawe et al., 2016). It was further supported by the historical ubiquity of antibody detection in production flocks (Biggs et al., 1972; Chubb and Churchill, 1968; Ianconescu and Samberg, 1971; Witter et al., 1971). However, we found virus on only one third of farms. It may in fact be present on the other two thirds of farms at densities below our detection threshold or at times when samples were not collected, or it may instead be that modern farm practices have led to changes in the distribution of the virus such that it is no longer ubiquitous on chicken farms. Many features of poultry farming have changed in recent decades that could have altered Marek's disease virus ecology, including vaccination strategies and cohort durations (Kennedy et al., 2015). Recent studies in Australia (Ralapanawe et al., 2016; Walkden-Brown et al., 2013b), and Ethiopia (Bettridge et al., 2014) have suggested that Marek's disease virus may no longer be ubiquitous in those locations. Our study suggests that this trend may be more general, extending to commercial poultry farming the United States. Introducing non-vaccinated sentinel birds could be a way to directly challenge this finding. If confirmed, this suggests that selective forces acting during sporadic outbreaks or acting in flocks with low prevalence of infection may play an important role in the evolution of the virus.

The importance of random effects (i.e. operation, farm, house, flock, and sample) in explaining the data suggests that substantial variation in virus dynamics can be explained by factors that co-vary with these random effects. For example, bird breeds, vaccination details, and average cohort durations may explain some of the variation between operations. Ventilation rates, clean out efficiency, and other hygiene factors may explain some of the

variation between farms. Structural differences and wind patterns may explain some of the variation between houses. Microbial communities, developmental plasticity and stochastic effects of virus transmission may explain some of the variation between flocks. Lastly, spatial clustering of virus may explain some of the variation between samples. Our model analysis showed that between about one quarter and three quarters of the variation in Marek's disease virus detection probability was attributable to the combined effect of production type and operation. This suggests that factors outside the control of individual farm operators may play a large role in Marek's disease virus dynamics. It also suggests that any intervention strategy intended to control virus evolution is likely to be ineffective unless implemented through top-down measures.

The observation that seasonality explained only a small portion of variance in Marek's disease virus prevalence contrasts with observations that Marek's disease associated condemnation in broiler chickens has had clear seasonal patterns in the past (Walkden-Brown et al., 2013b; Witter, 1996). However, seasonal patterns in condemnation have become less pronounced in recent years (Kennedy et al., 2015). The data we report here are consistent with the theory that this decrease in seasonality is attributable to an overall decline in prevalence, resulting in stochastic outbreaks playing a relatively larger role in dynamics than seasonal forcing (Kennedy et al., 2015).

An interesting question is whether virus populations are persisting within individual houses and farms, or instead going through repeated extinction and recolonization events. Our observation in the longitudinal data that there was a strong autocorrelation in virus

concentration within houses over time (Appendix A.12) contrasted with the observation that virus densities were often undetectably low in one cohort but emerged as detectable in the next (figs. 6 and 7). This reemergence might be due either to recolonization events or to the epidemiological amplification of virus persisting within the house at undetectable concentrations. Genetic techniques could be used to determine the relative contributions of these two factors.

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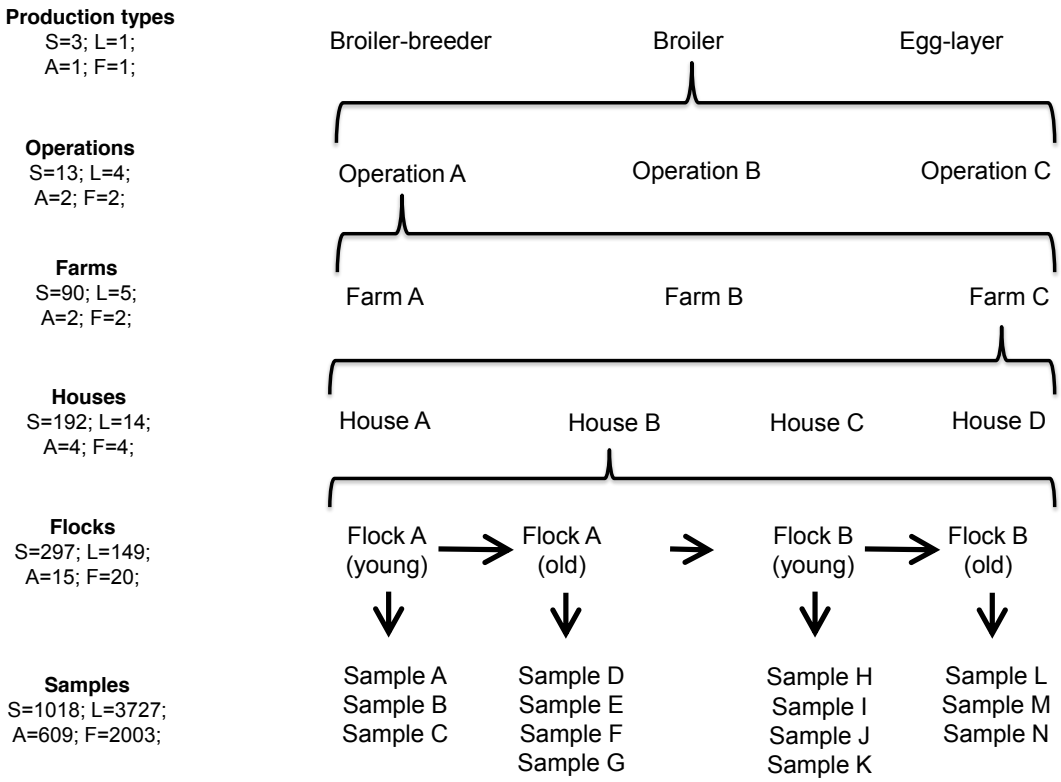

Figure 1: Depiction of the structure of poultry farming in our study. Multiple samples were collected from a single flock, multiple flocks were reared in a single house over time, multiple houses were located on a single farm, multiple farms were associated with a single operation (company), and multiple operations were rearing chickens that typically belonged to a single production type. This created a nested hierarchical structure in the data. The total number of levels sampled for each hierarchy are shown as "S" for the surveillance data, "L" for the longitudinal data, "A" for the air tube data, and "F" for the feather tip data.

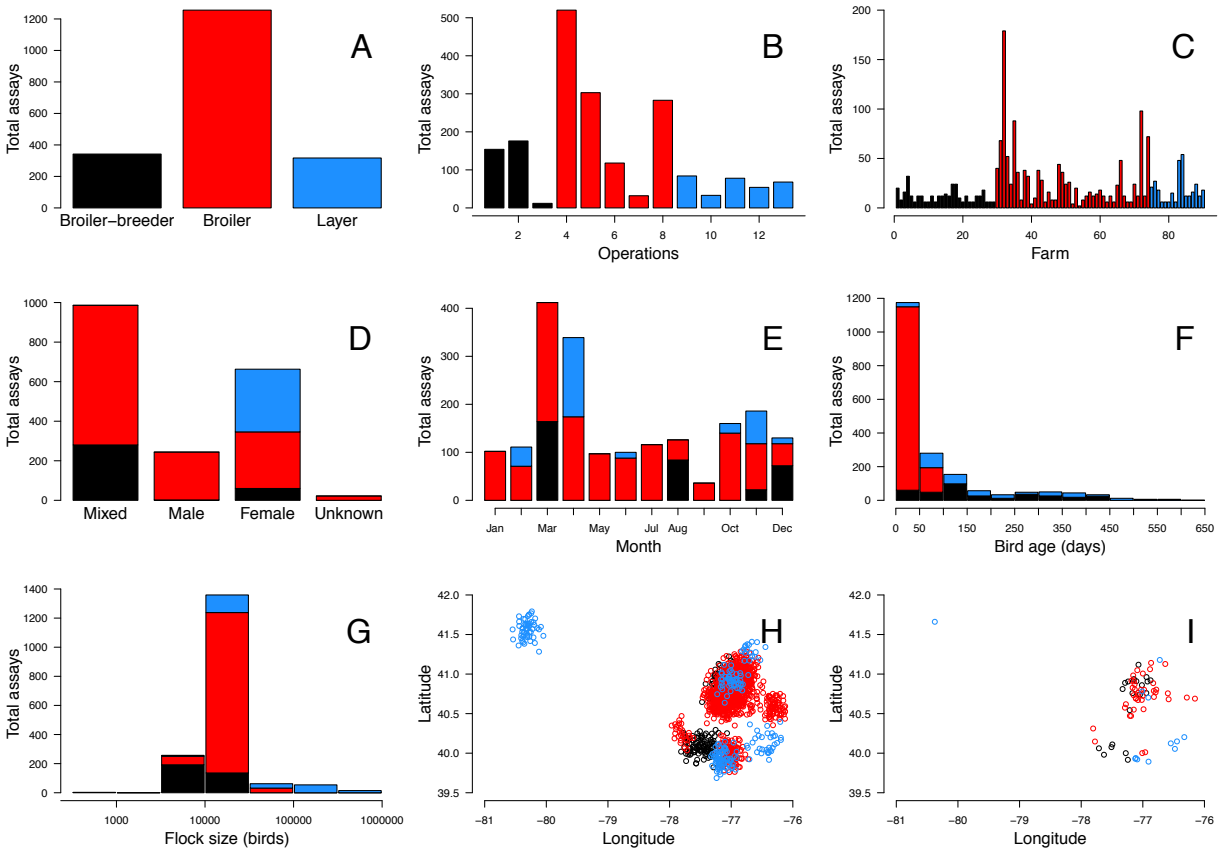

Figure 2: Summary plots of the surveillance data depicting the number of assays that were performed as a function of production type (A), operation (B), farm (C), sex (D), month of the year (E), bird age (F), and flock size (G). Also depicted are the approximate locations of origin of each assay (H) and each farm (I). Note that to maintain farm location anonymity, normal random variables with mean 0 and standard deviation 0.1 were added to the points when plotting latitude and longitudes in H and I. In all plots, black color depicts breeder facilities, red color depicts broiler facilities, and blue color depicts layer facilities.

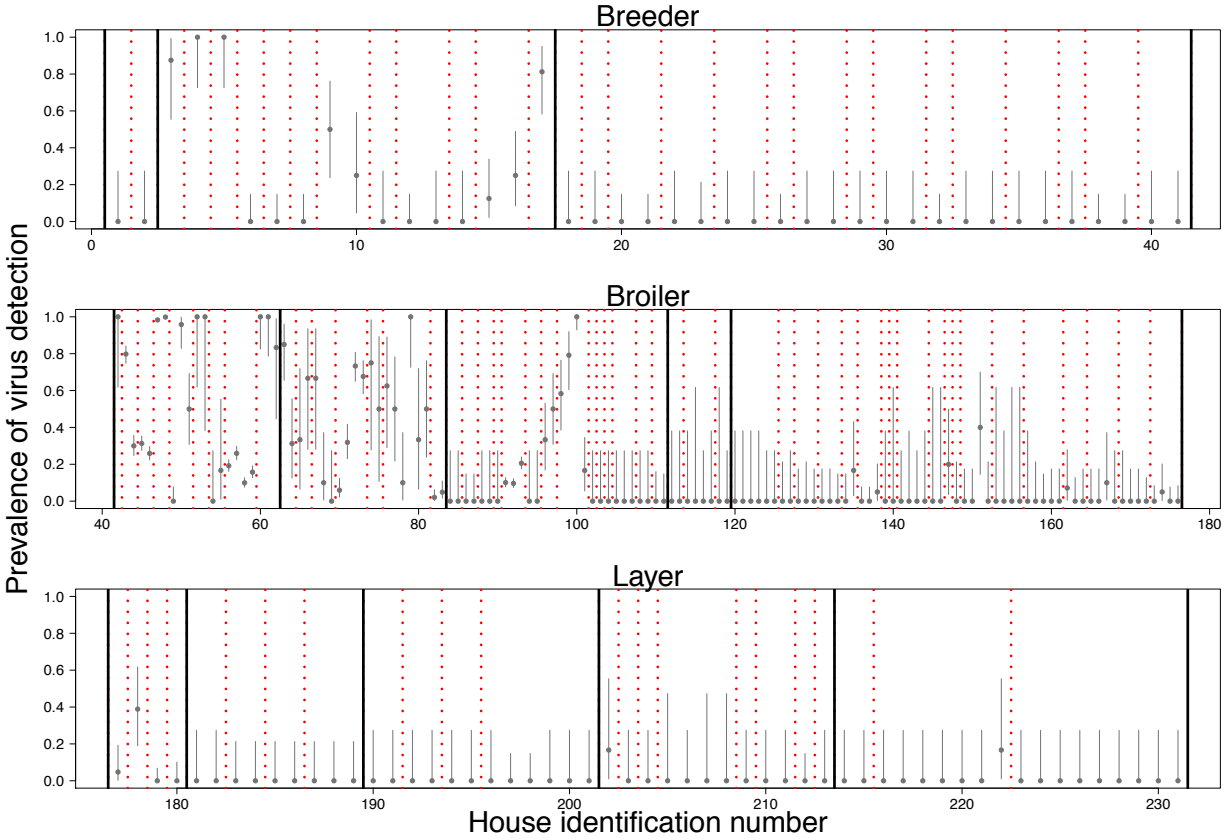

Figure 3: Fraction of tests with detectable virus separated by house. Different rows depict different production types (top–breeders, middle–broilers, bottom–layers). Solid black lines separate different operations. Dashed red lines separate different farms. Grey bars show 95% confidence intervals on the mean (Appendix A.10). Confidence intervals vary between houses because of variable sample sizes. Note that prevalence estimates are from the raw data, not corrected to account for potential confounding effects from bird age, collection date, flock, or sample.

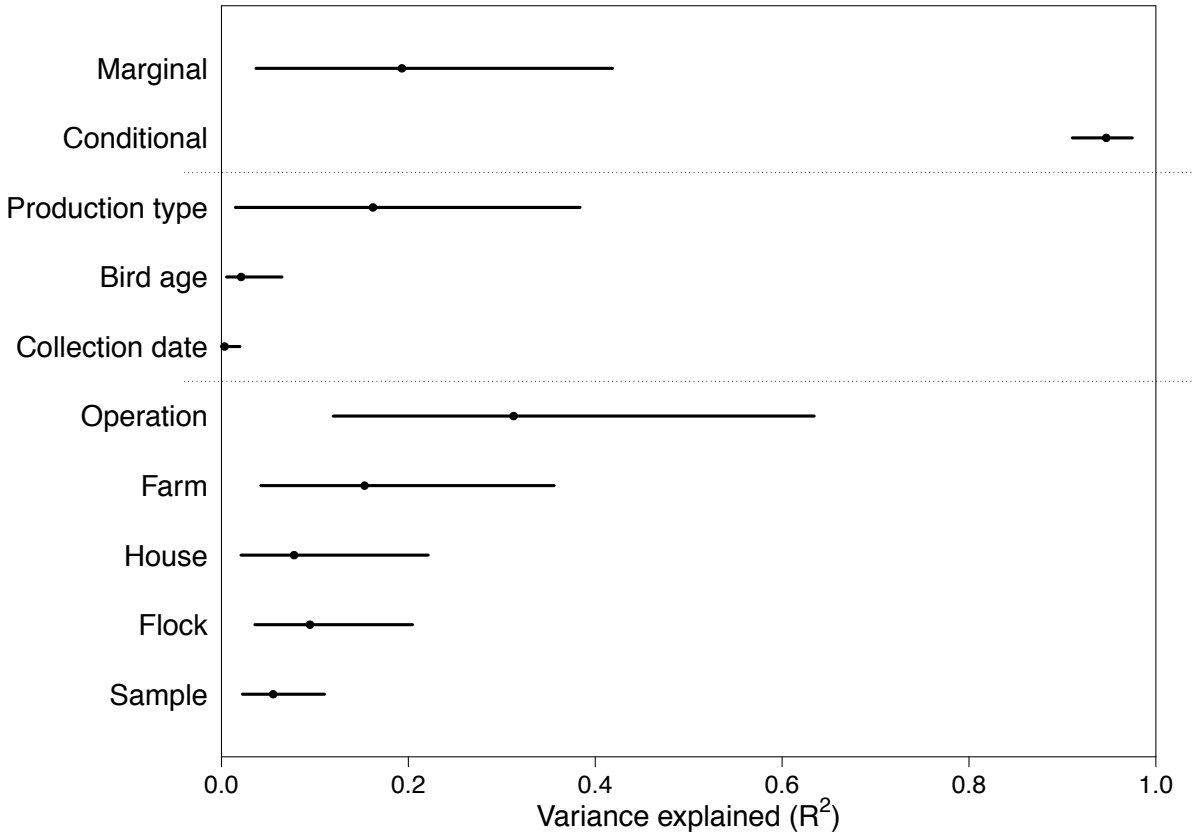

Figure 4: Fraction of variance on the latent scale attributable to each model factor. Points are median values and lines are 95% credible intervals. Marginal and conditional R^2 values represent the variance explainable by all fixed effects, and all fixed plus random effects respectively. Note that only the values for the best model (Table 1) are shown.

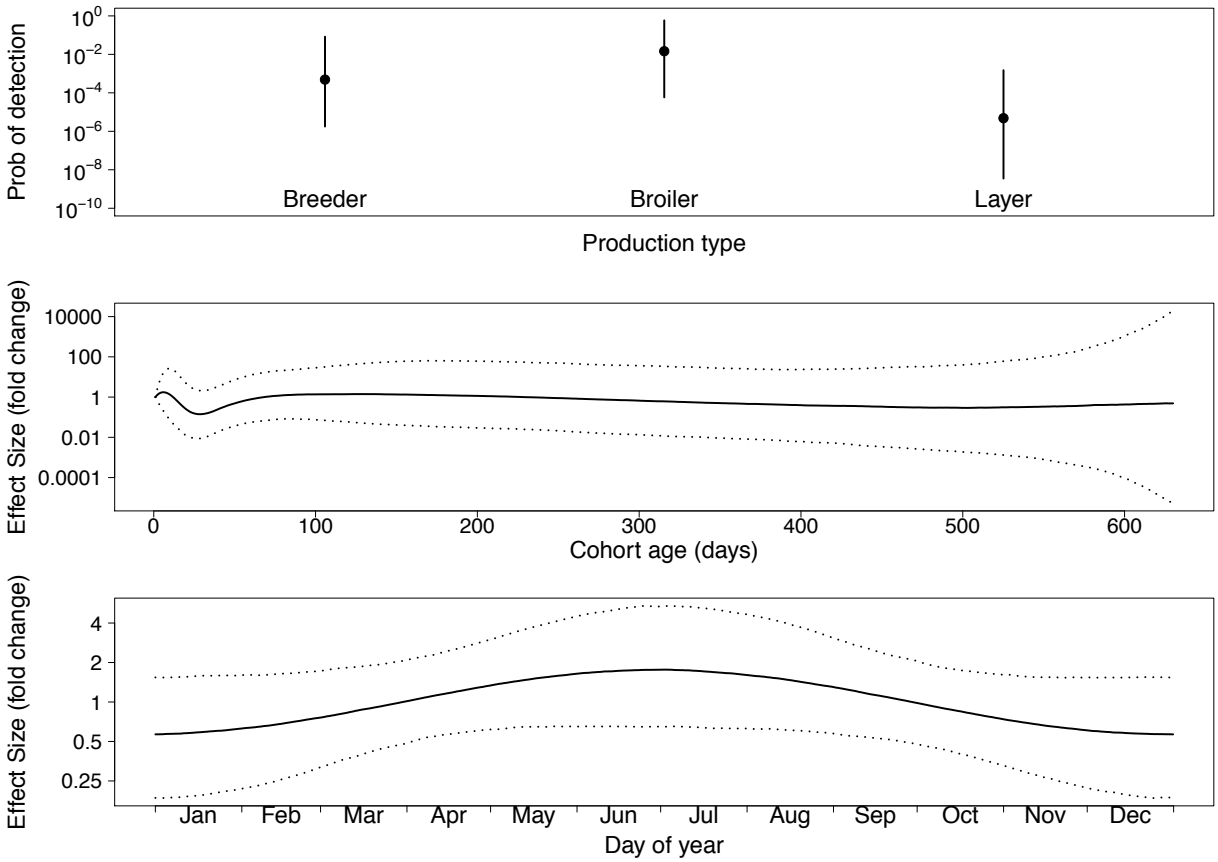

Figure 5: Effect sizes for fixed effects. The top panel shows the median and 95% credible interval for the three production types. The middle panel shows the median and 95% credible envelope for the effect of bird age on the probability of detecting virus in a dust sample. The bottom panel shows the median and 95% credible envelope for the effect of collection date on the probability of detecting virus.

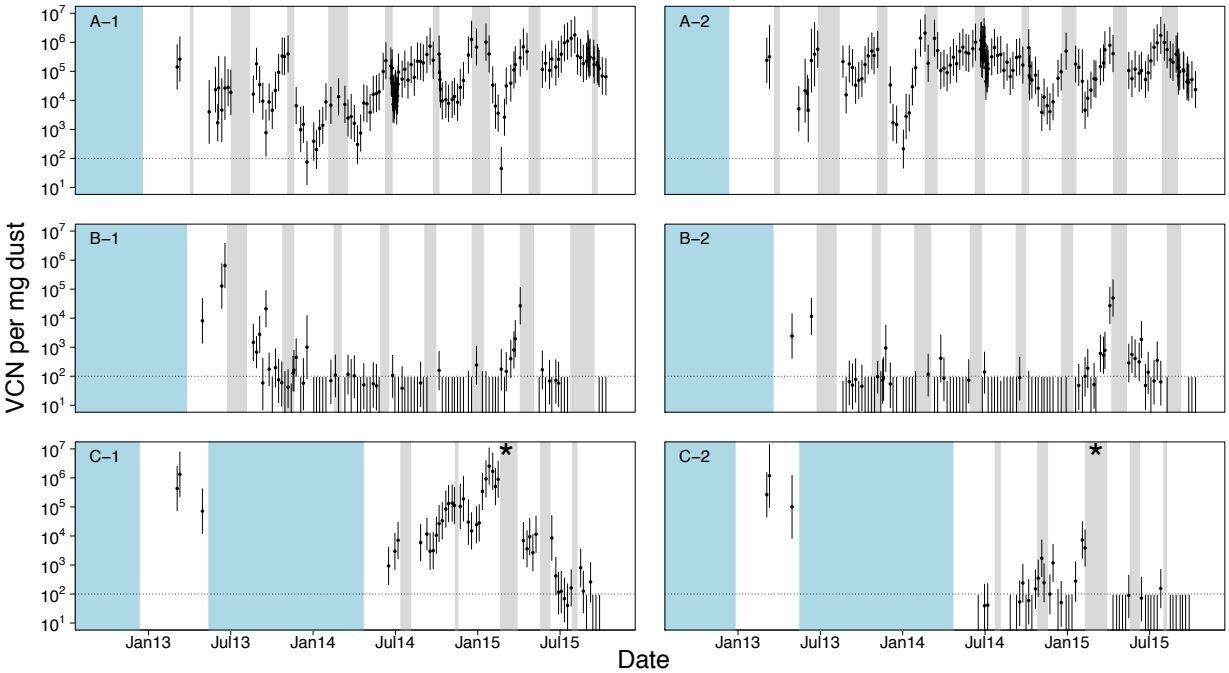

Figure 6: Longitudinal surveillance data for three broiler farms in Pennsylvania. Each panel is labelled "X-Y", where "X" gives a unique farm identification, and "Y" gives a house number on that farm such that each two character label is unique. Note that each of these farms had two houses. All of these farms began associated with the same operation, but farm "C" changed operations in the middle of our surveillance. The timing of this change is denoted by an asterisk in the plot. All farms followed an "all-in, all-out" policy meaning that houses had discrete periods of rearing and down time. To represent the presence or absence of birds, white intervals cover periods when birds were present, grey intervals cover periods when birds were absent, and blue intervals cover unknown periods. Each point represents the log-mean virus concentration for that set of dust samples. Error bars are 95% confidence intervals calculated as explained in Appendix A.10. The dotted horizontal line shows the approximate qPCR limit of detection for a single test.

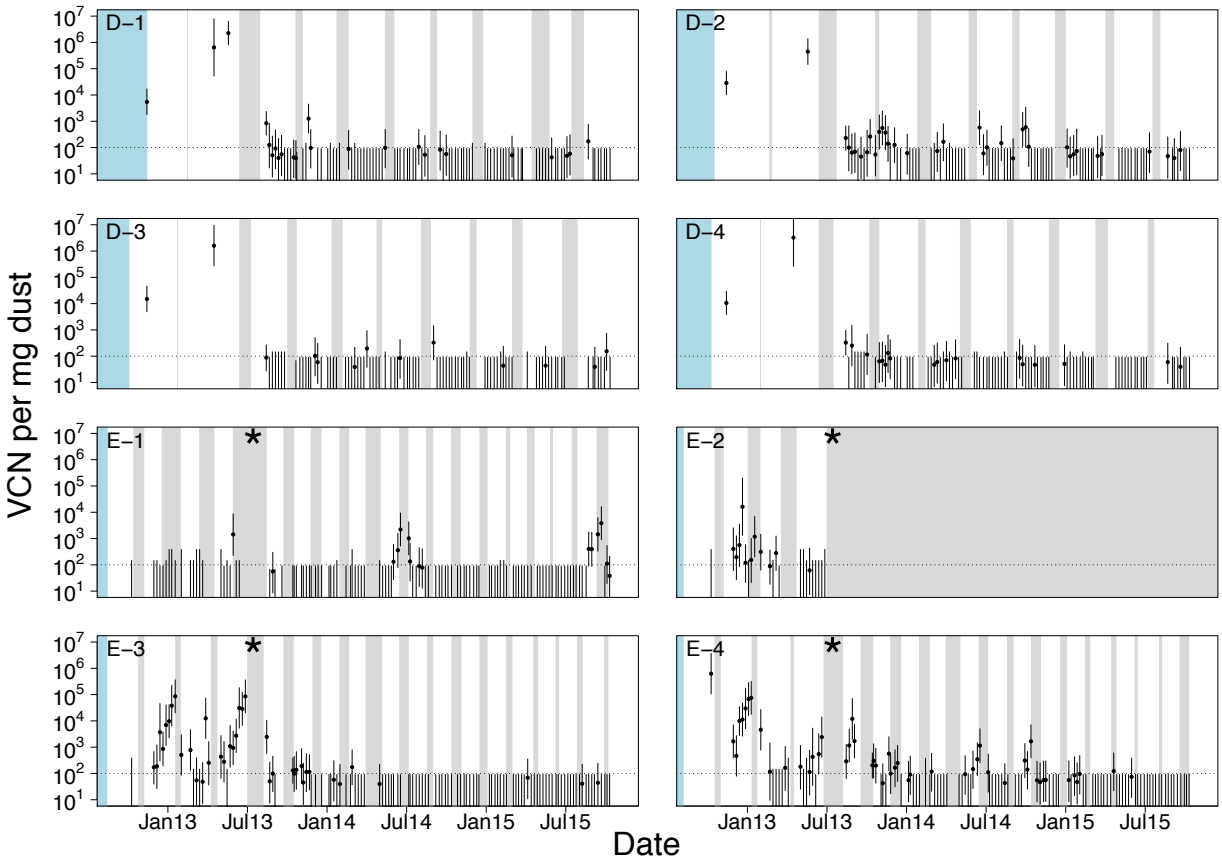

Figure 7: Longitudinal surveillance data for two additional broiler farms in Pennsylvania. Symbols, colors and layout as in fig. 6. Each of these farms had four houses. Farm "D" was association with the same operation as the farms in fig. 6, but farm "E" was not. Note also that farm "E" changed operations during our surveillance period, the timing of which is marked with an asterisk.

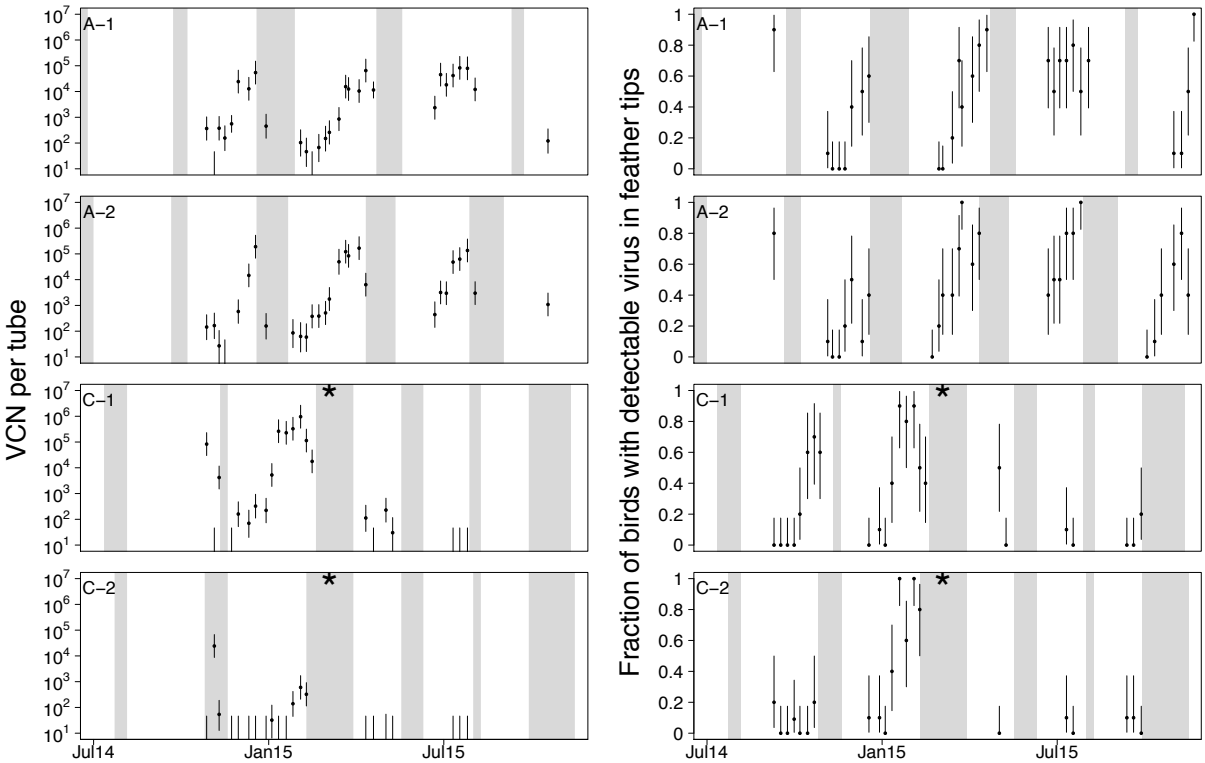

Figure 8: Air tube data (left column) and feather tip data (right column) for two broiler farms in Pennsylvania. Symbols, colors and layout as in fig. 6. Note that the dynamics in the air tube data and feather tip data are highly similar to one another, and are highly similar to that of the corresponding houses in the surveillance data (fig. 6).