Transmission from vaccinated hosts can cause dose-dependent reduction in pathogen virulence

Short title: Vaccination and the transmission of pathogen virulence.

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Abstract

Many livestock and human vaccines are leaky as they block symptoms but do not prevent infection or onward transmission. This leakiness is concerning as it increases vaccination coverage required to prevent disease spread, and can promote evolution of increased pathogen virulence. Despite leakiness, vaccination may reduce pathogen load, affecting disease transmission dynamics. However, the impacts on post-transmission disease development and infectiousness in contact individuals are unknown. Here, we use transmission experiments involving Marek's disease virus in chickens to show that vaccination with a leaky vaccine substantially reduces viral load in both vaccinated individuals and unvaccinated contact individuals they infect. Consequently, contact birds are less likely to develop disease symptoms or die, show less severe symptoms, and shed less infectious virus themselves, when infected by vaccinated birds. These results highlight that even partial vaccination with a leaky vaccine can have unforeseen positive consequences in controlling the spread and symptoms of disease.

Introduction

Vaccination is routinely used as an efficient and economical way to control the spread and symptoms of infectious diseases in humans and livestock. Vaccines vary in their protective properties[1,2], and while some completely block infection, others only prevent disease symptoms but not infection or onward transmission. The latter are termed 'leaky' or 'imperfect' vaccines. Leaky vaccines are commonly used to prevent or alleviate disease symptoms in livestock, and are becoming more prevalent among human vaccines[3]. Leakiness allows pathogen populations to persist even at high levels of vaccination coverage[4], and reduced mortality of vaccinated individuals can lengthen their infectious period and hence promote the evolution of increased pathogen virulence[5]. A better understanding of the overall impacts on populations of vaccination with leaky vaccines is therefore urgently needed.

The underlying hypothesis in this paper is that vaccination, even with leaky vaccines, not only has direct positive effects on vaccinated individuals, but also indirect positive effects on individuals in the same contact group. Often only a fraction of a population receives the direct benefits of vaccination, due to incomplete coverage and heterogeneity in vaccine responses[15-17]. However, vaccination even with a leaky vaccine often reduces pathogen load in infected individuals[5-12], with potential consequent reduction in the exposure dose of susceptible individuals. Transmission experiments, in which infected 'shedders' are placed in contact with uninfected 'contact' individuals and transmission recorded, have revealed that lower shedder pathogen load reduces transmission in some cases[5,13-14], but not all[10]. Measures of vaccine effectiveness can include these indirect benefits for unvaccinated individuals, through dose-dependent reduction in transmission rates from infected vaccinated individuals[18]. However, beyond transmission effects, lower exposure dose can also decrease pathogen load in newly infected hosts[19-21], potentially leading to decreased pathogen

virulence[19-20,22-27] and infectiousness in these secondary cases. These downstream effects of leaky vaccines on disease development and spread are currently poorly understood. Here, we use transmission experiments with vaccinated versus sham-vaccinated shedders and only unvaccinated contact individuals, to examine how a leaky vaccine affects both transmission and subsequent pathogen virulence and load (and hence, potentially, infectiousness) in contacts.

Gallid alphaherpesvirus 2, more commonly referred to as Marek's disease virus (MDV), is a highly oncogenic herpesvirus of poultry causing worldwide annual losses of 1-2 billion USD[28]. It is an airborne pathogen, spreading via inhalation of virus-laden "chicken dust", which accumulates through shedding of infectious feather follicle epithelia[29]. Marek's disease (MD) symptoms include peripheral nerve enlargement, tumours in a variety of organs, wing and leg paralysis, and iris lymphoma causing pupil irregularities, as well as death. Infected birds remain infectious for life, and the virus can remain viable in the environment for many months. Higher MDV ingestion dose has been reported to increase disease progression[27,30], but has not been linked to vaccination or exposure dose under natural transmission. On top of clear welfare concerns, MD causes production losses at inspection due to a drop in egg production of laying hens[31], and symptoms known as "leukosis" leading to meat condemnation. Leukosis has other causative agents but is primarily due to MDV in chickens[32].

Management of MD led to development of the first widely-used anti-cancer vaccine, the related live turkey herpesvirus *Meleagrid alphaherpesvirus 1*, commonly referred to as HVT[33]. In the US, vaccination of all commercial poultry has been routine since the 1970s. However, from the 1950s to the present day there have been several jumps in MDV

virulence[34], each causing more severe symptoms and reducing the symptom-blocking effects of existing vaccines. Several generations of vaccines have been developed to combat this increased virulence, all of which are leaky and may in fact have contributed to continuing virulence evolution[5]. Currently, widespread vaccination leads to low production losses in the US commercial poultry industry. However, vaccination is not routine worldwide, and may vary considerably in quantity and quality[35], leading to incomplete effective vaccine coverage within a flock.

High variability in virulence among MDV strains[36], in genetic resistance among chicken strains[37], and in vaccine effectiveness[38], combined with well-developed empirical methods for examining host infection and disease[39], make MDV in chickens an ideal model system to examine the relationships between vaccination with leaky vaccines and pathogen load, transmission, and subsequent virulence in non-vaccinated birds.

The overall aim of this study was to assess how vaccination with a leaky vaccine affects pathogen transmission and subsequent disease development in unvaccinated contact individuals. To investigate this, we carried out transmission experiments for MDV in chickens, where vaccinated or sham-vaccinated shedder birds inoculated with MDV were placed in contact with unvaccinated naïve contact birds (Fig 1). We chose unvaccinated contacts to avoid confounding effects of vaccination on contact bird resistance and shedder transmission ability. We investigated to what extent vaccination reduces both MDV transmission and subsequent disease severity in contacts, and asked whether the effects of shedder vaccination on contacts were mediated by lower shedder viral load. We found that shedder vaccination led to a large reduction in contact disease symptoms, and confirmed that this effect was mediated by viral load.

Fig 1. Schematic overview of one "lot" (2 lots per replicate, one for each shedder vaccination status) of the MD transmission experiment. In each lot, shedder birds were all either HVT-vaccinated or PBS sham-vaccinated. All contacts were unvaccinated. The experiment comprised 16 replicates, each consisting of one lot in which three infected vaccinated shedders were placed in 48 hour contact with 15 naïve unvaccinated contacts at 2 time points, and one equivalent lot with sham-vaccinated shedders (4 additional sham-vaccinated lots were added as two of these had only 2 shedders due to early death). In total, there were thus 1080 contacts and 106 shedder individuals distributed into 72 contact bird groups. Contact bird groups each had roughly equal numbers of males and females. All indicated time points (not to scale) are relative to the day of shedder inoculation with wild-type virus. Open and closed symbols represent uninfected and infected chickens, respectively. For all birds, necropsy was carried out to determine the presence and severity of disease symptoms (tumours and peripheral nerve enlargement) at 8 weeks post-infection (shedders) or post-contact (contact birds), or upon death/euthanasia, whichever was the sooner.

Results

Establishing the transmission model and sampling times. Given the novelty of the experimental setup, appropriate contact duration and sampling times to examine shedder vaccination effects needed to be established in pilot experiments prior to the main trial. Pilot experiment methods and results are presented in Supplementary Information. As little as 4 hours of contact between inoculated shedders and uninfected contacts was sufficient for most contact birds to become infected and show visible disease symptoms by 8 weeks post-contact (Fig S1). A contact duration of 2 days was subsequently chosen to ensure ample shedding time and to standardize time available for shedding of feather follicle epithelia by the shedders. Both vaccinated and sham-vaccinated shedders were positive for small quantities of virus in feather follicle epithelia by 7 days post-infection (hereafter DPI), but this feather viral load (hereafter FVL) had increased considerably by 10-12 DPI (Fig S2). When shedders were moved to a new set of contacts every 2 days from 10-20 DPI, the proportion of infected contacts, as measured by qPCR from feather and blood samples collected 14 days post-contact (hereafter DPC), was consistently high (Fig S3). However, while contact with sham-vaccinated shedders also consistently led to high incidence of disease symptoms at necropsy, contact with vaccinated shedders led to lower proportion of diseased contacts, in particular at the early contact periods. These temporal trends coincided with differences in shedder FVL, with higher overall FVL in sham-vaccinated birds, reaching a peak around 12 DPI, and lower FVL peaking around 20 DPI in vaccinated shedders (see Fig S2). Both groups of shedders then remained positive for virus in feathers for the 8-week duration.

Informed by the pilot data, we chose 13 and 20 shedder DPI as standardized contact start times in the main experiments (Fig 1) to capture the aforementioned temporal variation in vaccine effects, and a 2-day contact period between shedders and contacts. Fourteen DPC

was chosen as the time for contact bird blood and feather sampling, as it proved ample for build-up of FVL in infected contacts while minimizing among-contact transmission (Fig S4). Viral loads were highly correlated between blood and feathers (main experiment contact birds only, correlation coefficient r = 0.73) and were typically higher and more often above the detection threshold in feathers, as shown previously[40]. Hence, we focused on viral load in feathers for all analyses, due to the dual benefits of the typically above-threshold level viral loads and the fact that feathers are the infectious tissue, hence increasing the likely association with infectiousness. Examination of the presence and severity of disease symptoms (tumours and peripheral nerve enlargement) at necropsy took place at 8 weeks post-infection (shedders) or post-contact (contacts), or when moribund, if this occurred earlier. The subsequent results only refer to analyses of data from the main experiment illustrated in Fig 1.

Vaccination blocks shedder disease symptoms without blocking infection. As expected, all shedder birds were positive for MDV as determined by qPCR, and vaccination almost universally blocked the development of disease symptoms at necropsy. Eighty out of 86 sham-vaccinated shedders (93%; 4 out of 90 birds excluded due to early death from other causes) were MD-positive at necropsy, while only 5 out of 80 (6%) vaccinated shedders were MD-positive.

Shedder vaccination does not block transmission but reduces contact bird disease development and pathogen load. The complete set of contact bird analysis results are presented in Table S1 (Supplementary Information). Overall, vaccination of shedders did not block virus transmission, but dramatically reduced the negative impacts of infection in contact birds. Almost all contacts became infected regardless of shedder vaccination status or

DPI, with 100% (all 572 birds) contact bird infection for sham-vaccinated shedders and 97.4% for vaccinated (442 out of 454). This difference, albeit small, was significant, with contacts of sham-vaccinated shedders 0-0.28 times as likely to remain uninfected as contacts of vaccinated (Fisher exact test: 16.82, $p<0.001$, odds ratio = 0, 95% C. I. 0, 0.28). However, fewer infected contacts developed visible disease symptoms or died within 8 weeks (Fig 2a), and of those showing visible symptoms, shedder vaccination was associated with less severe contact bird symptoms, including fewer tissues with tumours and less severe enlargement of peripheral nerves, as illustrated by non-metric multidimensional scaling (Fig 2b).

Fig 2. Summary of shedder impacts on contact birds. (A) Impact of shedder vaccination status on contacts at 13 and 20 shedder DPI. Contacts positive for virus in qPCR from samples taken at 14 DPC were classified as infected. “Diseased” individuals showed visible symptoms (peripheral nerve enlargement and/or tumours) at necropsy, 8 weeks post contact or upon death. “Dead” contacts were those that died or were humanely euthanized before the end of the 8-week experimental period, were infected, and were positive for disease symptoms at necropsy. HVT = vaccinated shedders; PBS = sham-vaccinated shedders. (B) Non-metric multidimensional scaling plot, for diseased contacts only, of relationships between contact bird disease severity (number of tissues with tumours; VNE = vagus nerve enlargement; SNE = sciatic nerve enlargement; BNE = brachial nerve enlargement), contact bird sex, and shedder vaccination status and FVL. Points are individual contact birds. Arrow distance along each axis represents its nonparametric Kendall’s tau correlation with that axis. Variables differ qualitatively (binary, continuous or ordinal) and so correlation coefficients and hence arrow lengths may not be directly comparable. Shedder DPI effects not shown (see Results text and Table S1).

Infected contacts were much less likely to show visible disease symptoms at necropsy after contact with vaccinated (232 out of 437 contacts; 53%) than sham-vaccinated (558 out of 569; 98%) shedders (Table 1). Disease symptoms in infected contacts were also more likely in the 20 DPI than 13 DPI contact groups (mixed-model logistic regression: $z = 4.5$, $p < 0.0001$), but this temporal effect was smaller when shedders were sham-vaccinated

(vaccination status by DPI interaction; $z = -2.3$, $p < 0.05$). Males were marginally less likely to show visible disease symptoms than females ($z = -1.9$, $p = 0.05$).

Table 1 | Effects of shedder vaccination status on contact bird disease symptoms, mortality and feather viral load for a model also including contact bird sex and shedder DPI, but excluding DPI by vaccination status interaction. Full results, including models with the interaction, are in Table S1.

Contact bird response	Shedder vaccination coefficient (SE)[6]	Test statistic[7]	p value
Disease status[1]	8.19 (1.50)	5.45	< 0.0001 ***
Mortality[2]	1.74 (0.20)	8.76	< 0.0001 ***
N tissues with tumours[3]	0.50 (0.13)	3.71	< 0.0005 ***
Vagus nerve enlargement[4]	0.22 (0.23)	0.94	0.35
Brachial nerve enlargement[4]	1.30 (0.26)	5.01	< 0.0001 ***
Sciatic nerve enlargement[4]	1.30 (0.24)	5.36	< 0.0001 ***
Feather viral load[5]	1.98 (0.11)	18.3	< 0.0001 ***

[1]Infected contacts (qPCR) only. Logistic regression. Coefficient = mean log odds ratio for presence of contact disease symptoms when exposed to sham-vaccinated relative to vaccinated shedders.

[2]Infected contacts (qPCR) only. Cox proportional hazards. Coefficient = log hazard ratio of contact death at a given time point associated with sham-vaccinated relative to vaccinated shedders.

[3]Diseased contacts (necropsy) only. Poisson GLM. Coefficient = mean difference in number of contact tissues containing tumours with sham-vaccination.

[4]Diseased contacts (necropsy) only. Ordinal logistic regression. Coefficient = proportional log odds of an increase in contact nerve enlargement ranking with sham-vaccination.

[5]All contacts. Least square mean difference in contact bird $\log_{10}$(viral load + 1e-5) with sham-vaccinated relative to vaccinated shedders.

[6]Positive = increase in contacts when exposed to sham-vaccinated relative to vaccinated shedders, except for Feather viral load (> 1 = increase with sham-vaccination).

[7]t statistic for linear regression, z statistic for all other models.

Mortality rates were also much lower among infected contacts exposed to vaccinated shedders (Fig 3), with those exposed to sham-vaccinated shedders being 6 times more likely to die per unit time (95% C. I. 3.9, 8.4; Table 1). Controlling for vaccination effects, contacts exposed to shedders at 20 DPI were almost twice as likely to die as those exposed to shedders at 13 DPI (95% C. I. 1.3, 2.1; $z = 3.6$, $p < 0.0005$).

Fig 3. Cox proportional hazards estimated survival probability curves for all combinations of shedder vaccination status and days post-inoculation (DPI). Shaded areas represent 95% confidence intervals. HVT = vaccinated shedders; PBS = sham-vaccinated shedders. Note that all mortality up to 7 days post contact (DPC) was assumed to be chick mortality unrelated to MD, and these individuals were excluded from all analyses.

Among contacts positive for disease symptoms at necropsy, shedder vaccination led to significantly lower disease severity (number of tissues with tumours, and enlargement of 3 peripheral nerves; see Fig 2b) for all individual symptoms except vagal nerve enlargement (Table 1). There was no evidence for an increase in contact bird disease severity between shedder DPI 13 and 20 for either vagus nerve (mixed-model ordinal logistic regression: $z = -0.1$, $p = 0.89$) or tumours ($z = 1.2$, $p = 0.21$), but marginal evidence for greater brachial nerve enlargement ($z = 1.9$, $p = 0.06$) and a significant increase in sciatic nerve enlargement ($z = 3.1$, $p < 0.005$) associated with the later exposure time. Regardless of the shedder vaccination status and exposure time, disease severity was significantly higher in contact females than males for all symptoms (Fig 2b; Table S1).

Next, we tested the extent to which shedder vaccination status also influenced contact FVL as an indicator of the infectiousness of contact birds, which has potentially important knock-on

effects for epidemiological dynamics. Infectiousness is likely to be determined by the amount of virus shed into the environment. Across all individuals, contact bird FVL at 14 DPC was much higher when exposed to sham-vaccinated than vaccinated shedders (Table 1, Fig 4). Contact FVL was also higher when exposed to shedders at 20 DPI than 13 DPI (mixed-model linear regression: $t = 4.9$, $p < 0.0001$).

Fig 4. Box and whisker plot of shedder and contact feather viral loads at 13 and 20 DPI. Horizontal bars are medians. Boxes cover the 1st to 3rd quartile, and vertical lines extend to maxima and minima except in the presence of outliers (filled circles). Shedder feather samples were taken at the start of each contact period, contact samples at 14 DPC. A small value (1e-5) was added to feather viral load values prior to log transformation, and hence the $\log_{10}$(viral load) for birds negative for virus was -5.

In summary, contact birds exposed to vaccinated shedders still became infected, but were considerably less likely to develop disease, experienced milder symptoms and lower mortality, and had lower feather viral loads.

Shedder vaccination effects on contacts are mediated by feather viral load. We hypothesized that the effects of shedder vaccination on contacts, described above, were mediated by a reduction in shedder FVL with vaccination, leading to a reduction in contact exposure dose with knock-on effects for disease development. To test this hypothesis, we followed the protocol for mediation analysis using regression, outlined in the Statistical Analysis section of the Methods.

Sham-vaccinated shedders had much higher FVL than vaccinated (mixed-model linear regression: $t = 13.35$, $p < 0.0001$; Fig 4). There was a highly significant increase in shedder

FVL at 20 DPI over 13 DPI ($t = 7.49$, $p < 0.0001$), but the highly significant interaction between vaccination status and DPI ($t = -5.03$, $p < 0.0001$) revealed that this temporal change only occurred in vaccinated shedders.

Replacing shedder vaccination status with shedder FVL as a covariate in the statistical models for contact birds, revealed that the effects of shedder FVL on contacts followed the same pattern as the effects of shedder vaccination status. Higher shedder FVL led to a small but significant increase in contact bird infection probability (univariate logistic regression: log odds = 0.76, $z = 3.0$, $p < 0.005$), with predicted infection probability increasing from 0.946 at the lowest shedder FVL values to 0.997 at the highest. Higher shedder FVL led to greater contact bird disease prevalence and severity, greater mortality, and higher contact FVL (Table 2).

Table 2 | Effects of shedder feather viral load on contact bird disease symptoms and feather viral load, for a model also including contact sex and shedder DPI, but not including shedder vaccination status. Full results in Table S1.

Contact bird response	Shedder viral load slope (SE)	Test statistic[6]	p value
Disease status[1]	3.83 (0.62)	6.17	< 0.0001 ***
Mortality[2]	0.93 (0.11)	8.76	< 0.0001 ***
N tissues with tumours[3]	0.34 (0.09)	3.57	< 0.0005 ***
Vagus nerve[4]	0.24 (0.16)	1.51	0.13
Brachial nerve[4]	0.91 (0.17)	5.4	< 0.0001 ***
Sciatic nerve[4]	0.90 (0.16)	5.57	< 0.0001 ***
Feather viral load[5]	0.83 (0.08)	10.33	< 0.0001 ***

[1]Infected contacts (qPCR) only. Logistic regression. Coefficient = increase in log odds ratio for presence of contact disease symptoms with 1 unit increase in shedder FVL.

[2]Infected contacts (qPCR) only. Cox proportional hazards. Coefficient = increase in log hazard ratio of contact death at a given time point with 1 unit increase in shedder FVL.

[3]Diseased contacts (necropsy) only. Poisson GLM. Coefficient = increase in number of contact tissues containing tumours with 1 unit increase in shedder FVL.

[4]Diseased contacts (necropsy) only. Ordinal logistic regression. Coefficient = increase in proportional log odds of contact nerve enlargement ranking with 1 unit increase in shedder FVL.

[5]All contacts. Increase in contact FVL with 1 unit increase in shedder FVL.

[6]t statistic for linear regression, z statistic for all other models.

Including shedder FVL in a model alongside vaccination status reduced, but did not always remove, the significance of vaccination status for all contact bird disease variables (Table S1). This indicated that shedder FVL at least partially explained the impacts of shedder vaccination on infected contacts. However, the further addition of contact FVL and sum of contact groupmate FVL (the latter to account for possible among-contact infection during the 8-week experimental period) as predictors fully explained the effects of shedder vaccination on contact disease and survival, rendering shedder vaccination status non-significant in all models (Fig 5). The results imply that shedder vaccination effects on contacts are fully mediated by FVL of shedders and infected contacts.

Fig 5. Diagrammatic representation of the mediating effects of viral load on contact bird binary disease status. Each arrow colour represents a different multiple regression analysis, with arrows pointing from predictors to response. Arrow thickness represents regression coefficient (all significant or marginal relationships were positive). The diagram shows that the effect of shedder FVL on contact FVL (see Table 2) fails to fully explain the shedder vaccination effect, which remains significant when both variables are included (purple arrows). The same is true for the cumulative FVL of all infected group-mates (yellow arrows). However, the three FVL predictor variables completely remove the

effect of shedder vaccination status (blue arrows) in explaining contact disease status (see Table 1), and this is true for all other contact disease variables (Table S1). This implies that shedder vaccination effects on contacts are fully mediated by FVL of infected individuals in a contact group. Significance is indicated by asterisks: *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$, ns = not significant. Marginally non-significant p-values are presented numerically. All results presented here are from models excluding the DPI by vaccination status interaction, but including sex and DPI main effects (not shown, see Table S1).

Discussion

We used controlled experiments involving natural virus transmission to reveal that vaccination with a leaky vaccine, which only marginally reduces transmission, can significantly reduce post-transmission disease development and mortality among unvaccinated contact individuals. Our analysis confirms that this effect is mediated by a reduction in exposure dose experienced by susceptible individuals when exposed to vaccinated shedders, leading to lower pathogen load and concomitant reduced symptoms in contact birds. The primary objectives of vaccination of livestock with leaky vaccines are to improve animal welfare and to reduce production losses caused by disease symptom development. Our results show that even partial vaccination against MD can substantially reduce disease symptoms and mortality in the whole flock, leading to universally positive impacts on animal welfare and productivity, and these conclusions may extend to leaky vaccines used in other systems.

One of the key findings of this study was that shedder vaccination effects on MD symptom prevalence and subsequent mortality within each contact group were fully explained by the cumulative FVL of all infected group members, measured at a relatively early stage of the epidemic, prior to onset of contact-contact transmission. This would suggest that contacts exposed to vaccinated shedders experienced overall lower cumulative exposure dose, including from other infected contacts, over the course of 8 weeks, resulting in milder symptoms and lower mortality. This negative feedback on the environmental pathogen burden strongly advocates the application of MD intervention strategies that reduce either within-host or environmental virus load, even if only moderately[41]. In general, depending on the relationship between exposure dose and subsequent within-host replication in any

particular system, targeting reduction in pathogen load in intervention strategies may have greater positive knock-on effects than currently assumed.

Increased disease severity with higher virus inoculation dose has been shown previously for MD in chickens[27,30], but not with natural transmission and not linked to interventions such as vaccination. The route of infection is known to alter the extent of infection and number of diseased tissues[42], hence it is important to mimic the field situation closely in order to accurately predict the outcome of vaccination and other intervention strategies in this and other systems. It is also important to measure pathogen load specifically in the infectious tissues where possible, or to measure shedding directly, as tissues may differ in the strength of association between their pathogen load and infectiousness. Vaccine experiments routinely establish the protective effect of vaccination on infection or disease progression, and pathogen shedding, in vaccinated individuals themselves[43], and occasionally also examine onward transmission. The novelty and primary focus of this study was to determine the effects of vaccination of 'shedder' individuals on disease progression and infectiousness specifically in newly infected, unvaccinated, contact individuals. To date, little is known about these potential 'downstream' effects of vaccination, and the majority of epidemiological models that predict the consequences of vaccination on disease spread and pathogen evolution assume that these don't exist[3,44]. Our findings that vaccination affects downstream pathogen load and host survival and hence, potentially, onward pathogen transmission, in a dose-dependent manner may have profound consequences for such predictions. Particularly in systems where an individual's infectiousness is strongly influenced by its pathogen load, existing estimates of the required vaccine coverage for achieving so-called herd immunity, i.e. for preventing disease spread within a population, may be upwardly biased. Based on current estimates, herd immunity is assumed to require

high coverage when vaccines are leaky[18,45]. Complete vaccine coverage is not typical for all infectious diseases throughout the world, and even where it is routine, vaccine administration can vary in quality. Furthermore, high variation in vaccine responsiveness may render a significant proportion of vaccinated animals effectively non-immunized[46]. The results of our study suggest that partial vaccination or high prevalence of vaccine non-responders may impose less risk with respect to disease invasion and persistence than anticipated from existing theory[4,47-48]. Prediction of the coverage required for herd immunity would benefit from an understanding of the downstream effects of vaccination-induced changes in exposure dose and their effects on individuals' infectiousness and survival in any particular system. These insights would also be useful for the development of dynamic epidemiological models that incorporate dose-dependent transmission effects and the impact of interventions on these[19,24,49-51].

Vaccination with leaky vaccines has been implicated in the evolution of increased pathogen virulence, primarily because vaccination reduces host mortality without preventing disease spread, allowing infectious hosts more time to transmit virulent pathogen strains[3,5,44]. The best evidence for this effect comes from studies of MDV[5]. Our results, showing that vaccination reduces negative impacts of MD by lowering pathogen load, do not contradict this hypothesis. However, the existence of vaccine-induced downstream effects of exposure dose on host infectiousness, recovery and survival, is likely to alter the strength of selection favouring more virulent strains. Furthermore, the lower pathogen load and slightly reduced rate of spread revealed here – the latter effect potentially being stronger in other systems - would lower the effective population size of the virus, therefore lowering the probability of establishment and fixation of new beneficial mutations such as those increasing virulence[52]. This may partially explain why years pass between reported increases in MDV virulence,

despite 59 billion chickens being reared annually worldwide[34,53]. The results of this study therefore suggest that existing models of virulence evolution would benefit from incorporation of dose response effects on downstream disease severity and mortality such as those detected here.

Vaccination is often not the only available intervention technique, and others such as improved animal husbandry techniques or genomic selection of genetically more resistant individuals[54-56] may also be available and cost-effective. The mediating effect of pathogen load can be used as a link for comparison between the effects of different interventions on pathogen dynamics and disease severity. Chickens are known to vary genetically in their resistance to MD[54], and hence a next step in understanding the benefits of interventions in this system would be to compare vaccination effects with host genetic effects. Furthermore, more virulent virus strains tend to result in higher viral shedding rates and may differ in their response to vaccination[5]. Hence, future studies to test the validity of our findings for multiple MDV and vaccine strains are warranted.

With the increasing development of leaky vaccines for treatment of human as well as livestock infectious diseases[3], there is great benefit in improving prediction of their consequences for host welfare, pathogen dynamics and virulence evolution. The currently neglected downstream, post-transmission effects that were revealed in this study are likely to impact all of these important facets of infectious disease biology, and hence disease management strategies. They therefore merit greater attention in future vaccine-related studies.

Methods

Transmission experiments. Experiments were carried out at USDA, ARS, USNPRC, Avian Disease and Oncology Laboratory (ADOL), East Lansing, USA, during 2018. All experiments used $15I_5$ x 7_1 white leghorn chickens, a F1 hybrid cross of MD-susceptible 15I5 males and 7_1 females[57]. These maternal antibody-negative chickens were reared from a SPF breeding flock housed in isolators that have received no MD vaccination or exposure. The flock was negative for MDV antibodies and also for exogenous avian leukosis virus and reticuloendotheliosis virus, as established by routine surveillance testing. All bird experiments were approved by the ADOL Animal Care and Use Committee and were carried out in negative pressure Horsfall-Bauer isolators. Birds were monitored at least daily throughout the experiment and were humanely euthanized upon reaching established humane endpoint criteria.

The experiments involved two types of shedders, with shedder birds either vaccinated at hatch via intra-abdominal (IA) inoculation with 2,000 PFU of HVT (*Meleagrid alphaherpesvirus* 1)[33], or sham-vaccinated with PBS (phosphate-buffered saline). Each shedder bird was then challenged with 500 PFU of virulent MDV (strain JM/102W) at 5 days post-vaccination (0 DPI). Each contact group of birds within each replicate consisted of 3 shedder birds of the same vaccination treatment (HVT or PBS) to be placed in contact with 15 unvaccinated, uninfected contacts (Fig 1). The 3 shedders were placed with the first group of 15 uninfected contacts at 13 DPI for 48 hours, before being removed back to their isolator at 15 DPI. They were then placed with a second group of 15 contacts at 20 DPI until 22 DPI. Contact chicks were hatched weekly so that all contact birds were within 4 days of age when shedders were first introduced. There were 16 replicates consisting of paired lots of shedder birds (one lot with 3 vaccinated shedders put into contact with 15 contacts at the two time

points, and the other with 3 sham-vaccinated shedders), and 4 further sham-vaccinated only replicates. These additional replicates were carried out due to early death of 2 sham-vaccinated shedders involved in the earlier replicates.

Shedders were then monitored until 8 weeks post-infection and contacts until 8 weeks post-contact, and mortality (death or euthanasia) recorded. Necropsy was carried out at 8 weeks or upon death, whichever was the sooner, to determine the presence and severity of MD symptoms.

Blood (100 μl) and primary feather samples were taken from shedders at the start of each contact period (13 and 20 DPI) and from contacts at 14 DPC. Based on earlier experiments, 14 DPC was sufficient for build-up of virus in blood and feathers but early enough to avoid cross-contamination from other contact birds (Supplementary Information). DNA samples isolated from feather pulp and peripheral blood lymphocytes (PBLs) were used for qPCR to determine virus load. Each measurement was taken from a unique sample.

DNA from each tissue type was isolated using the Puregene DNA isolation kit (Gentra System, Minneapolis, MN) followed by a multiplex PCR using methods as previously described[58]. The TaqMan assay used FAM-TAM probes for MDV gB and VIC-TAM probes for the cellular GAPDH. Results were reported as the ratio of MDV gB copies per GAPDH copies, estimated using standard curves consisting of 10- fold serial dilutions of plasmids containing either MDV gB or GAPDH. Amplifications were performed at Michigan State University, USA, using the ABI Quant Studio 7Flex BI 7500.

Statistical analyses. Forty-two of 1080 contacts were removed from the dataset prior to analysis due to chick mortality (death up to 7 days old), with some further filtering for data quality and death by other causes. Final sample sizes were 211 (shedder FVL as response), 1005 (infected contacts only), 789 (diseased contacts only) and 1023 (all contacts regardless of infection or disease status). The transmission experiments were analysed using various linear and generalized linear mixed models in R 3.6.0[59], depending on the type of the response variable (Table 3). Regression analyses followed the logic of process analysis[63] to assess the role of pathogen load in mediating shedder vaccination effects on contacts, details below. Non-metric multidimensional scaling for Fig 2b was carried out in PC-ORD v. 7.0[64] statistical software. All statistical tests were two-sided. No adjustments were made for multiple comparisons.

Table 3 | Summary of modelled response variables.

Response variable	Description	Source	Coefficient interpretation	Statistical model	Data subset[6]
Disease status	Binary presence/absence of visible disease symptoms	Necropsy	Log odds	Logistic regression (GLM binomial errors)[1]	Infected
Mortality	Day of death/euthanasia or last day of study	Daily observations	Log proportional hazard ratio	Right-censored Cox proportional hazards[2]	Infected
N tissues with tumours	Number of tissues with visible tumours	Necropsy	Log relative risk	GLM Poisson errors[3]	Diseased

Nerve enlargement	Qualitative ranking of nerve enlargement (0-4)	Necropsy	Log proportional odds	Ordinal logistic regression[4]	Diseased
Viral load	$\log_{10}$(Ratio of virus to GAPDH quantity + 1e-5)	qPCR	Mean relative quantity	Ordinary linear regression[5]	All

[1]R function glmer in lme4 package[60]; logit link.

[2]R function coxph in survival package[61].

[3]R function glmer in lme4 package; log link.

[4]R function clmm in ordinal package[62]; logit link.

[5]R function lmer in lme4 package; identity link.

[6]Infected = positive for virus in qPCR of one or both of feather and blood samples; Diseased = presence of visible disease symptoms (tumours and/or peripheral nerve enlargement) at necropsy; All = all contact individuals including uninfected.

First, we tested the direct treatment effect (shedder vaccination status) on the outcome variables (contact disease variables, Table 3). The model formulae also included as fixed effects contact bird sex and shedder DPI, and a vaccination status by DPI interaction, which was removed if non-significant. Replicate, and contact group nested within replicate, were included as random effects in all models except for the survival analysis, for which contact group and replicate were included as clustering variables. Each contact individual was treated as a data point. For this and all subsequent analyses, testing contact feather viral load (FVL) as response involved all contact individuals, infected or uninfected (Table 3). Contact binary disease status and mortality analyses involved infected (from qPCR) contacts only, and disease severity variables (tumours and nerve enlargement) involved diseased (from necropsy) contacts only.

Second, we carried out a mediation analysis, for which we tested all intermediate steps in the following proposed causal chain (see Fig 5): we hypothesized that the impacts of shedder vaccination status on the various contact infection and disease variables were primarily mediated by the vaccine effect on shedder feather viral load (FVL). More specifically we hypothesized that shedder vaccination directly reduces shedder FVL and consequently also the exposure dose of contacts. The resulting lower exposure dose may reduce the probability of becoming infected and/or may lead to lower ingestion dose, and consequently also to lower viral load in infected contacts. Lower contact viral load reduces the probability in infected contacts of developing visible disease symptoms or dying within the 8-week experimental period, and also reduces disease severity among individuals positive for symptoms at necropsy. Eight weeks is also sufficient time for infected contacts to become infectious themselves, and for disease development to occur in contacts infected by other contacts. Hence it was necessary to also consider the FVL of infected group mates alongside shedder and contact FVL in the mediation analysis.

We began the mediation analysis by testing whether shedder FVL explained a similar amount of contact bird disease variation as shedder vaccination status, by replacing shedder vaccination status with shedder FVL in the model formula described in the first step above. We then tested to what extent shedder FVL was affected by vaccination, and then to what extent contact FVL and the sum FVL of each contact bird's groupmates (hereafter denoted as groupmate FVL) were affected by vaccination and shedder FVL. Thus, for contact FVL and groupmate FVL as response variables, the model formulae were the same as described in the first step above, with the addition of sum of shedder FVL for each contact group as a fixed effect. Conversely, when shedder FVL was tested as a response variable, we used each individual shedder feather sample as a data point, and hence there were two data points per

shedder individual (13 and 20 DPI). For this test we used the same fixed effects model formula as described in the first step, above, while replicate and shedder individual were included as random effects, the latter to account for repeated measures.

The values for contact FVL at 14 DPC were calculated as $\log_{10}$(contact FVL + 1e-5) for each individual. The contact groupmate FVL variable was the sum of FVL at 14 DPC of all 15 contacts in a group, minus the value for the focal individual. This variable was also analysed as $\log_{10}$(groupmate FVL + 1e-5). For shedder FVL as a predictor, we calculated $\log_{10}$(sum(shedder FVL + 1e-5)) across the 3 shedders, from feather samples collected at the start of the contact period with each group of 15 contacts (13 and 20 DPI).

Third and finally, we tested whether shedder vaccination status exerted any effect on contact disease variables when controlling for mediating effects (shedder, contact, and contact groupmate FVL). If shedder vaccination status were to be rendered non-significant when tested alongside FVL variables, this would support the hypothesis that shedder vaccination impacts on contact disease were fully mediated by their effects on FVL. We first added shedder FVL alone to the basic model described in step 1, above, to test whether this variable was an effective bioindicator of shedder vaccination effects in secondary cases (infected contacts). We then further added contact and groupmate FVL to the model. Same-individual viral load is expected to be the strongest indicator of disease status, and so we expected shedder vaccination status and shedder and groupmate FVL to become non-significant in this model.

Data availability

The datasets generated and analysed during the current study are available in the Edinburgh DataShare repository, https://doi.org/10.7488/ds/2598.

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Author contributions

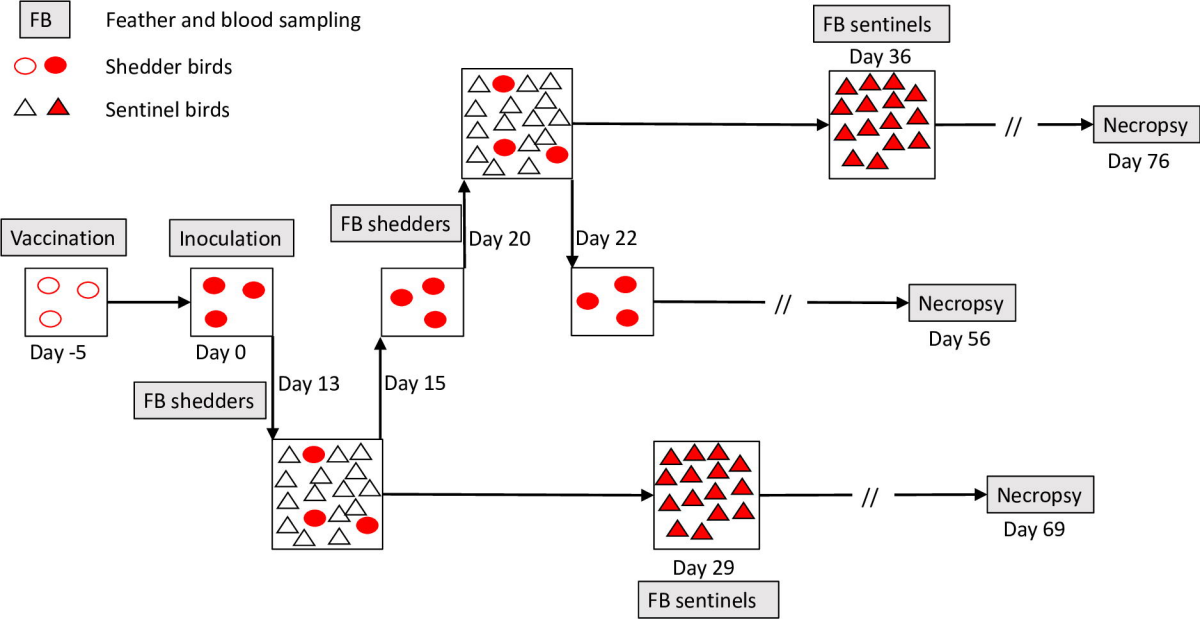
JRD led project conceptualization, experimental design and funding acquisition, alongside ADW, HHC and OA. Experimentation and data curation were carried out by JRD (lead), JKM and HHC. RIB took the lead in statistical analysis with contributions from MCT and ADW. RIB led interpretation of results and manuscript writing, alongside ADW, JRD, HHC, MCT. All authors contributed to reviewing and editing the manuscript.

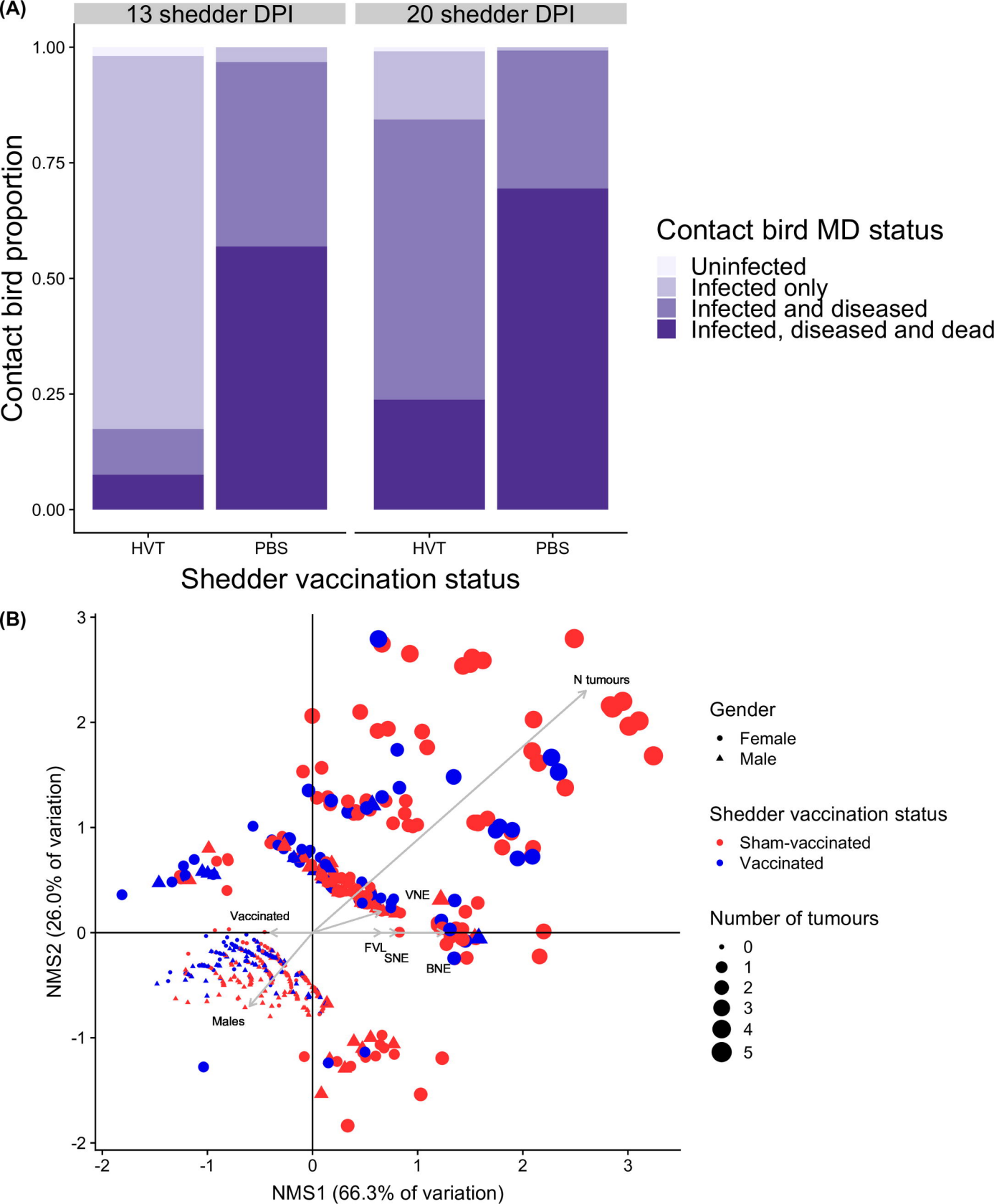
Competing interests

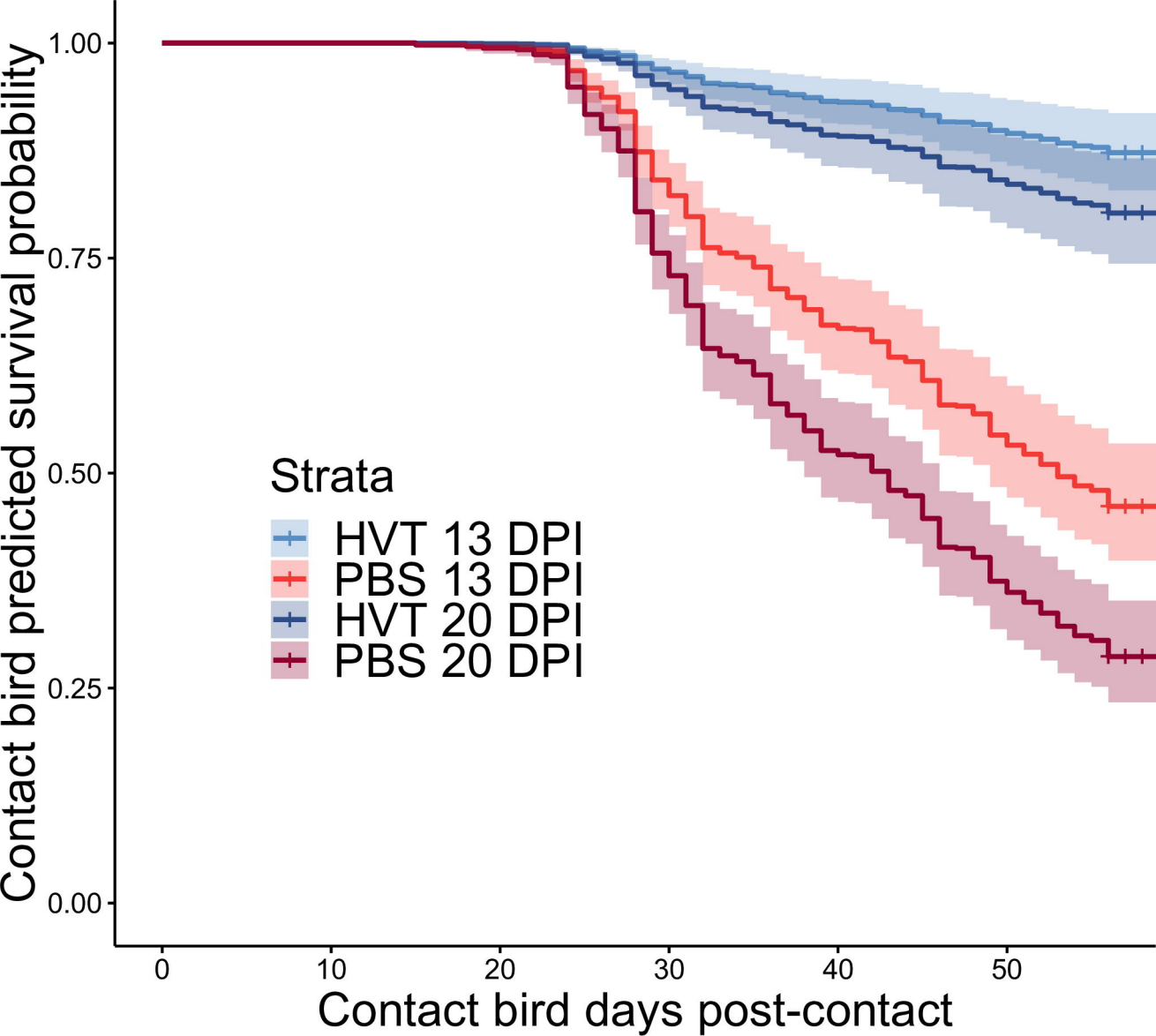
The authors declare no competing interests.

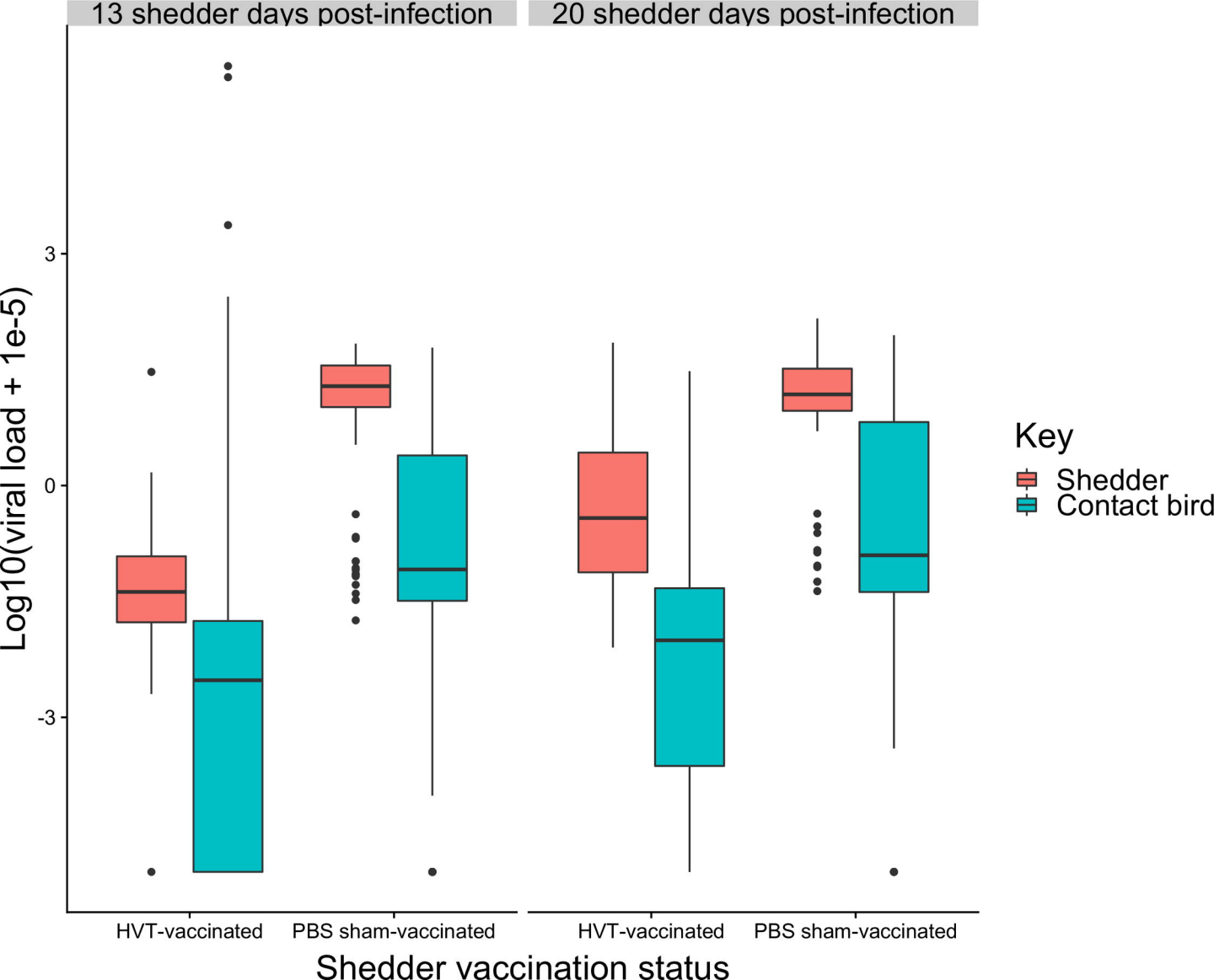
Materials & Correspondence

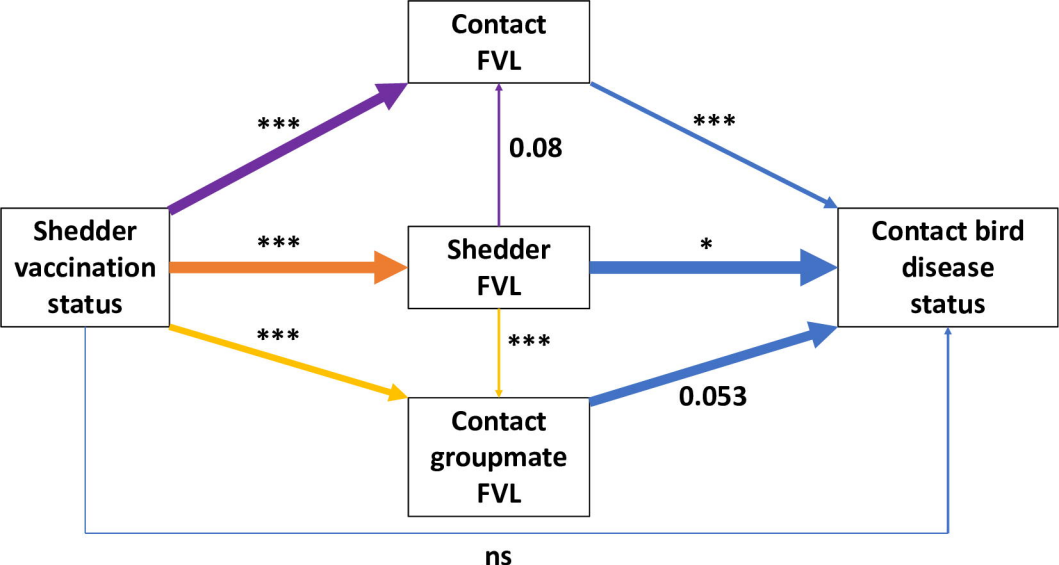
Requests for physical materials and raw data should be addressed to JRD. Requests for analysis scripts and outputs should be addressed to ADW or RIB.


FB
Feather and blood sampling
Shedder birds
Sentinel birds
Vaccination
Day -5
Inoculation
Day 0
FB shedders
Day 13
FB shedders
Day 15
Day 20
Day 22
FB sentinels
Day 36
Necropsy
Day 76
Necropsy
Day 56
Day 29
FB sentinels
Necropsy
Day 69


(A)
13 shedder DPI
20 shedder DPI
Contact bird proportion
1.00
0.75
0.50
0.25
0.00
HVT
PBS
HVT
PBS
Shedder vaccination status
Contact bird MD status
Uninfected
Infected only
Infected and diseased
Infected, diseased and dead
(B)
NMS2 (26.0% of variation)
NMS1 (66.3% of variation)
N tumours
VNE
Vaccinated
FVL
SNE
BNE
Males
Gender
Female
Male
Shedder vaccination status
Sham-vaccinated
Vaccinated
Number of tumours
0
1
2
3
4
5


Contact bird predicted survival probability
1.00
0.75
0.50
0.25
0.00
Strata
HVT 13 DPI
PBS 13 DPI
HVT 20 DPI
PBS 20 DPI
0
10
20
30
40
50
Contact bird days post-contact


13 shedder days post-infection
20 shedder days post-infection
Log10(viral load + 1e-5)
3
0
-3
HVT-vaccinated
PBS sham-vaccinated
HVT-vaccinated
PBS sham-vaccinated
Shedder vaccination status
Key
Shedder
Contact bird


Contact FVL
***
0.08
***
Shedder vaccination status
***
Shedder FVL
*
Contact bird disease status
***
***
0.053
Contact groupmate FVL
ns