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VACCINATION AND REDUCED COHORT DURATION CAN DRIVE VIRULENCE EVOLUTION: MAREK'S DISEASE VIRUS AND INDUSTRIALIZED AGRICULTURE

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Marek's disease virus (MDV), a commercially important disease of poultry, has become substantially more virulent over the last 60 years. This evolution was presumably a consequence of changes in virus ecology associated with the intensification of the poultry industry. Here, we assess whether vaccination or reduced host life span could have generated natural selection, which favored more virulent strains. Using previously published experimental data, we estimated viral fitness under a range of cohort durations and vaccine treatments on broiler farms. We found that viral fitness maximized at intermediate virulence, as a result of a trade-off between virulence and transmission previously reported. Our results suggest that vaccination, acting on this trade-off, could have led to the evolution of increased virulence. By keeping the host alive, vaccination prolongs infectious periods of virulent strains. Improvements in host genetics and nutrition, which reduced broiler life spans below 50 days, could have also increased the virulence of the circulating MDV strains because shortened cohort duration reduces the impact of host death on viral fitness. These results illustrate the dramatic impact anthropogenic change can potentially have on pathogen virulence.

KEY WORDS: **Failed vaccines, industrialization, pathogen evolution, poultry disease, virulence-transmission trade-off.**

The intensification of agriculture in the 20th century transformed the landscape for infectious agents of farm animals. This provides immense opportunities for evolutionary ecologists trying to understand pathogen adaptation. Marek's disease virus (MDV), a tumor-inducing airborne poultry pathogen, evolved substantially higher virulence over the second half of last century. Until the 1950s, strains of MDV circulating on poultry farms caused a mildly paralytic disease, with lesions largely restricted to peripheral nervous tissue. Death was relatively rare. Today, hyperpathogenic strains induce lymphomas in a wide range of organs and mortality rates of up to 100% (Witter 1997, 1998; Nair 2005; Osterrieder et al. 2006). It seems likely that the radical intensification of poultry farming drove this virulence evolution, but it is unclear which of many ecological changes were responsible. Here, we assess two of the leading contenders: altered broiler poultry life span and widespread vaccination.

EVOLUTION
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During the early part of the 20th-century broiler birds lived for over 75 days until the desired weight was reached. By the end of century, advances in genetics and nutrition had halved that life span (Morrow and Fehler 2004; Sheppard 2004). Over that same period, Marek's disease (MD) became a significant problem that today costs the global poultry industry about $2 billion annually (Morrow and Fehler 2004). Control is mostly via vaccination of chickens with live persistent vaccines. Vaccinated hosts are still able to become infected with, and transmit, MDV (Witter and Lee 1984; Islam et al. 2006; Islam and Walkden-Brown 2007; Islam et al. 2008; Atkins et al. 2011). All three MDV serotypes are used as live vaccines, singly or in combination. In order of increasing potency (a more effective immune response) and protection (fewer clinical signs), these are HVT (Herpesvirus of turkeys), MDV serotype 2 (MDV-2, usually used in bivalent form in combination with HVT), and an attenuated form of MDV serotype 1 (MDV-1) known as Rispens after the scientist who isolated it (Bublot and Sharma 2004). HVT has been used worldwide since the early 1970s, bivalent vaccines since the early 1980s and, and in the United States, Rispens since the early 1990s when the HVT and bivalent vaccines were unable to control the highly virulent MDV strains that had begun to circulate in vaccinated flocks (Witter 2001). By 2004, nearly all broiler birds were being routinely vaccinated against MDV either in ovo or at one-day old (Morrow and Fehler 2004).

The leading evolutionary theory of virulence evolution is the parasite-centered Ôvirulence trade-offÕ theory (Levin and Pimentel 1981; Anderson and May 1982a; Frank 1992; Read 1994; Alizon et al. 2009; Restif 2009). This posits that there are fitness benefits and costs associated with virulence. The benefits (why selection favors virulent strains at the expense of the benign) are assumed to be increased transmission stage production per unit time and/or longer time to immune clearance. The cost of virulence (why natural selection penalizes excessively virulent strains) is assumed to be the truncation of the infectious period by host death. Since natural selection maximizes fitness, it should favor strains that maximize propagule shedding by balancing the fitness costs and benefits of virulence. There have been a number of empirical results suggesting that this functional form may be a reasonable assumption (Anderson and May 1982a; Lipsitch 1997; Mackinnon and Read 1999; de Roode et al. 2008) for such pathogen infections as myxoma in rabbits (Anderson and May 1982a; Dwyer et al. 1990; Fenner and Fantini 1999), malaria in mice (Mackinnon and Read 1999), malaria in chickens (Paul et al. 2004), *Pasteuria ramosa* in *Daphnia magna* (Jensen et al. 2006), HIV in humans (Fraser et al. 2007), *Ophryocystis elecktroscirrha* in monarch butterflies (de Roode et al. 2008), and MDV in chickens (Atkins et al. 2011).

Other models of virulence evolution have been proposed, and the most appropriate model certainly depends on the biology of the disease in question (Bull 1994; Frank 1996; Boots and Sasaki 1999; Dieckmann 2002; Ebert and Bull 2003; Bull and Ebert 2008; Alizon et al. 2009; Restif 2009). Indeed both Fraser et al. (2007) and de Roode et al. (2008) have adapted host–pathogen specific fitness functions for HIV-humans and *O. elecktroscirrha*-butterfly systems respectively. Fraser et al. (2007)) report a genetic trade-off by calculating transmissibility as a function of set point viral load, and evaluating the intermediate virulence at which lifetime transmission potential is maximized. Similarly, de Roode et al. (2008) calculate relationships between within-host replication, virulence and transmission to build a pathogen fitness function specific to the life cycle of Monarch butterflies.

If MDV follows a transmission-life span trade-off, two hypotheses could explain why MDV became more virulent over the second half of the 20th century. First, MDV vaccines, which reduce the likelihood of death but do not prevent transmission, would have relaxed selection against virulence by greatly reducing the fitness costs (i.e., host death). Thus, more virulent strains with higher transmissibility could spread because vaccination would protect their hosts from premature host death (Gandon et al. 2001). A different hypothesis is that the marked reductions in broiler life spans as a result of cohort duration would have decreased the host life span, which would benefit a more virulent virus having increased transmission but not at a fitness cost because the host would die before MDV could kill it (Anderson and May 1982a; Sasaki and Iwasa 1991; Day 2002; Nidelet et al. 2009).

Here, we quantitatively evaluate whether these two evolutionary explanations can account for increasing MDV virulence in broiler barn settings.

Methods

We defined two fitness measures: Infectious potential, W (the average virus shed during a single infection), and the reproductive number, R_0 (the average number of secondary infections from an infected host in a completely susceptible population). The virulence of an MDV strain was measured by the virulence score, v, which gives the mean percentage of HVT- and bivalent-vaccinated maternal antibody positive birds dying or developing gross clinical signs within eight weeks of infection (Atkins et al. 2011). This metric is similar to the "gold-standard" pathotyping system of MDV strains (Witter 1997; Witter et al. 2005) and was used in previous parameter estimation (Atkins et al. 2011) based on pathotyping experiments (Renz et al. 2012; Walkden-Brown et al. 2012). Although it is not a direct measure of host mortality rate, we have previously shown virulence score to be negatively associated with host life span (Atkins et al. 2011). Virulence has been measured in other systems in different ways (Read 1994; Day 2002) (e.g., time until parasite-induced death (Ebert and Mangin

1997), instantaneous mortality rate (Anderson and May 1982a), and case fatality (Fenner and Fantini 1999).

MDV BIOLOGY

MDV is transmitted indirectly via inhalation of contaminated dust particles. Once infection occurs, the virus enters a cytolytic stage before birds become latently infected (around six to seven days postinfection) (Baigent and Davison 2004). A second cytolytic stage may then follow, before the final transformation stage (when latently infected cells form tumor cells). Live virus is shed from the feather follicle epithelium as keratinized dust. One study detected virus in primarily infected birds around seven days postinfection (Baigent et al. 2005). Birds begin shedding virus after about a week, although the timing depends on vaccination status (Atkins et al. 2011). Once birds become infectious, they continue shedding until death (Baigent and Davison 2004; Islam and Walkden-Brown 2007). Birds do not recover from MDV infection. Vaccination reduces death rates, clinical signs, and can reduce shedding (Islam et al. 2001; Baigent et al. 2006; Islam et al. 2008).

MODEL OF VIRAL SHEDDING

Within a cohort, a bird can be removed from the population by natural mortality (any cause of death other than MDV-related), by MDV-related death, and by final removal (along with the other birds at the end the cohort duration on day T_c). The life span and viral shedding of an individual depend on the infecting virus strain virulence, v, and the vaccine status, j, of an individual. Fully productive MDV infection only occurs once infection has spread to the feather follicle epithelium whence virus is shed into the environment in association with feather dander. This is incorporated into the model by assuming a possible delay from infection until limited shedding, then another delay until full shedding. Therefore, it is assumed that once infected, a bird undergoes an exposed phase for a period of T_{s1} days, after which the bird shed virus at a rate $a_1(v, j)$ (viral copy number/mg dust) from day $T_{s1} + 1$ postinfection until day $T_s + T_{s2}$ and then at rate $a_2(v, j)$ (viral copy number/mg dust) from day $T_s + T_{s2} + 1$ until the end of the cohort duration at T_c, unless it dies and is removed from the population.

MODEL OF HOST LIFE SPAN

The probability that a bird survived until t days in the cohort is the probability of not dying from either MDV or background mortality until that time. The daily background mortality probability per bird is denoted by μ. The probability of a bird life span being t days (with death due to MDV) is $f(t|v, j)$, given infecting virus virulence, v and vaccination status j (Atkins et al. 2011). Therefore, the probability of dying from MDV on or before time t is $P(T \leq t) = F(t|v, j) = \sum_{T=1}^{t} f(T, v, j)$. The probability of survival until t days within the cohort is therefore $L(t|v, j) = (1 - \mu)^{t-1}(1 - F(t - 1|v, j))$.

MODEL OF DANDER SHEDDING

The amount of dander shed daily by a broiler bird of age t days is calculated to be $d(t, T_c) = 368 \exp(-P_{T_c}/t^{1.64}) + 10.8$ (mg) (Supporting Information), where P is a function of the cohort duration, T_c. The parameter P_{T_c} changes how quickly the dander levels asymptote. The amount of dander is assumed to directly relate to the size of the bird. The value of P_{T_c} changes to reflect the speed that a bird grows. A bird selected to be raised in a short cohort duration grows faster than one selected to be raised in a longer cohort duration. Therefore, when a different cohort duration is used, P_{T_c} was re-estimated (Supporting Information).

PARAMETERIZATION OF MDV MODEL

Previously, we found that MDV strains with a greater virulence score have a higher viral shedding rate, and kill their hosts quicker (Atkins et al. 2011). Thus, MDV may be an example of a virulence-transmission trade-off. Our previous work estimated primary and secondary latent (uninfectious) periods, and primary and secondary virus shedding rates as a function of both virulence score and bird vaccination status (Table 1). To complete the parameterization of the fitness measures in this study, we have also estimated the dander shed for a broiler bird, and the transmissibility of the virus (Table S1).

Infectiousness potential, W

The maximum amount of an MDV strain that can be shed by a single bird over its lifetime is defined as its infectiousness potential, W. This metric is similar to what other authors have calculated and called "lifetime transmission success" (e.g., Jensen et al. 2006).

Combining results above, the average quantity of virus produced in a bird's lifetime (given infection at $t = 0$, infecting strain virulence v and vaccine status j) is

$$\begin{aligned} W(T_c, v, j) = {} & a_1(v, j) \\ & \sum_{t=T_{s1}+1}^{T_{s1}+T_{s2}} (1 - \mu)^{t-1}(1 - F(t - 1|v, j))d(t, T_c) \\ & + a_2(v, j) \\ & \sum_{t=T_{s1}+T_{s2}+1}^{T_c} (1 - \mu)^{t-1}(1 - F(t - 1|v, j))d(t, T_c). \end{aligned} \quad (1)$$

Reproductive number, R_0

The calculation of W, the maximal expected amount of virus produced by a single bird over the course of its life, does not take into

Table 1. Parameter values used in the fitness calculations. Personal communications refer to Dr. Nick Sparks, Scottish Agricultural College (NS), and Prof. Stephen Walkden-Brown, University of New England (SWB).

Parameter	Symbol	Value(s)	Reference
Primary viral shedding rate	$a_1(v, \text{sham})$	$8.65 \times 10^4 - 1.19 \times 10^5 v_T$	Supporting Information
(VCN/mg dust)	$a_1(v, \text{hvt})$	$-5.4 \times 10^3 + 3.54 \times 10^4 v_T$	Supporting Information
	$a_1(v, \text{biv})$	$1.77 \times 10^2 + 1.21 \times 10^4 v_T$	Supporting Information
Secondary viral shedding rate	$a_2(v, \text{sham})$	$-2.39 \times 10^7 + 8.45 \times 10^7 v_T$	Supporting Information
(VCN/mg dust)	$a_2(v, \text{hvt})$	$-2.39 \times 10^7 + 7.42 \times 10^7 v_T$	Supporting Information
	$a_2(v, \text{biv})$	$-2.39 \times 10^7 + 5.39 \times 10^7 v_T$	Atkins et al. (2011)
Dust produced per chicken	$d(t, T_c)$	$368 \exp(-P_{T_c}/t^{1.64}) + 10.8$	Supporting Information
(age t days, mg)			
Total Mortality per flock (%)	D_{total}	3.6-6.8	Sheppard (2004)
Maximum dust concentration	E	7.15	Takai et al. (1998)
(mg/m^3)			
Mortality probability on day t	$F(v, t, j)$	$1 - \exp(-(t/\lambda(v, j))^r)$	In text
Height of barn (m)	h	2.5	SWB, pers.comm., 2008
Vaccination treatment	j	sham, hvt, biv	
Probability bird alive on day t	$L(t\|v, j)$	$(1-\mu)^{t-1}(1 - F(v, t-1, j))$	In text
Probability MDV infection	$p\left(\frac{M_e(t,T_c,v,j)}{V(S_0,s_d)}, j\right)$	$\alpha(j)\frac{M_e(t,T_c,v,j)}{V(S_0,s_d)}$	In text
(per bird per day)			
Growth scaling constant	P_{T_c}	$-T_c^{1.64} \ln\left(\frac{d(45,45)-10.8}{368}\right)$	Supporting Information
Weibull shape parameter	r	4.18	Atkins et al. (2011)
2008			
Cohort duration (days)	T_c	30, 50, 70, 90	Sheppard (2004)
Delay until viral shedding	T_{s1}	4.7	Supporting Information
(days)			
Delay until second viral	$T_{s2}(\text{sham})$	9.92	Supporting Information
shedding rate (days)	$T_{s2}(\text{hvt})$	9.92	Supporting Information
	$T_{s2}(\text{biv})$	$29.44 - 26.46 v_T$	Supporting Information
Virulence score (%)	v	0-100	Atkins et al. (2011)
Transformed virulence score	v_T	$\arcsin\sqrt{0.01v}$	In text
Barn volume (m^3)	$V(S_0, s_d)$	$S_0 wh/s_d$	In text
Final bird weight (kg)	w	2.5	Sheppard (2004)
Average total virus produced by host	W		
Daily transmission probability	$\alpha(\text{sham})$	8.97×10^{-9}	Supporting Information
(per VCN/m^3)			
	$\alpha(\text{hvt})$	1.47×10^{-9}	Supporting Information
Fraction dust left in barn on day t	$\gamma(t, T_c, s_d)$	see Methods	In text
Weibull scale parameter	$\lambda(v, \text{sham})$	$4.54 - 0.53 v_T$	Atkins et al. (2011)
	$\lambda(v, \text{hvt})$	$4.89 - 0.53 v_T$	Atkins et al. (2011)
	$\lambda(v, \text{biv})$	$4.98 - 0.53 v_T$	Atkins et al. (2011)
Non-MDV daily death probability	μ	$1 - T_c 1 - D_{total}$	In text

account the transmission potential of that virus. There are several reasons why infectiousness potential of a strain might not be sufficient to calculate fitness: first, dander and dust get removed from the poultry barns; second, infected dust titers are based on PCR, which counts genomes, not necessarily live viable virus; lastly, infectious processes after inhalation might be density dependent. In this section, we deal with these complications by incorporating transmission parameters estimated from experimental data. Using these transmission parameters, R_0 can be calculated for each strain under a different set of environmental conditions. R_0 is the number of individuals that are directly infected by a single infected bird in an otherwise susceptible population, where transmission of virus between birds is indirect through the dust.

REDUCTION IN TRANSMISSIBLE VIRUS

In floor-reared broiler barns, there is an equilibrium of aerial contaminants with the continued production and removal of dust and airborne material (Wathes 1994, 1998). There have been

implementations of Optional Exposure Limits (OELs) in North America and Europe to reduce dust pollutants to a reasonable level within broiler barns (Donham et al. 2000). We assume the dust concentration stays constant once this limit had been reached. The new dust (and virus) is assumed to be produced at the start of each day, was thoroughly mixed with the old dust and a proportion removed to regain the equilibrium level of dust.

If the limit for the density of dust in the barn was set to E (mg/m^3), the fraction of remaining dust at each time point t is therefore

$$\gamma(t, T_c, s_d) = \min\left[\frac{EV(S_0, s_d)}{\min[\sum_{s=1}^{t-1} S_0 d(s, T_c), EV(S_0, s_d)] + S_0 d(t, T_c)}, 1\right]. \quad (2)$$

where the volume, V, of the barn can be calculated by the initial number of birds, S_0, and the stocking density, s_d (kg/m^2) given the finishing weight, w (kg), is fixed for all calculations. Studies around Northern Europe have revealed the density of inhalable dust in broiler buildings (sampled at around twenty eight days into the cohort duration) (Takai et al. 1998). The mean inhalable dust ranged from 3.8–10.4mg/m^3. The mean value of 7.25 mg/m^3 is used for the concentration limit of dust in the atmosphere, E (and varied in the Supporting Information).

Therefore, we can calculate the effective amount of virus produced by a single bird, $M_e(t, T_c, v, j)$ (VCN) still remaining in the atmosphere by time t. If we suppose that $m(t, T_c, v, j)$ virus is released into the atmosphere by a single bird at time t, we assume this amount will be thoroughly mixed with the virus already in the dust atmosphere at day $t-1$. On day t a fraction, γ, will remain once the dust has been removed, so the effective amount of virus at time t is

$$M_e(t, T_c, v, j) = \gamma(t, T_c, s_d)[M_e(t-1, T_c, v, j) + m(t, T_c, v, j)]. \quad (3)$$

CALCULATION OF REPRODUCTIVE NUMBER

Most calculations of R_0 are derived from assumed infinite (and continuous) populations occurring over continuous time and described using differential equations. However, MDV infection in broiler farms requires an individual-based approach with R_0 calculated in a more heuristic way. Indeed Keeling and Grenfell (2000) describe the formulation of R_0 in such a manner and give a method for the calculation under the individual-based setting. We formulate an MDV-specific R_0 based on Keeling and Grenfell (2000) by summing the secondary infections over the total infectious time period of the index case. The number of secondary infections per day is the product of the probability the index case is alive, the probability of infection per susceptible individual, and the number of susceptible individuals. Therefore

$$R_0(T_c, v, j, s_d) = \sum_{t=T_s+1}^{T_c} S(t) p\left(\frac{M_e(t, T_c, v, j)}{V(S_0, s_d)}, j\right) L(t|v, j), \quad (4)$$

where p is the daily probability of transmission to a single uninfected bird. The transmission probability for a viral concentration in the air (M_e/V) is calculated from transmission experiments (Supporting Information). The probability that the index case is alive (L) is calculated from survival analysis results assuming a Weibull distributed life span (Atkins et al. 2011). We assume that the number of susceptibles stays approximately constant throughout the cohort duration, $S(t) = S_0$ (i.e., the number of infecteds remains small compared to the cohort size). Although some authors use R_e to denote the reproductive value in a partially immune population, we denote the reproductive number as R_0 regardless of population vaccination status for notational clarity. R_0 does not change with the number of individuals in the barn (Supporting Information).

Results

INFECTIOUSNESS POTENTIAL, *W*

We calculated the virulence score at which the maximum W was reached, for different cohort durations and vaccine treatments (Fig. 1a). Decreasing the cohort duration and introducing vaccination (or moving from HVT to bivalent vaccine) both increased the virulence score, which yielded the greatest value of W. These results suggest that both factors could have selected for increased virulence of MDV isolates. We also calculated the reduction in cohort duration, which gave the same increase in optimal virulence score as the introduction of HVT or bivalent vaccination (Fig. 1b). For example, introducing HVT vaccination into a cohort of unvaccinated birds with a cohort duration of 50 days had the same effect on optimal virulence score as reducing the cohort duration to 40 days. The background mortality had a negligible effect on the fittest virulence score (results not shown).

In the above analysis, we assumed cohort duration had an effect on both the maximum potential life span of the bird and the rate at which the bird sheds dust into the environment, because we assumed shorter lived birds would grow faster and so shed propagules at a higher rate earlier. To disentangle these two possible effects of changing cohort duration, we removed the latter effect by assuming that the function calculating the daily dust produced by a bird does not change with the cohort duration (eq. S1). Therefore, every bird would maintain the same growth regardless of the cohort for which it was raised. With this assumption, changing the cohort duration only alters the maximum potential life span of each bird. We found that the qualitative results were the same: reducing cohort duration and introducing vaccination (or moving from HVT to bivalent vaccine) both increased the

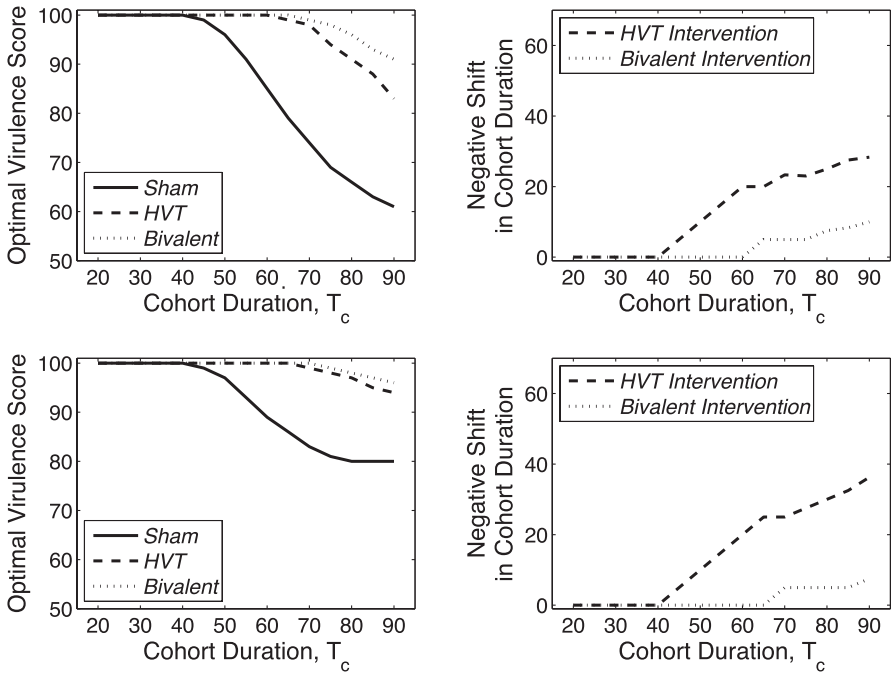

Figure 1. Infectiousness potential (*W*) calculations. Background (non-MDV) mortality rate was set at 0.0005 per bird per day. (a) The optimal virulence score for a given cohort duration and host vaccine treatment. Decreasing cohort duration and increasing vaccine potency increased the virulence score which yields the highest value of *W*. (b) The effect of introducing a new vaccine on the increase in virulence score measured in terms of the reduction in cohort duration to provide an equivalent increase in virulence score. A shift from no vaccine to HVT vaccine (dashed) or from HVT vaccine to bivalent vaccine (dotted) at a given cohort duration (*x*-axis) increased the optimal virulence score by the same as a reduction in cohort duration (*y*-axis), see main text for more information. (c) Same figure as in (a) but where dust shed function is not changed with cohort duration. (d) Same figure as in (b) but where dust shed function is not changed with cohort duration.

virulence score, which yielded the greatest value of W. However, the optimal virulence score for all cohort durations and vaccination scenarios increased compared to previous results (Fig. 1c and d compared to Fig. 1a and b).

REPRODUCTIVE NUMBER, R_0

We calculated the virulence score at which the maximum R_0 was reached, for different cohort durations and vaccine treatments (Fig. 2a and b). Decreasing the cohort duration and introducing HVT vaccination both increased the virulence score, which yielded the greatest value of R_0 (Fig. 2c). These results suggest that both factors could have selected for increased virulence in MDV isolates. We also calculated the reduction in cohort duration, which gave the same increase in optimal virulence score as the introduction of HVT vaccination (Fig. 2d). Introducing HVT vaccination had the same effect on the optimal virulence as reducing the cohort duration by between 15 and 65 days depending on the cohort duration into which vaccination was introduced. For example, introducing HVT vaccination into a cohort of unvaccinated birds with a cohort duration of 50 days had the same effect on optimal virulence score as reducing the cohort duration to 26 days. An equivalent shift in optimal virulence when HVT vaccination was introduced at cohort durations shorter than 35 days could not be gained from a reduction in cohort duration alone.

Decreasing the maximum dust concentration permitted in the barn decreased the fitness score of all virulence scores, but it did not alter the optimal virulence score (Supporting Information). The background mortality and stocking density both had a negligible effect on the optimal virulence score (results not shown).

Again, similar to the results for W, we decoupled the two effects of changing cohort duration by assuming that the function calculating the daily dust produced by a bird does not change with the cohort duration. Therefore, every bird would maintain the same growth regardless of the cohort for which it was raised. We found that the results were the same as calculated previously (results not shown).

Discussion

We calculated two evolutionary fitness measures for MDV strains: the infectiousness potential (W) and the reproductive number (R_0). We used both these measures to quantify the optimal virulence to which MDV should evolve. We analyzed whether vaccination and reduced host bird life span could have been

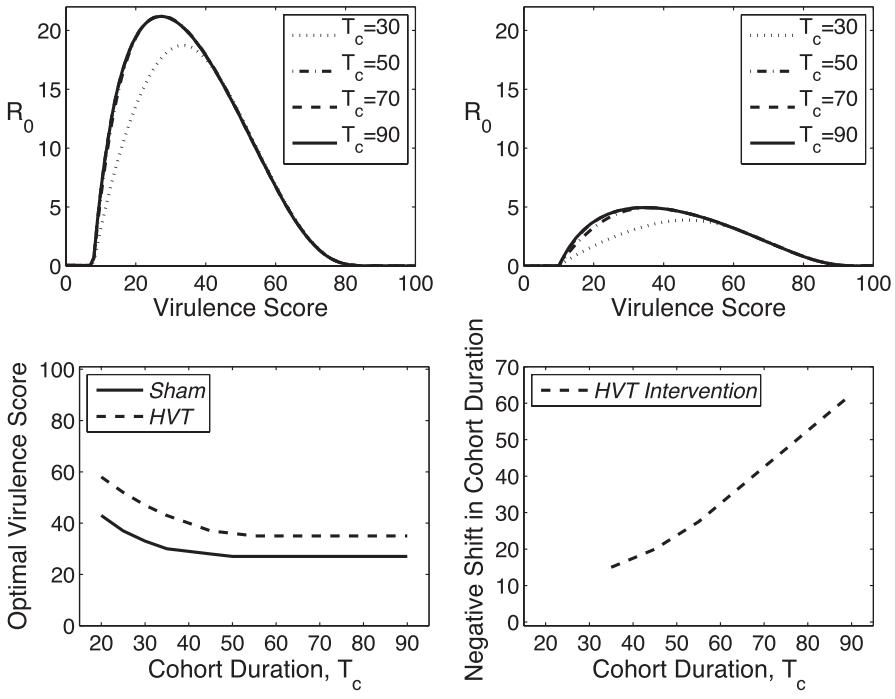

Figure 2. Reproductive number, R_0, calculations. Background (non-MDV) mortality rate was set at 0.0005 per bird per day and stocking density was set at 35 kg/m^2. (a) The reproductive number of an unvaccinated host infected with MDV with a given virulence score (*x*-axis) growing in a cohort of different durations. (b) The reproductive number of an HVT-vaccinated host. (c) The optimal virulence score for a given cohort duration and host vaccine treatment. Decreasing cohort duration and increasing vaccine potency increased the virulence score, which yielded the highest value of R_0. (d) The relative effect of cohort duration and the introduction of new vaccines on the optimal virulence score. A shift from no vaccine to HVT vaccine at a given cohort duration (*x*-axis) increased the optimal virulence score by the same as a reduction in cohort duration (*y*-axis).

responsible for the increase in the virulence of MDV isolates since the 1950s. So far as we are aware, this study is the first quantitative analysis testing these hypotheses.

The reproductive number, R_0, is arguably a better measure of fitness than the infectiousness potential because it includes transmissibility between individual hosts. Nonetheless, we included W as a fitness measure for three reasons: first, because this is also a commonly used metric for fitness in experimental evolution; second, because no transmissibility data for bivalent-vaccinated hosts were available so R_0 could not be calculated in this case; and third, because the different predictions, which flow from using W or R_0, highlight the importance of constructing host–pathogen specific fitness measures if one wants to quantitatively evaluate the drivers of selection.

Our previous work reports a trade-off association between virulence and transmission for MDV (Atkins et al. 2011). The study we report here shows that MDV evolution does indeed accord with results from trade-off models, with fitness maximized at intermediate levels of virulence. We have examined the effects of this genetic virulence-transmission trade-off by exploring the two phenotypic associations: vaccination lengthening host life span and reducing viral shedding, and reduced cohort duration increasing bird growth rate and limiting host life span duration. Introducing vaccination (or increasing the potency of the vaccine) and reducing the cohort duration of hosts both led to a rise in the optimal virulence. Therefore, assuming that strains would have evolved to maximize their fitness, both vaccination and a reduction in bird cohort duration could have led to more virulent MDV strains. Reducing the life span of a broiler reduces the duration of viral shedding but also increases the rate at which virus is shed (because the birds are grown at a faster rate and therefore produce more dander). Conversely, vaccination increases the duration of viral shedding but decreases the rate at which virus is produced (as well as reducing the overall infection rate). These mechanisms of host life span and viral shedding rate (and transmission rate in the case of vaccination) act dynamically to dictate the virulence at which fitness is maximized. We have focussed on broilers because this is the sector that has undergone the most change and expansion.

Our previous work estimated infectious periods of birds infected with MDV as a function of both virulence score and vaccination status. Available data limited the statistical analysis to a small range of discrete virulence scores ($v = 16.5$, 36, 46). Therefore, interpolation and extrapolation have been used to incorporate those results over the full virulence spectrum (v 0,100). Therefore, we highlight this as a possible caveat for the W results,

where the optimum virulence falls outside the range investigated experimentally. In addition, we have tried to include the important aspects of a broiler barn in our fitness calculations, not least threshold limits for dust levels and using data-derived epidemiological parameters.

Most mathematical models idealize populations by assuming all individuals are both genetically and phenotypically identical, and that transmission occurs homogeneously throughout the entire population. Interestingly, although we assume these same principles in our model, we would argue that an airborne and highly transmissible virus infecting a genetically homogeneous host population in a dust-ridden broiler barn would be an example where these mathematical model assumptions are nearly consistent with reality. While a couple of other studies have developed pathogen-specific fitness functions (Fraser et al. 2007; de Roode et al. 2008), so far as we know our study is the first to develop such a framework for vaccinated hosts. This allows us to quantitively assess the likelihood that vaccination may be a driver for virulence evolution.

The broiler industry has been able to reduce the cohort duration of broilers by increasing the growth rate of birds. We have accounted for this increased growth rate as a function of cohort duration in our analysis. However, this plausible assumption allows cohort duration to dictate both the maximum life span of the birds and their growth rate. We have therefore disentangled these two effects of reducing cohort duration on the virulence selection by allowing the growth rate to remain unchanged regardless of cohort duration. Under these conditions, the fitness measure, W, predicts a higher optimal virulence score than if growth rate was accounted for. Experimental conditions equate to a 45-day maturation period for birds. Therefore, for cohort durations of longer than 45 days, maintaining a fixed growth rate corresponds to birds shedding dust (and virus) at a greater rate than if the growth rate were altered with cohort duration. Now, for cohort durations longer than 45 days, a bird is assumed to shed disproportionately much more dust in the first half of its life span than in the second half, as it grows much more quickly to its finishing weight. Therefore, the optimal fitness is achieved by increasing virulence to maximize the increased virus shedding at the start of the bird's life span. This result is in keeping with evolutionary theory stating that increasing the lag time between the start of pathogen transmission and the start of host mortality will select for higher pathogen virulence (Day 2003). When the same procedure is applied to the R_0 results, we found that optimal virulence score is not changed. This result stems from the fact that we have included explicit transmission and dust dynamics in to the R_0 model. Even though birds in cohorts longer than 45 days shed more dust daily, this dust is now removed by the OELs. Thus, allowing birds to shed more dust does not have any impact of their daily transmissibility.

MDV vaccines are examples of imperfect vaccines that reduce clinical signs of disease but do not completely block onward transmission (Supporting Information, Atkins et al. 2011). Current evolutionary theory suggests such vaccines may cause evolution of increased virulence (Gandon et al. 2001, 2003). Our finding is consistent with this hypothesis. Improved genetics and feeding techniques have allowed the broiler industry to halve the life span of broiler birds from 70 to 35 days while growing to the same finishing weight. Current evolutionary theory has shown that a shorter host life span (increased host mortality rate) can lead to increased virulence evolving in the pathogen population (Anderson and May 1982b; Sasaki and Iwasa 1991; Day 2002). Our finding is also consistent with this theory.

We examined the relative impact of vaccination compared with cohort duration reduction. Using the results from maximizing R_0, introducing HVT vaccination into a cohort of 75 days led to the same increase in virulence as a reduction in cohort duration of 45 days. Over the course of 30 years (1960–1990), in the United States, two vaccines were introduced (HVT then bivalent) and cohort duration was reduced by about 30 days (75 days to about 45 days). This suggests that the strength of selection by both vaccination and cohort duration reduction are of the same magnitude. However, again looking at the results from maximizing R_0, reducing cohort duration to above 50 days did not have an effect on reducing the optimal virulence, suggesting that vaccination may have been the predominant driver of MDV virulence evolution in the first instance.

Nevertheless, MDV vaccines were initially developed to combat the increased bird morbidity and mortality caused by MDV at the time. If this initial rise in disease burden, or incidence, was to do with virus evolution, it may not have been due to vaccination. However, it could have been initiated by a reduction in broiler cohort duration. One alternative explanation for this initial rise in losses is that the industrialization of poultry housing may have led to larger on-farm bird population densities, which may have selected for higher virulence (Day and Proulx 2004).

There have been numerous examples of pathogen virulence evolution. The most extensively studied case of virulence evolution is myxoma (Fenner and Fantini 1999). When successfully introduced into the rabbit population in Australia in 1950–1951, the myxoma virus caused exceedingly high fatality, with nearly all animals dying, and most before two week after infection (Fenner et al. 1956). By 1964 the case fatality in naïve rabbits had dropped to around 80% and rabbits were surviving longer with the disease, around four weeks (Fenner and Fantini 1999). Evidence from both empirical and modeling studies suggests that during this time after myxoma introduction, the virus evolved to its evolutionary stable strategy such that it killed its host at a rate to maximize its between-host fitness (Anderson and May 1982a; Dwyer et al. 1990; Fenner and Fantini 1999). However, the

trajectory of virulence evolution may be complex and have multiple selection drivers (Sabelis and Metz 2002). Relevant to this study, there have been virulence evolution reported as a result of vaccination (Gandon and Day 2008; Read and Mackinnon 2008). For instance, pathogens may be evolving in response to widespread vaccination when there is evidence that vaccine-escape genotype types are found more often in vaccinated hosts, and these mutants are also increasing in vaccinated populations. While these escape types have the potential to pose a risk for virulence management, there are often additionally associated with a change in virulence from the wild type. Widespread vaccination against hepatitis B, pertussis, pneumococcal disease, infectious bursal disease have led to a rise in virulence of the vaccine-escape types. Widespread vaccination against diphtheria may have led to a decrease in virulence of the circulating bacteria, as the vaccine essentially selected for non-toxin producing strains.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Table S1. Transmission to unvaccinated birds: Maximum likelihood estimates for quantities from the hypergeometric distribution.

Table S2. Transmission to HVT-vaccinated birds: Maximum likelihood esti- mates for quantities from the hypergeometric distribution.

Figure S1. Dust shedding: The amount of dust shed over time by a broiler chicken (black line) and the fitted function, $d(t)$ (red line).

Figure S2. Transmission: Five-day probabilities for infection for different atmospheric virus concentrations (measured in VCN per m^3).