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Quantification of heterosubtypic immunity between avian influenza subtypes H3N8 and H4N6 in multiple avian host species

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Low-pathogenicity avian influenza virus (LPAIV) can lead to epizootics that cause economic losses in poultry or the emergence of human-infectious strains. LPAIVs experience a complex immunity landscape as they are endemic in numerous host species, and many antigenically distinct strains co-circulate. Prevention and control of emergence of detrimental strains requires an understanding of infection/transmission characteristics of the various subtypes in different hosts, including interactions between subtypes. In order to develop analytical frameworks for examining control efficacy, quantification of heterosubtypic immunity interactions is fundamental. However, these data are scarce, especially for wild avian subtypes in natural hosts. Consequently, in this study, three host species (mallards, quail and pheasants) were infected with two LPAIV subtypes isolated from wild birds: H3N8 and H4N6. The recovered hosts were also reinfected with the alternate subtype to measure the effects of heterosubtypic immunity. Oropharyngeal and cloacal swabs were collected and viral RNA load was quantified by real-time RT-PCR. For secondary infections in recovered hosts, peak viral load was up to four orders of magnitude lower and shedding length was up to 4 days shorter. However, both the magnitude and presence of heterosubtypic immunity varied across specific host species/subtype combinations. Using a mathematical model of virus replication, the variation in virus replication dynamics due to host individuals was quantified. It was found that accounting for individual heterogeneity is important for drawing accurate conclusions about treatment effects. These results are relevant for developing epidemiological models to inform control practices and for analysing virus replication data.

INTRODUCTION

Wild birds are the main source of novel strains of avian influenza viruses that emerge and circulate in domestic poultry and livestock (Webster *et al.*, 1992). In the USA, haemagglutinin (H) subtypes 1–14, as well as H16, (Krauss *et al.*, 2007; Nolting *et al.*, 2012; VanDalen *et al.*, 2008; Webster *et al.*, 2007) can be found in wild birds, with H3 and H4 subtypes being the most prevalent (Krauss *et al.*, 2004; Piaggio *et al.*, 2012; Webster *et al.*, 2007). Both H3 and H4 (among other subtypes) have been shown to emerge (Campitelli *et al.*, 2002) and co-circulate in domestic poultry (Panigrahy *et al.*, 2002; Pepin *et al.*, 2012), causing significant losses to poultry operations (Capua & Alexander, 2004; Lupiani & Reddy, 2009; Saif *et al.*, 2008). A recent study of low-pathogenicity avian influenza viruses (LPAIVs) in Chinese live-bird markets highlighted that H3 and H4 subtypes tend to co-circulate and are found consistently in numerous host species with an overabundance in domestic ducks (Pepin *et al.*, 2012). This phenomenon of antigenically distinct subtypes co-circulating in a number of different host species creates a complex immunity landscape and high levels of individual variation in transmission probability. Consequently, it is difficult to interpret and predict incidence patterns. Understanding interactions between co-circulating subtypes is fundamental to developing quantitative tools that can be used for identifying appropriate methods of prevention and control, as well as for anticipating epizootics that could increase spillover risk to domestic poultry.

One primary way in which subtypes can interact is indirectly through immunity. Studies that have examined the effects of heterosubtypic immunity in avian influenza viruses have revealed that prior infection tends to offer some degree of protection against other subtypes (Berhane *et al.*, 2010; Costa *et al.*, 2010a; Fereidouni *et al.*, 2009; Imai *et al.*, 2007; Jourdain *et al.*, 2010). However, our understanding of

Two supplementary figures and two tables are available with the online version of this paper.

heterosubtypic immunity on epidemiological outcomes remains very limited, as studies that have quantified the magnitude of cross-protection are few and experimental designs/analyses were variable. Furthermore, previous studies that measured heterosubtypic immunity were conducted in a single host species (mostly mallards), making it difficult to understand cross-protective effects across multiple hosts. Also, most focused on cross-protection of LPAIVs against high-pathogenicity strains, which may be expected to differ from the effects of LPAIVs on each other.

Here, we have provided quantitative estimates of the degree to which heterosubtypic immunity between LPAIVs could impact on viral infections in various host species. We also used a model of virus replication dynamics to understand the importance of variation among host individuals on virus replication in secondary infections. Our analyses provide much-needed tangible data for epidemiological models of LPAIV dynamics.

RESULTS

Primary infections differ by virus subtype and host species

Viral infections were characterized as total viral load, peak load and detection period [DP; the number of days when viral RNA concentrations $>10^1$ 50% egg infective dose (EID_{50}) equivalents per swab were detected]. Only data from swabs with the highest RNA loads were included in this analysis (cloacal for ducks and oropharyngeal for pheasants and quail). Based on a two-factor analysis of variance (ANOVA), there was a significant interaction of subtype and host species for total viral load and DP in primary infections, and an effect of subtype on peak load in all host species (Table S1, available in JGV Online). These results indicated that the relative difference between subtypes in total viral loads and DPs varied with host species, but one subtype (H4) had a significantly higher peak load than the other (H3) in all three host species. Mean peak loads of H4 in all three host species ranged from $10^{4.0}$ to $10^{4.2}$ EID_{50} equivalents per swab (medians $10^{4.0}$–$10^{4.3}$), and mean and median DPs were between 7 and 8 days (Fig. 1). However, on average, H3 replicated to significantly lower peak titres ($10^{3.3}$ EID_{50} equivalents per swab, median $10^{3.6}$) for shorter periods (4.9 days, median 5.0 days) in pheasants relative to the other two host species (mean $10^{3.7}$ EID_{50} equivalents per swab, median 6–7 days). Thus, there was a significant difference in infection parameters between H3 and H4 in pheasants but not in ducks or quail. Data from the alternate swab types (oropharyngeal for ducks, cloacal for pheasants and quail) are presented in Fig. S1.

Magnitude of heterosubtypic immunity between virus subtypes varies by host species and subtype

In order to examine the magnitude of heterosubtypic immunity, infection data from secondary infections were subtracted from the mean values in primary infections. The effects of virus subtype and host species on the magnitude of heterosubtypic immunity were examined using separate one-way ANOVAs (see Methods for specific tests). Heterosubtypic immunity for a given experiment was assessed within the ANOVA framework by examining whether the 95 % confidence intervals of the means of the difference data overlapped zero. Due to financial limitations, the experimental design was unbalanced and thus not all reciprocal heterosubtypic immunity experiments were performed (see Table 1). In quail, where both types of cross-secondary infections were carried out, the magnitude of heterosubtypic

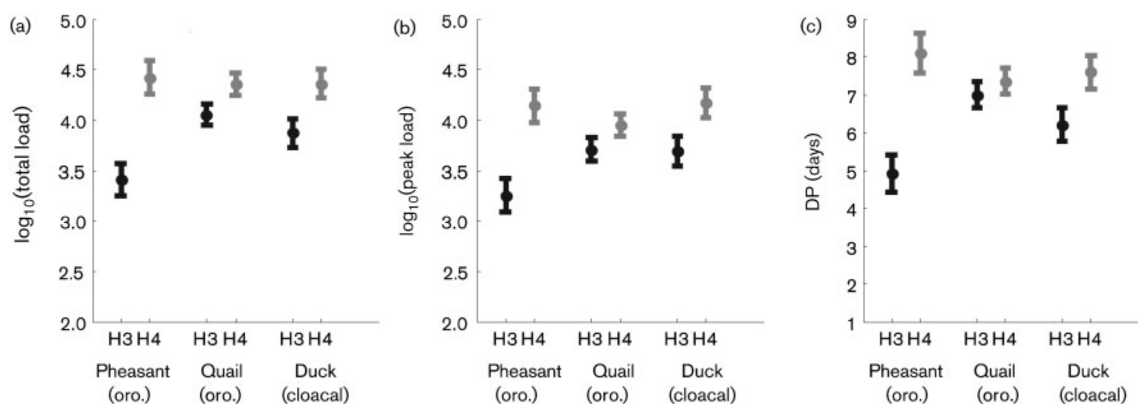

Fig. 1. Viral infection parameters for primary infections in different subtypes and host species. Data are means ± SEM. (a) Total viral load (sum of daily titres). (b) Peak load (maximum viral titre). Viral loads in (a) and (b) are on a log scale. (c) DP [time span that viral loads were above 10^1 EID_{50} equivalents ml^{-1} (last day − first day)]. Viral subtypes, host species and sample types are indicated along the *x*-axes. Sample sizes for experiments by host are shown in Table 1. Only data from the sample types with the highest viral loads (oropharyngeal for quail and pheasants, and cloacal for ducks) are presented. See Fig. S1 for data from the other samples. Multiple comparisons were conducted within the ANOVA framework presented in Table S1. oro., Oropharyngeal.

Table 1. Experimental design and sample sizes

Subtype infection*	Pheasant		Quail		Duck	
	F	M	F	M	F	M
Primary: H3N8	12	0	15	9	5	10
Secondary: H4N6	0	0	7	7	5	10
Primary: H4N6	11	0	9	16	5	10
Secondary: H3N8	12	0	7	8	0	0

*Secondary infection was carried out 20 days after the primary infection.

immunity by H4 on H3 secondary infection was significantly stronger than the opposite (Fig. 2, Table S2). For H3-infected quail that were infected previously by H4, total viral load was three orders of magnitude lower and the detection period was 4.3 days shorter, on average, compared with primary H3 infections. However, primary infection with H3 provided only minimal protection against H4 (H4 total loads were only one order of magnitude lower than H4 in primary infections and there was no difference in DP).

Interestingly, heterosubtypic immunity was significantly different (Table S2) and, in fact, reversed in the other host species (Fig. 2). Secondary infections with H4 had much lower viral loads (by four orders of magnitude) and much shorter infections (by 4 days) in ducks that had been infected previously with H3 (Fig. 2). Secondary infections with H3 in pheasants that had been infected previously with H4 were not significantly different from primary infections of H3. Thus, H4 provided strong heterosubtypic immunity against H3 in quail but not pheasants, and H3 provided strong heterosubtypic immunity against H4 in ducks but not quail. When heterosubtypic immunity occurred, peak loads were up to four orders of magnitude lower and infections were up to 4 days shorter but, even under the largest reductions due to heterosubtypic immunity, peak loads were still detectable ($10^{2.5}$) and infections lasted 3.3 days on average.

Variation from individual ducks is highly significant and important for determining statistical significance of virus replication rates

Although infection history explained a significant amount of variation in the data, much of the variation was left unexplained (see R^2 in Tables S1 and S2). Thus, we modelled virus replication dynamics to identify sources of variation in viral population growth parameters while accounting for the effects of heterosubtypic immunity. Models included a replication rate parameter (r) and an asymptotic viral load parameter (a, equivalent to total viral load), as well as two parameters for testing the difference of r and a between treatments (i.e. parameters R and A; see Methods). Sources of variation included individual bird and sex, and were included in the model as 'random effects' (i.e. as error correlation, the idea being that data points from a single individual or sex are correlated and thus have similar error magnitudes relative to the model predictions). The following three models with the four parameters were constructed: no random effects, random effects of individual bird and random effects of sex. Only duck and quail data were used in these analyses as they produced adequate viral loads in both types of sample (oropharyngeal and cloacal swabs) and because the pheasant experiments did not include males.

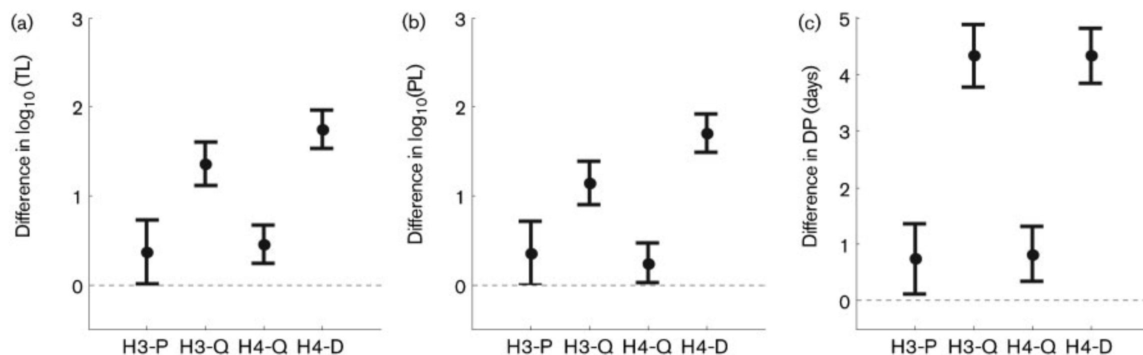

Fig. 2. Effects of heterosubtypic immunity on viral infection parameters. Data are means ± SEM (note that we have not presented 95 % confidence intervals, as quail data were used in two different tests) of the difference between mean performance in primary infections and performance in secondary infections (where hosts had recovered from an infection by the alternate subtype). Values above zero indicate the magnitude of heterosubtypic immunity. For example, a value of 2 for H3 indicates that secondary infections with H3 in hosts that were previously infected with H4 resulted in two orders of magnitude lower total viral load relative to primary infections of H3. Infection parameters (a–c) are as in Fig. 1. TL, Total load; PL, peak load. Host species are indicated on the *x*-axes: P, pheasants; Q, quail; D, ducks. Sample sizes for treatments by host are shown in Table 1. Only data from the sample types with the highest viral loads (oropharyngeal for quail and pheasants, cloacal for ducks) are presented. See Fig. S1 for data from the other samples. Multiple comparisons were conducted within the one-way ANOVA frameworks presented in Table S2.

The structure of the model provided a very good description of the data (R^2>0.96 for all models). Note that variation explained by random effects is not a component of R^2, rather random effects explain variation in the processes (i.e. *r* and *a*) being modelled. The best model included a random effect of host individuals, which accounted for 98 % of variation in viral-infection parameters in both host species (57 % for *a* and 41 % for *r* in ducks; 38 % for *a* and 60 % for *r* in quail) (Table 2). The magnitude of variation in total load (*a*) due to individual effects was up to 0.9 $\log_{10}$ (2 SD) in quail oropharyngeal data and 1.4 $\log_{10}$ in duck cloacal data (Table 2). In both datasets, the magnitude of variation in replication rate (*r*) was ~1.2 SD. Secondary infection of H4 in ducks recovered from H3 infection showed slower replication rates in cloacal but not oropharyngeal samples (Fig. 3). However, when variation from duck individuals was excluded from the model, replication rates of H4 in secondary infections in ducks were slower in both oropharyngeal and cloacal samples (results not shown). This highlights the importance of accounting for individual variation when assessing the statistical significance of treatment effects. Fig. S2 gives an example of the magnitude of variation between individuals and shows that the variation was widespread rather than being from only a few individuals.

Host sex causes variation in viral dynamics in cloacal samples but not oropharyngeal samples in ducks

The model with sex as a random effect performed better than the model with no random effects only in duck cloacal data (see ΔAIC and ΔBIC in Table 2), where sex explained up to 29 % of the variation in replication rates and total loads (12 % for *r* and 17 % for *a*). In duck cloacal samples, females tended to have lower total viral loads than males (Fig. S2). However, in the oropharyngeal duck samples and both sample types in quail, sex did not explain any variation.

Table 2. Random effects on parameters *a* and *r* in the non-linear growth model

Statistics for model results from Fig. 2 are in bold. ΔAkaike Information Criterion (ΔAIC) and ΔBayesian Information Criterion (ΔBIC) for models with individual duck or sex are relative to a model with no random effects. Note that higher ΔAIC (ΔBIC) values indicate a better fit. Models for each sample type from quail and duck data are presented separately. Pheasant data were excluded, as they did not produce viral RNA consistently in cloacal samples and because only females were included. Experiment definitions: H4 vs H3→H4 compares data for H4 in primary infections versus secondary infections; H4 are data for H4 primary infections, H3→H4 are H4 data from secondary infections. The random effects of sex could not be disentangled from treatment effects in the H4 vs H3→H4 analyses; thus, these effects were also examined in primary and secondary infections separately.

Experiment	Random effects	Quail					Duck				
		ΔAIC*	ΔBIC*	SD†	% Var‡	Corr§	ΔAIC*	ΔBIC*	SD†	% Var‡	Corr§
Cloacal											
H4 vs H3→H4	Duck ID: *a*	**889**	**876**	**1.29**	**87**	**−0.42**	**1746**	**1733**	**0.70**	**57**	**0.53**
	Duck ID: *r*			**0.40**	**8**				**0.59**	**41**	
H4 vs H3→H4	Sex: *a*	−6	−19	0.00	0	−0.01	117	104	0.32	17	1.00
	Sex: *r*			0.00	0				0.27	12	
H4	Sex: *a*	13	3	0.44	19	−1.00	45	34	0.18	14	1.00
	Sex: *r*			0.04	0				0.16	11	
H3→H4	Sex: *a*	5	−5	0.18	3	1.00	66	55	0.46	23	1.00
	Sex: *r*			0.20	4				0.24	6	
Oral											
H4 in H3→H4	Duck ID: *a*	**701.7**	**690**	**0.46**	38	**0.08**	**1660**	**1647**	**0.54**	**36**	**0.62**
	Duck ID: *r*			**0.58**	**60**				**0.71**	**63**	
H4 in H3→H4	Sex: *a*	−0.44	−16.2	0.05	1	1.00	−1	−14	0.07	1	1.00
	Sex: *r*			0.03	0				0.09	2	
H4	Sex: *a*	0.42	−10.1	0.06	1	1.00	−6	−17	0.00	0	0.00
	Sex: *r*			0.53	74				0.00	0	
H3→H4	Sex: *a*	−6	−14.3	1.80E−05	0	0.00	3	−1	0.12	4	1.00
	Sex: *r*			5.70E−06	0				0.11	3	

*Difference in AIC or BIC from the same model but without random effects.
†SD estimate for random effects; 2 SD of fixed effect estimate *r* (or *a*) includes 95 % of *r* (or *a*) estimates for the population of random-effect units ($\log_{10}$ titre).
‡Percentage of all random variation on parameter estimates *a* and *r*.
§Correlation of random effects.

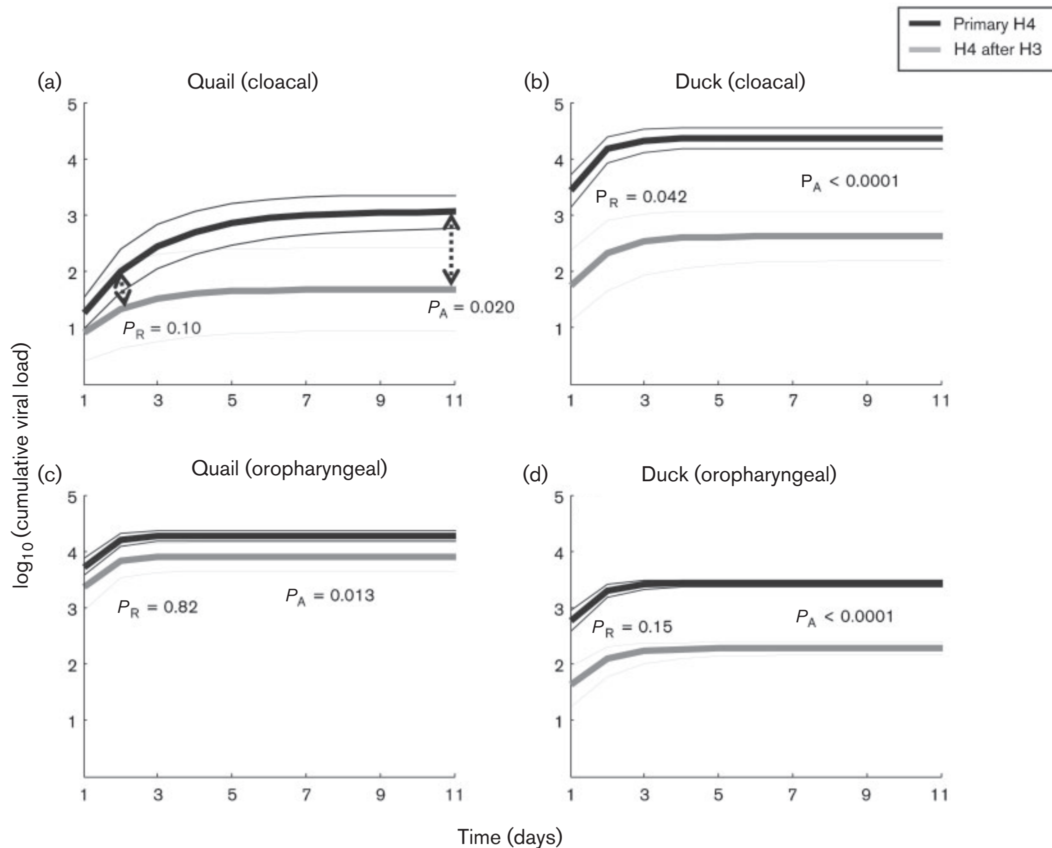

Fig. 3. Predicted viral dynamics. H4 in primary (black) and secondary (grey) infections where hosts were previously infected with H3. Results for quail (a, c) and ducks (b, d) were compared for both types of samples: cloacal (a, b) and oropharyngeal (c, d). The cumulative viral load was predicted from the best model, which included random effects from individual hosts. Fixed effects in the model included *a*, *r*, *A* and *R* (see Methods). The levels of significance are indicated for parameters *A* (P_A) and *R* (P_R), which are the difference between treatments for parameters *a* and *r*. Thin lines indicate the SEM around predicted viral loads. Note that in models that excluded effects of duck identification, *R* was significant at a $P<0.05$ level in both duck samples (b and d) but not in quail samples (model predictions not shown).

Correlation of viral infection parameters differs across host species

In duck samples, the replication rate and total load parameters were positively correlated (e.g. high *r* also meant high *a*), whereas in quail samples they were either negatively correlated (e.g. high *r* meant low *a*) or uncorrelated (Table 2). This difference highlights the fact that the growth curve function (i.e. the shape of the growth curve) was different in the two bird species.

Individual variability in secondary infections is not due to immunity from primary infections

We investigated whether individual variation in viral load in secondary infections was due to the immune response in primary infections by regression. Only quail data were included in this analysis due to sample limitations. We did not find a significant relationship for H4 viral loads in hosts that had been infected previously by H3 ($P=0.13$ for *F*-test of the full model relative to the mean of the data; Fig. 4a), despite the strong effect of individual variation in replication parameters (Table 2). However, we did find a significant non-linear relationship for H3 viral loads in hosts that had been infected previously by H4 ($R^2=0.60$; $P=0.004$ for *F*-test of the full model relative to the mean of the data; Fig. 4b). For secondary infections of H3, the non-linear relationship ($y=x+x^2$; Fig. 4b) was a better model of the data than a negative linear relationship ($y=x$; $P=0.004$ for *F*-test of the non-linear versus linear model).

DISCUSSION

The co-circulation of different avian influenza subtypes is ubiquitous. Our understanding of how heterosubtypic immunity impacts on the dynamics of LPAIVs in different host species remains weak, despite the fact that the risk of an epizootic cannot be estimated from single-subtype dynamics. This gap is due partly to the absence of quantitative measures

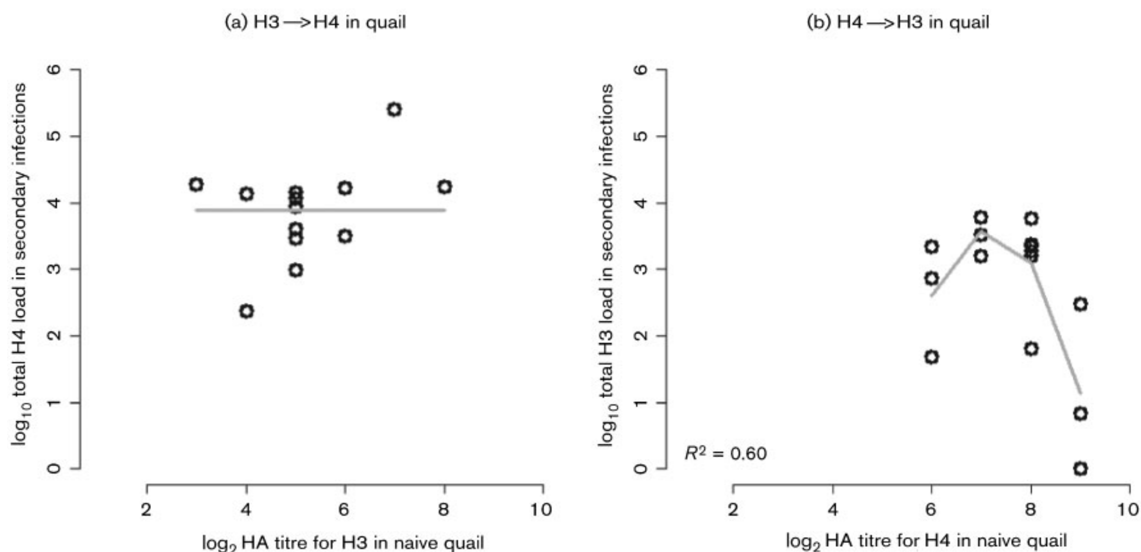

Fig. 4. Effects of immune response on viral loads in secondary infections. Total viral loads in secondary infections were plotted against the HA titres in primary infections. Viral loads were on a $\log_{10}$ scale and HA titres were on a $\log_2$ scale to reflect the dilution method. Circles show the data, whilst grey lines show the fitted regression model.

of heterosubtypic interactions. We found that recent prior infection with a different subtype was correlated with a decrease in peak viral load of up to four orders of magnitude and shedding duration of up to 4 days (~50 %).

Although H4 replicated to higher titres for longer periods in all host species, the strength of heterosubtypic immunity was not consistent across hosts. H4 was not protective against infection with H3 in pheasants, but it was strongly protective in quail. Likewise, although H3 performed similarly in ducks and quail, it was barely protective against H4 infection in quail but strongly protective in ducks. This suggests that cross-protective effects cannot be predicted directly from primary infection patterns in individual species due to an interaction between the host species and viral subtypes. In order to predict the magnitude of cross-protective effects in multiple host species, more experiments are needed to understand the basis of this interaction.

Individual variation in infectiousness and susceptibility are important determinants of epidemiological patterns and virus persistence (Kramer-Schadt *et al.*, 2009; Lloyd-Smith *et al.*, 2005). An understanding of the prominence and magnitude of individual variation is needed for structuring and parameterizing epidemiological models that can be used to predict epidemics and assess the efficacy of preventions and controls. Using the model of virus replication dynamics, we found that individual ducks can vary in total load by up to almost three orders of magnitude (i.e. 2 SD $=1.4 \log_{10}$ total load, which is a range of 2.8 $\log_{10}$). This is consistent with recent studies showing that birds may differ substantially in total viral loads due to host age or foraging behaviour (Costa *et al.*, 2010b; Hoye *et al.*, 2012; VanDalen *et al.*, 2010). Thus, the assumption of homogeneous host populations, which underlies many epidemiological models, is a simplification that may not be appropriate for studying LPAIV transmission and could lead to erroneous estimation of strain fitness (i.e. R_0) or disease risk assessment.

Individual variability in secondary infections could be a function of immunity in primary infections. We found that when H3 infection occurred before H4 infection, individual variation in H4 viral loads was not explained by immunity to H3. However, when H4 infection occurred before H3, there was a significant relationship of immunity and viral loads (although this relationship was non-linear). One difference between these infection sequences is that H3 primary infection resulted in lower viral loads and haemagglutination (HA) titres relative to H4 [$\log_2$ HA titres (mean $\pm$ SD): 5.2 ± 1.3 vs 7.6 ± 1.1]. Thus, H4 is faced with weaker cross-immunity following H3 infection compared with H3 following H4 infection. This relatively low level of immunity is apparently not enough to explain individual variability in subsequent infections. In contrast, H3 is faced with stronger cross-immunity when infecting hosts that were infected previously by H4. In this case, we saw a negative relationship between cross-immunity and H3 viral load at high HA titres but, again, no relationship at lower HA titres. Thus, when cross-immunity is strong ($\log_2$ HA titres >7), it can explain some of the variability in viral load during secondary infections but has no explanatory power when cross-immunity is weak.

A second important implication of the high variability between individuals concerns data analysis and interpretation. Most studies that test differences between treatments

on viral loads do not use a growth function or include random effects (e.g. as in the analyses presented in Figs 1 and 2). When possible, it is always favourable to use the full dataset in statistical analyses (as in Fig. 3), rather than summaries of the data (as in Figs 1 and 2, which do not account for individual heterogeneity or host sex), in order to reduce the risk of false conclusions. In studies that aim to compare the full dynamics of virus replication (i.e. the growth curve), individual random effects are important to include. Random effects are essentially a correction for error terms that are not independent, which reduces the chance of falsely rejecting the null hypothesis. Thus, when variation due to individuals is high and unaccounted for, this circumstance could lead to erroneous conclusions about the significance of treatment effects. For example, in the duck oropharyngeal data, there was no significant difference in replication rates when individual duck variation was included in the model, but replication rates were significantly lower in secondary infections when the random effect was excluded from the model. Thus, most of the variation in replication rate in duck oropharyngeal swabs was attributed to individual ducks rather than the effects of heterosubtypic immunity. Exclusion of this random effect from the model may have led to the misguided conclusion that replication rates in secondary infections were slower.

Although we expected to find quantitative differences in viral loads between cloacal and oropharyngeal samples, as others have found (Jindal *et al.*, 2010; VanDalen *et al.*, 2010), we were surprised that sample type impacted on the nature of random effects due to individual duck and sex. Most obvious was the significant effect of sex in duck cloacal samples but not oropharyngeal samples. One interpretation of this is that there could be a difference between the sexes in shedding rates by cloacal routes but not by oropharyngeal routes. Thus, cloacal samples provide more accurate estimates of the viral growth curve that is relevant to total transmission in the population, and host heterogeneity due to sex is important to consider. A balanced design with equal numbers of males and females may be optimal for estimating shedding rates. Alternatively, it is possible that a behavioural or morphological difference between the sexes could introduce contamination in cloacal samples of one sex and not the other.

Our analyses emphasize that individual variation in within-host dynamics should be incorporated into transmission models of LPAIVs and statistical analyses of treatment effects. One way to include these effects in transmission models is through individual variability in infectiousness (Paull *et al.*, 2012), which can result in fewer outbreaks with higher severity relative to populations where all individuals are equally infectious (Lloyd-Smith *et al.*, 2005). We have provided quantitative estimates for introducing these effects in models. We also showed that heterosubtypic immunity can be strong and inconsistent across hosts, for at least the two strains we used in our experiments. Similar research on other strains in a number of different host species is needed to understand the prevalence and magnitude of heterosubtypic immunity in LPAIVs in their natural habitat.

METHODS

Viral strains and hosts. The two strains of avian influenza, A/mallard/CO/P66F1-5/08 (H4N6) and A/wildbird/CA/187718-36/08 (H3N8), were collected from wild birds as part of a US national surveillance system for avian influenza initiated in 2006 (Deliberto *et al.*, 2009; USDA, 2006). Prior to use, both viruses were inoculated in mallards and passaged once in the allantoic cavity of 9–11-day-old specific-pathogen-free embryonated hen eggs at 37 °C. Allantoic fluid was harvested and pooled, and aliquots were stored at −80 °C prior to titration. Viral titres were determined as EID_{50} (Szretter *et al.*, 2006) using the method of Reed & Muench (1938).

All experiments were approved by the Institutional Animal Care and Use Committee of the US Department of Agriculture, Animal and Plant Health Inspection Service, Wildlife Services, National Wildlife Research Center (NWRC), Fort Collins, CO, USA (approval nos NWRC 1620 and 1751). Mallards (*Anas platyrhynchos*) were purchased from Field Trial Gamebirds in Fort Collins, CO. Ring-necked pheasants (*Phasianus colchicus*) were purchased from Premiere Bird Barn in Kiowa, CO, and Northern bobwhite quail (*Colinus virginianus*) were purchased from R&R Game Birds in Lamar, CO. At the start of experimentation, mallards were 5–6 months old, quail were 8–9 months old and pheasants were ~4 months old. During testing, all birds were housed at the NWRC in the outdoor animal research facility in a Biosafety Level 2 indoor aviary as cohorts of two pheasants or three mallards per pen; quail were housed individually in cages. The number of birds per experiment is indicated in Table 1. Pens and cages were equipped with a shallow water bowl and food bowl, and mallards were provided with an artificial pond, which was a 375 l oval stock tank filled with water. Food, water and grit were provided daily. Control birds (i.e. not used for inoculation) were also housed in the indoor aviary but were separated from inoculated animals with a floor-to-ceiling plastic Zipwall around the perimeter of their pen. Virus was not detected in any of the six control birds (two birds of each species). All birds were confirmed to be negative for influenza A virus antibodies prior to testing by either agar gel immunodiffusion or bELISA (FlockCheck AI multiscreen antibody test kit; IDEXX Labratories), performed as described by the manufacturer.

Infection protocol. Control birds were mock inoculated orally with 1 ml amniotic allantoic fluid diluted in BA-1 viral transport medium [M199-Hank's salts, 1 % BSA, 350 mg sodium bicarbonate l^{-1}, 2.5 mg amphotericin B ml^{-1} in 0.05 M Tris/HCl (pH 7.6), with 100 U penicillin ml^{-1} and 100 mg streptomycin ml^{-1}]. The exposed group was inoculated orally with 10^5 EID_{50} of the target strain in 1 ml. Oropharyngeal swabs and cloacal swabs were collected daily for 10 days (pheasants and quail) or 19 days (mallards). Viral RNA was undetectable in all birds after 10 days. All swab samples were placed in 1 ml BA-1 medium and stored at −80 °C until laboratory testing. A serum sample was collected from each bird at ~2 weeks post-inoculation (p.i.) for antibody screening. Birds that were positive for influenza A virus antibodies (confirmed by ELISA) were used in the secondary infection experiments (see Table 1 for experimental design). Inoculation methods for the heterosubtypic immunity experiments were the same as inoculations for naïve birds and were conducted 20 days after infection with the first subtype.

Analysis of samples. All samples were tested for the presence of influenza A virus RNA by real-time RT-PCR, performed with an ABI 7900 Real-time PCR system (Life Technologies). Viral RNA was extracted from samples using a MagMAX-96 AI/ND Viral RNA Isolation kit (Ambion). Primers and probes were specific for the

influenza A virus matrix gene and were developed previously (Spackman *et al.*, 2003). Thermocycling conditions were as described by Agüero *et al.* (2007). Calibrated controls with known viral titres (10^2–10^5 EID_{50} ml^{-1}) were also used to construct four-point standard curves for PCR data. Sample viral RNA quantities were extrapolated from the standard curves and considered as EID_{50} equivalents ml^{-1} (VanDalen *et al.*, 2010). The detection threshold of the assay was 10^1; values below this were considered to be 0. Serum samples were screened for antibodies to influenza A virus via agar gel immunodiffusion or ELISA as described above.

Haemagglutination inhibition titrating. We quantified antibody levels by haemagglutination inhibition assays on sera collected following primary infections. Haemagglutination inhibition testing was conducted at the National Veterinary Services Laboratory in Ames, IA, USA, following the methods of Spackman (2008). Only quail samples were used, as samples from the other host species did not contain enough serum to be tested.

Hypothesis testing. Analyses were conducted in Matlab R2011b (Mathworks). Viral growth curve data were summarized into the following infection parameters: total viral load, peak viral load and DP. A two-factor ANOVA was used to compare the effects of subtype and host species in primary infections. To test the effects of heterosubtypic immunity between hosts and subtypes, three one-way ANOVAs were conducted on the differences between primary and secondary infections for the following comparisons: (i) secondary infection of H3 in pheasants versus quail; (ii) secondary infection of H4 in ducks versus quail; and (iii) secondary infection of H4 versus H3 in quail. Difference data were calculated as the mean of subtype X in primary infections minus each data point for subtype X in secondary infections (for each host species and subtype separately). Within each ANOVA test, multiple comparisons were carried out using Tukey's honestly significant difference test. Total and peak viral load data were transformed to a $\log_{10}$ scale for all analyses. For each bird species, we used data from the sample type (cloacal versus oropharyngeal) that produced the highest titres (oropharyngeal for quail and pheasants, and cloacal for mallards). This is consistent with the finding that LPAIVs tend to replicate mainly in the cloacal bursa and lower intestine in mallards (Daoust *et al.*, 2010) but not in quail or pheasants. Data from the other sample types are shown in Fig. S1. Lastly, we investigated whether the immune response in primary infections explained the variation in secondary infections by regressing total viral loads in secondary infections on HA titres in primary infections with the alternate subtype.

Model of viral dynamics. Analyses were conducted in R version 2.13.0 using the 'nlme' package. A non-linear, mixed (i.e. incorporating 'random effects', which are correlated errors) regression model was used to examine factors contributing to variation in viral dynamics, specifically host individual and sex. Note that this could not be carried out in the ANOVA framework (above), as each bird was only experimented on once and thus had only one total load, one peak load and one DP. Using a viral dynamic model, we could assess variation in virus replication rates at the level of individual birds, as each individual was sampled multiple times (i.e. daily). We conducted model selection on models with and without random effects of host individual and sex using ΔAIC and ΔBIC (which has a larger penalty for more parameters) to determine the difference in AIC or BIC between models with no random effects and those with random effects. A positive difference indicated that the model with random effects was better than the model without random effects, and larger differences indicated a better model. Data from each type of sample (cloacal versus oropharyngeal) were modelled separately. Viral titres over time were transformed to cumulative viral titres and then log transformed in order to smooth the data and manifest the important aspects of the viral trajectory: highest rate of increase (r, virus replication rate) and total viral load (a). Cumulative titres over time (Y) were modelled by a two-parameter exponential asymptote function:

$$Y = a(1 - \exp^{-rt})$$

where a is the asymptote (total viral load), r is the replication rate and t is the day p.i. Using the best model that included random effects of host individual (i.e. Fig. 3), we tested for differences between treatments in viral infection parameters by estimating two additional parameters.

$$Y = (a + xA)(1 - \exp^{-(r + xR)t})$$

Here, x is an indicator variable (0 or 1) for the treatment type, and parameters A and R are the differences between treatments in parameters a and r. Thus, estimates that overlap 0 indicated no difference between treatments.

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Table S1. Two-way ANOVA results for single infection data (oral swabs)

DF, Degrees of freedom.

Data	Factor	DF	*F*	*P*	R^2
Total load	Species	2, 102	2.2	0.11	
	Subtype	1, 102	28.3	<0.0001	0.26
	Species×subtype	2, 102	3.4	0.039	
Peak load	Species	2, 102	1.1	0.32	
	Subtype	1, 102	21.4	<0.0001	0.21
	Species×subtype	2, 102	2.7	0.076	
DP	Species	2, 102	1.2	0.29	
	Subtype	1, 102	21.3	<0.0001	0.28
	Species×subtype	2, 102	5.3	0.0065	

Table S2. One-way Anovas on difference data (mean of subtype X in single infections−subtype X in secondary infections)

DF, Degrees of freedom

Data	Comparison	DF	*F*	*P*	R^2
Total load	Q-H3 vs Q-H4*	1, 29	7.1	0.013	0.20
	P-H3 vs Q-H3†	1, 26	4.3	0.048	0.15
	D-H4 vs Q-H4‡	1, 29	18.8	0.0002	0.40
Peak load	Q-H3 vs Q-H4*	1, 29	6.8	0.015	0.19
	P-H3 vs Q-H3†	1, 26	2.7	0.11	0.10
	D-H4 vs Q-H4‡	1, 29	22.2	<0.0001	0.44
DP	Q-H3 vs Q-H4*	1, 29	26.1	<0.0001	0.48
	P-H3 vs Q-H3†	1, 26	18.6	0.0002	0.43
	D-H4 vs Q-H4‡	1, 29	14.2	0.0008	0.34

*Tests effect of subtype on magnitude of cross-protection in quail.

†Tests effect of host species on magnitude of cross-protection of H4 on H3 secondary infection.

‡Tests effect of host species on magnitude of cross-protection of H3 on H4 secondary infection.

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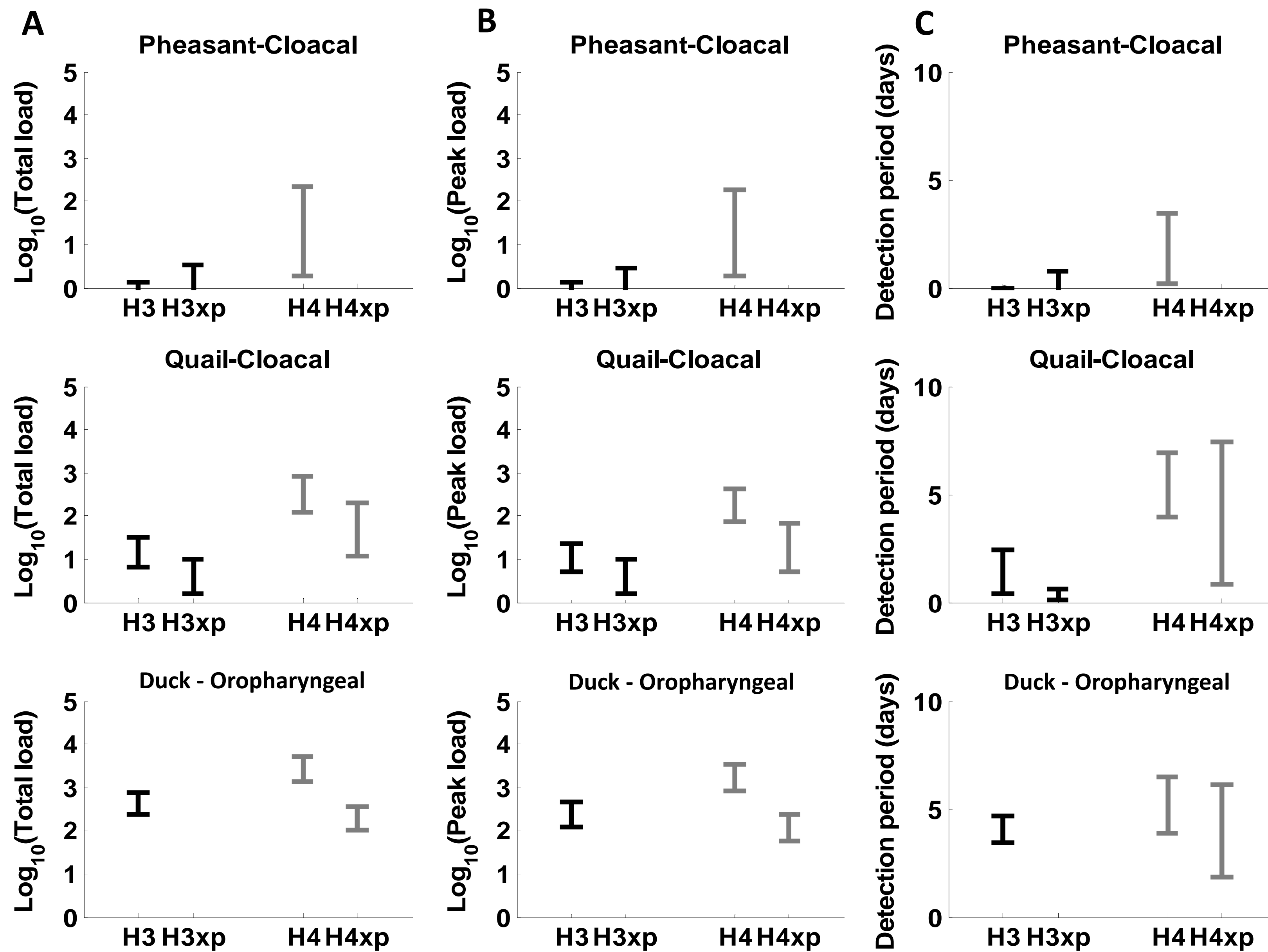

Fig. S1. Summary of infection parameters for each experiment in each host species. Data are from other sample types relative to those presented in Figs 1 and 2 (cloacal for pheasants and quail, and oropharyngeal for ducks). H3, H3 primary infections; H3xp, H3 secondary infections in birds previously infected with H4; H4, H4 primary infections; H4xp, H4 secondary infections in birds previously infected with H3. Host species and sample type are indicated above each plot. Infection variables are on the *y*-axes.

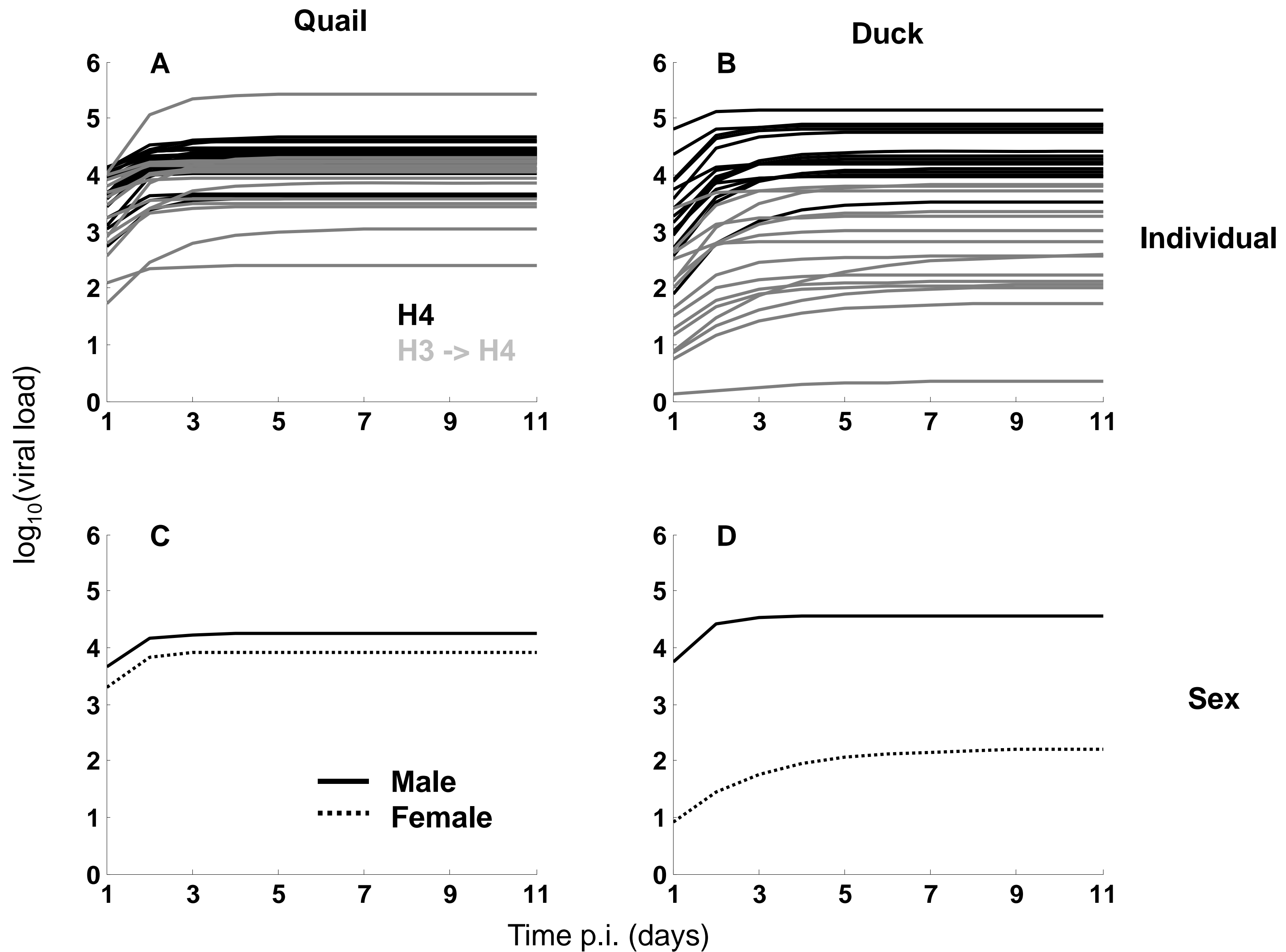

Fig. S2. Example of variation due to random effects of individuals and sex. Predicted viral load over time for each individual (a, b) and sex (c, d) for H4 and H3→H4 experiments in quail oropharyngeal samples (a, c) and duck cloacal samples (b, d). Plots (a) and (b) show the amount of variation due to individuals that underlies the predictions from fixed effects in Fig. 2. (a) corresponds to Fig. 3(c), and (d) corresponds to Fig. 3(b). The statistics for random effects are given in Table 2.