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Environmental transmission of influenza A virus in mallards

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ABSTRACT Influenza A viruses present a major challenge for animal and human health. They circulate widely in wild waterfowl and frequently spillover into poultry, emphasizing the need for risk-based surveillance in wild birds and an understanding of the relative importance of different transmission mechanisms. We addressed this objective with a replicated (N = 6) experimental infection study in which we serially exposed eight cohorts of four naïve contact mallards to an experimentally infected mallard and a shared water pool. Viral concentration in the water was a better predictor of transmission than several direct measures of viral shedding in the focal duck. Our data provide quantification of transmission probability and its variation throughout the infectious period of an infected duck. Our findings highlight the need to consider environmental surveillance in risk-based surveillance planning and provide realistic parameters for identifying optimal control strategies using epidemiological inference.

IMPORTANCE Wild birds are the natural reservoir hosts of influenza A viruses. Highly pathogenic strains of influenza A viruses pose risks to wild birds, poultry, and human health. Thus, understanding how these viruses are transmitted between birds is critical. We conducted an experiment where we experimentally infected mallards which are ducks that are commonly exposed to influenza viruses. We exposed several contact ducks to the experimentally infected duck to estimate the probability that a contact duck would become infected from either exposure to the virus shed directly from the infected duck or shared water contaminated with the virus from the infected duck. We found that environmental transmission from contaminated water best predicted the probability of transmission to naïve contact ducks, relatively low levels of virus in the water were sufficient to cause infection, and the probability of a naïve duck becoming infected varied over time.

KEYWORDS avian influenza, influenza A, environmental transmission, wild bird, mallard, *Anas platyrhynchos*, infectious dose

Avian influenza A virus (IAV) is a high-consequence pathogen that continues to threaten public and animal health globally. IAVs have very high evolutionary rates and infect a wide range of bird species, resulting in the continual emergence and re-emergence of novel viral strains (1, 2). For example, in 2014, H5Nx (2.3.4.4.b), highly pathogenic (HP) IAV strains, began spreading globally in wild birds and are now causing unprecedented outbreaks in captive and wild birds in dozens of countries (3). The recent re-emergence of HPIAVs (H5) viruses in US wild birds (4) resulted in 683 captive bird flocks affected across 47 states from January 2022 to December 2022 (5). This pattern of rapid and widespread distribution of IAV outbreaks in captive birds suggests that spillover from wild birds can be frequent and plays an important role in shaping the spatial dynamics of outbreaks in captive birds during re-emergence (3), and more recently, of free-ranging mammals (6).

Mallards (*Anas platyrhynchos*) act as an important reservoir for a wide diversity of IAV genotypes (7, 8) and contribute to the geographic spread of novel IAVs, given their

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ubiquity across the globe (8, 9). In mallards and many other species of wild birds, IAV is shed through the gastrointestinal tract in feces that can contaminate water sources or surfaces in the environment (10). Other individuals can become infected through oral uptake of contaminated water, soil, or fecal matter (11). It is widely believed that such environmental transmission is a predominant mechanism of transmission and persistence of IAV in wild bird populations (9, 10, 12, 13). Mallards and other dabbling ducks prefer shallow aquatic habitats, suggesting that environmental transmission is especially important for this group of IAV hosts because those habitats allow IAVs to reach high concentrations in the environment (14, 15). Rohani et al. (16) outlined several factors pointing to the likelihood of environmental transmission of IAV in dabbling ducks, including the demonstrated persistence of IAV in water (17, 18), high juvenile infection rates (1) indicative of exposure outside of family groups, and the failure of density-dependent transmission to appropriately capture transmission dynamics and interseasonal persistence (16, 19). While environmental transmission of avian IAVs is currently accepted as an important transmission mechanism, much of the evidence for and attempts to quantify environmental transmission mechanisms are indirect (e.g., 16, 20–23), limiting our ability to understand its role in the epidemiology of this important global pathogen. Understanding the relative importance of different transmission mechanisms of IAVs in wild bird hosts is fundamental for appropriately targeting control, developing better predictive models for risk assessment, and guiding effective risk-based surveillance (24).

Quantifying transmission processes in nature is challenging. Experimental infection studies offer an important tool to fill data gaps about natural transmission mechanisms (24, 25). Recent experimental infection studies have begun to provide direct evidence of environmental transmission of IAV in wild and domestic bird species and have laid the groundwork for quantifying the role and rates of environmental transmission in IAV infections (26–28). Individual-level variation in behavior and within-host infection dynamics can be high, necessitating high levels of replication for each dimension of interest (e.g., transmission mechanism, time, etc.), which rapidly expands the size of the study. For this reason, no studies to date have quantified transmission over time, which requires housing many individuals concurrently and separately, and is logistically challenging, e.g., reference (28), especially for HPIAVs that need to be studied in high-containment conditions. In this study, we leveraged an extensive captive animal facility to quantify transmission probability by environmental transmission, its relationship to viral concentration (dose-response), its variation throughout an individual's infection curve, and whether exposure levels to contact ducks predict subsequent infection dynamics in contact ducks. We conducted experiments with a low pathogenicity IAV (LPIAV) strain (H6N2) that is widespread, circulates at high prevalence, has a broad host range, frequently co-infects with a variety of subtypes including H5s, and has shown increased adaptation to poultry and mammals (29–34).

Our experimental design allowed us to infer how much transmission in each cohort of contact ducks was due to the level of oral, fecal, and cloacal viral shedding by the focal duck they were exposed to, as well as the viral load in the shared water pool. This design featured a replicated ($N = 6$) experiment in which we serially exposed eight cohorts of four naïve contact mallards over time to an experimentally infected mallard and a shared water pool (Fig. 1). After a short exposure to the focal duck, contact ducks were removed and housed separately to ensure that their infection was due only to exposure to the focal duck. The shared water pool was also cleaned, and new water was added in between each cohort's exposure to ensure contact transmission only included virus shed during the exposure period. Our data quantify the role of environmental transmission of IAVs in mallard ducks, document variation in transmission probability over time, and provide dose-response curves from water for improving risk assessment and planning effective surveillance (e.g., 12, 24).

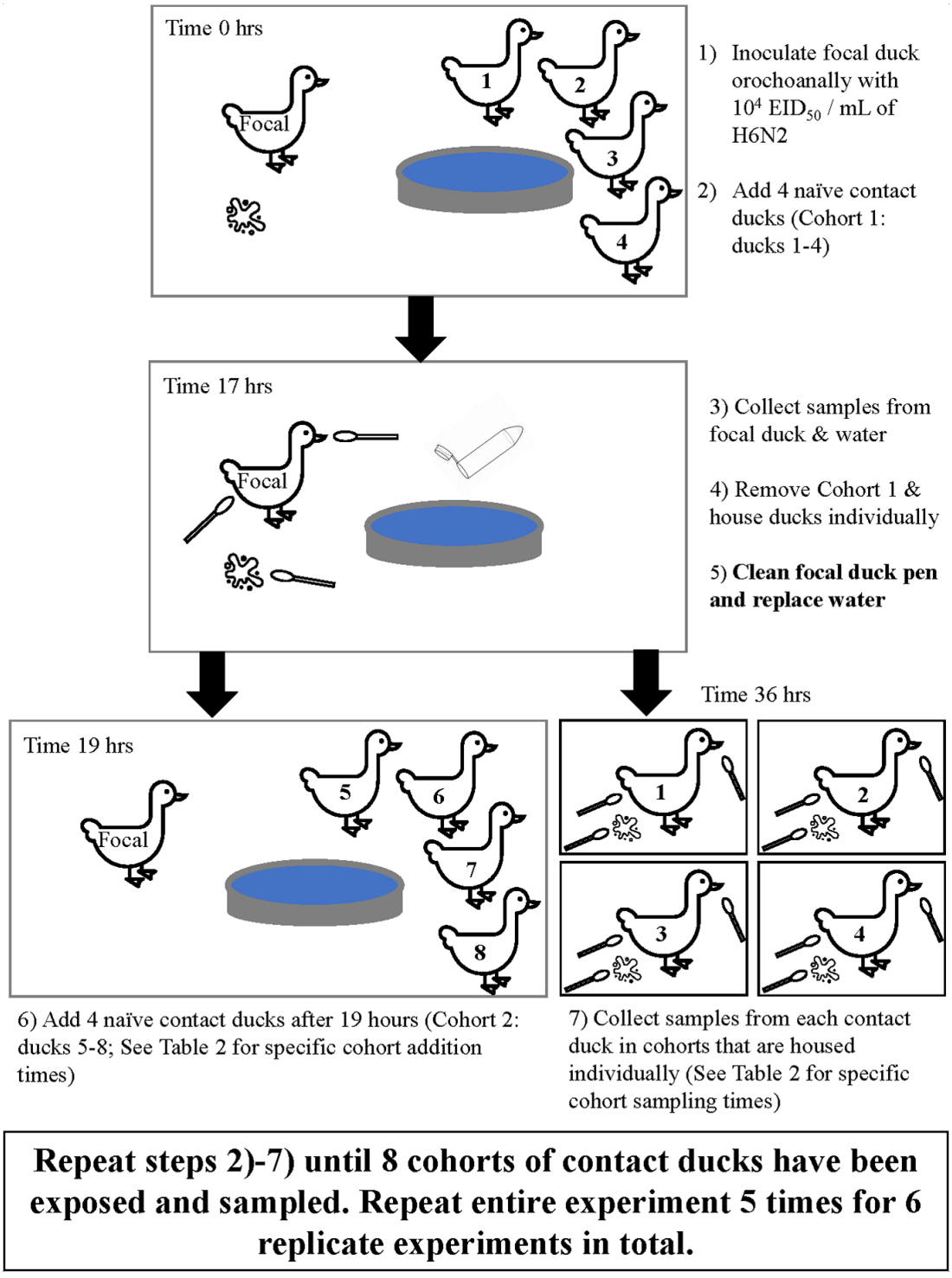

FIG 1 Schematic of experimental design. The process of adding and removing four new naïve contact ducks is repeated eight times for a total of 32 contact ducks exposed to each focal duck throughout the focal duck's infection. After each 17-hour exposure period, contact ducks were removed and housed individually. Before each new cohort was added, we cleaned the focal duck pen by hosing pen floors and sanitizing and refilling food bowls, water bowls, and water pools. Thus, each contact cohort was only exposed to the amount of virus shed by the focal duck within the 17-hour exposure period (i.e., viral shedding by the focal duck was not cumulative). The entire experiment was conducted six times with a new focal duck and new cohorts of contact ducks.

RESULTS

Shedding in focal ducks

The six experimentally inoculated focal ducks exhibited variation in viral shedding curves across sample types (Fig. 2; Fig. S1). Median peak viral loads were 7.2×10^4, 1.9×10^2, 4.5×10^3, and 4.6×10^4 EID_{50}/mL for fecal, oral, cloacal, and water samples respectively, and ranged across three orders of magnitude across the six focal ducks: (6.7×10^3 to 3.0×10^5—fecal), (5.3×10^1 to 5.6×10^2—oral), (1.6×10^2 to 1.6×10^5—cloacal), and (5.0×10^3 to 1.6×10^5—water) (Table S1). Duration of viral RNA detection tended to be the longest in fecal (median 8.3 days) and water (median 8.7 days) samples, but time to the peak viral

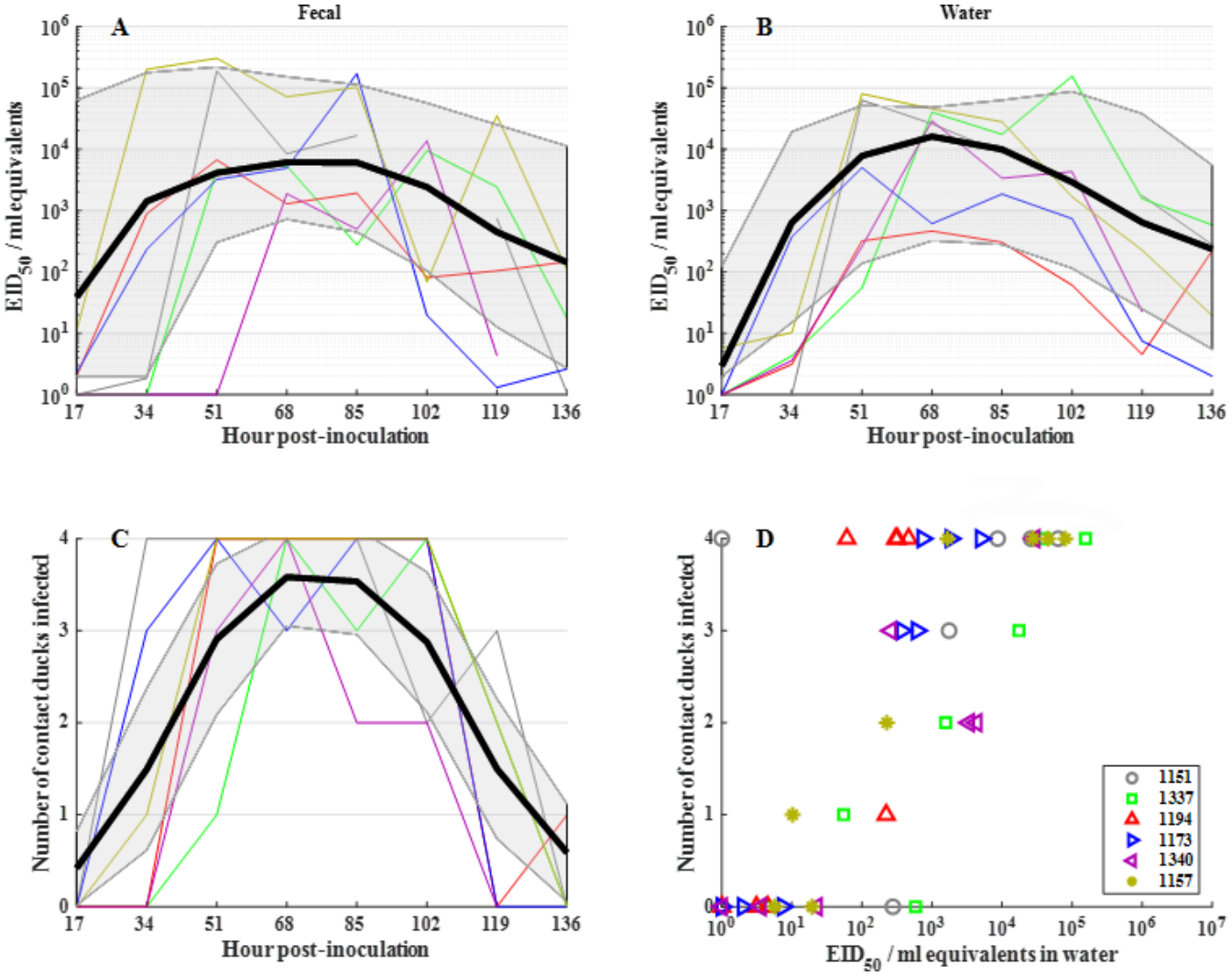

FIG 2 Descriptive plots of experimental infection data. (A) Viral RNA concentrations (calibrated to EID_{50}/mL) in fecal samples collected from inoculated focal ducks over time. (B) Viral RNA concentrations for water samples collected from water pools over time. (C) The number of contact ducks in each of the eight serially introduced cohorts of four ducks that became infected after being exposed to one of the six focal ducks for 17 hours. Each time (hours post-inoculation) on the *x*-axis represents the introduction time of one of the eight contact cohorts, and each colored line is for a different focal duck replicate of the experiment. (D) The number of contact ducks from a cohort that became infected was plotted against the viral RNA concentration in water at the end of the cohort exposure period. Each symbol type is associated with an individual focal duck, and each of the same symbols represents one of the eight cohorts associated with that focal duck. (A–D): Each colored line is for a different focal duck; colors are shown in D's legend. (A–B) Thick black line is the median with Gaussian smoothing over a 5-day window; shaded regions are the 95% credible intervals. (C) Thick black line is the mean with Gaussian smoothing over a 5-day window; shaded regions are the 95% confidence intervals.

load was the shortest in oral and water samples and longer in fecal and cloacal samples (median of 1.5 and 2.7 days, respectively, versus 4.0 and 3.5 days, respectively; Table S1).

Temporal trends in transmission probability to contact ducks

When viewing the raw data descriptively, it was apparent that the number of contact ducks that became infected rose and then fell sharply similar to average viral replication dynamics in focal ducks (Fig. 2C compared with Fig. 2A and B). Also, it appeared that transmission probability was equally low during the early and late phases of the focal duck viral growth curve but corresponded to different viral loads, e.g., in the early phase of a focal duck's infection when transmission probability was 0.25, viral loads in water were $<10^2$ EID_{50} equivalents/mL, but in the late phase when transmission probability was 0.25, viral loads were $\sim 5 \times 10^2$ EID_{50} equivalents/mL (compare Fig. 2B and C).

Viral load in water was the strongest predictor of transmission to contact ducks

Viral load in water showed the strongest correlation to the number of contact ducks infected across the range of sample viral loads (Fig. 2D; Fig. S2). Correspondingly, the logistic mixed effects regression models of contact-duck infection status as a function of water, fecal, cloacal, or oral viral RNA concentrations in focal ducks showed that models without viral loads in water were significantly less supported compared to models that included viral loads in water. Furthermore, models that included viral loads in water were not significantly improved by adding viral loads through other potential routes of uptake (i.e., direct oral contact, direct cloacal contact, or contact with fecal material on the ground from the focal duck, Table 1).

Dose-response curves through environmental transmission

The top regression models all contained a significant effect of pre- and post-peak viral loads on the transmission probability to contact ducks. This result was consistent with the descriptive data trends that showed that the quantitative relationship between viral loads in focal ducks and transmission probability to contact ducks depended on whether the focal duck's within-host infection dynamics were before or after their peak levels. While the slope of the relationship did not change significantly (Table 1), higher viral loads in water after the focal duck's peak shedding time were needed to reach the same transmission probabilities to contact ducks as those before the focal duck's peak shedding time (Fig. 3). In the pre-peak relationships, the model-predicted transmission probability was <0.1 on average when the viral concentration in water was 2 EID_{50} equivalents/mL (95% confidence interval: [1, 9]; Fig. 3), increased to 0.25 when the average concentration was 9 EID_{50} equivalents/mL (95% confidence interval: [2, 45]; Fig. 3), and was 0.5 when the concentration was 50 EID_{50} equivalents/mL (95% confidence interval: [10, 263]; Fig. 3). Viral concentrations that led to transmission probabilities as high as 0.75 or 0.9 were as follows: 275 EID_{50} equivalents/mL (95% confidence interval: [59, 1,778]) and 1.4×10^3 EID_{50} equivalents/mL (95% confidence interval: [275, 1.2×10^4]), respectively. The wide range in the dose-response relationship reflects variability in the amount of viral shedding by focal ducks as seen in their individual shedding curves in water (colored lines in Fig. 2B). For the same relationships after the peak viral loads in the focal ducks, the quantities were as follows: 18 [2, 78], 85 [15, 355], 457 [98, 2.0×10^3], 2.5×10^3 [589, 1.3×10^3], and 1.3×10^4 [2.9×10^3, 8.7×10^4] for transmission probabilities of 0.1, 0.25, 0.5, 0.75, and 0.9, respectively (Fig. 3). Thus, viral loads measured by RT-qPCR after the peak needed to be almost 10 times higher to reach the same transmission probability as the same viral loads up to and including the peak viral concentration time in the focal duck's viral replication curve.

TABLE 1 (A) Model selection results [difference in Akaike information criterion (AIC); ΔAIC) with absolute goodness of fit estimates (area under the curve; AUC) for models estimating transmission probability to contact ducks. All models have a random effect for focal ducks. (B) Coefficient estimates for the most parsimonious and competitive model with 95% confidence intervals. This model was used to infer the dose-response relationship (Fig. 3)[a]

A. Model selection			
Model	**AIC**	**ΔAIC**	**AUC**
Water + PrePost + (1|Focal)	107.9	0	0.90
Water × PrePost + (1|Focal)	109.3	1.4	0.91
Water + Cloacal + PrePost + (1|Focal)	109.5	1.5	0.91
Water + Fecal + PrePost + (1|Focal)	109.5	1.6	0.91
Water + Oral + PrePost + (1|Focal)	109.8	1.8	0.90
Water + Fecal + (1|Focal)	116.5	8.6	0.89
Water + (1|Focal)	116.5	8.6	0.90
Water + Cloacal + (1|Focal)	116.6	8.7	0.90
Water + Oral + (1|Focal)	118.4	10.5	0.90
Fecal + (1|Focal)	158.8	50.9	0.81
Cloacal + (1|Focal)	159.7	51.8	0.81
Fecal + PrePost + (1|Focal)	160.7	52.8	0.81
Fecal × PrePost + (1|Focal)	161.1	53.2	0.82
Cloacal + PrePost + (1|Focal)	161.7	53.7	0.81
Cloacal × PrePost + (1|Focal)	163.1	55.2	0.80
Oral × PrePost + (1|Focal)	206.7	98.8	0.76
Oral + (1|Focal)	215.7	107.8	0.69
Oral + PrePost + (1|Focal)	217.0	109.1	0.67
(1|Focal)	223.8	115.8	0.64
PrePost + (1|Focal)	225.7	117.7	0.57

B. Parameter estimates for the most parsimonious, top model			
Variable	**Mean**	**2.5%**	**97.5%**
Intercept	−2.93	−4.23	−1.63
Water	1.72	1.24	2.20
PrePost	−1.64	−2.75	−0.53

[a]PrePost is a two-level factor indicating whether the data for the number of contact ducks infected occurred before (and including) the peak viral concentration in the focal duck (Pre) versus after the peak viral concentration in the focal duck (Post)

DISCUSSION

We demonstrate that the dominant mode of transmission from mallard ducks infected with an endemic wild bird IAV strain occurs from shedding into and uptake from water. Viral shedding routes likely involved in direct contact (e.g., oral, cloacal, and fecal) explained comparatively less variation in transmission probability to contact ducks. Indirect transmission through feces on the ground likely occurred, but fecal loads did not significantly improve transmission models that included viral RNA concentrations in water.

The important role of transmission in water emphasizes the need to continue investing effort into optimizing environmental surveillance protocols (12, 24). Recent studies involving GPS tracking of wild waterfowl (including three mallards) suggest that even small water sources near or on captive bird premises can attract wild waterfowl visitation (35). Birds can become infected by drinking contaminated water or through natural behaviors that include wading and drinking (26, 36). A recent experimental infection study estimated R_0 (the number of infections that occur in a wholly naïve population from a single infected individual) from water contaminated with HPIAVs to be 3 on average, and as high as 4.2 (27), supporting our finding that environmental transmission is a major transmission mechanism for mallards.

Similar to Ahrens et al. (2022), we found that very low titers in water (<55 EID_{50} equivalents/mL of pre-peak viral concentration) produced a substantial transmission

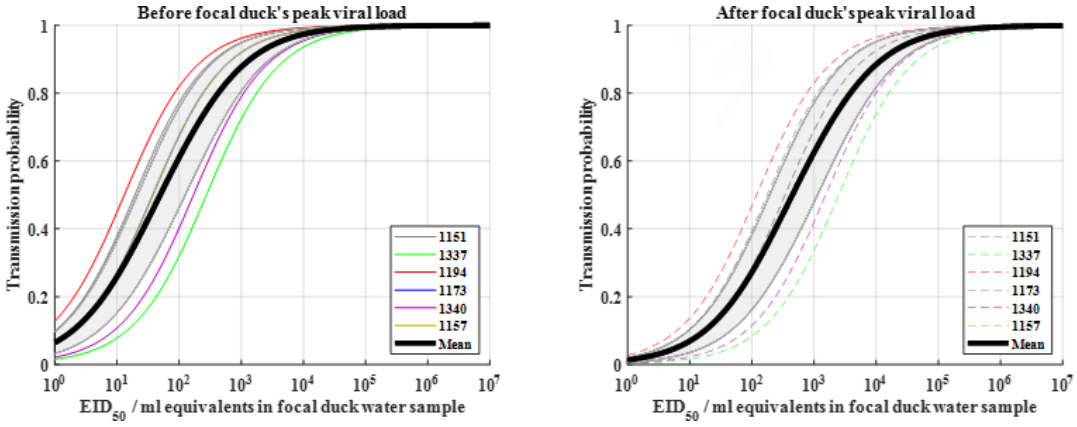

FIG 3 The predicted transmission probability from focal to contact ducks based on the concentration of virus in water for each focal duck replicate. The predicted 50% transmission probability occurs when the concentration of viral RNA in water is 182.0 EID_{50} equivalents/mL (95% confidence interval [38.0, 831.7]). The shaded region shows a 95% confidence interval of the mean.

probability (e.g., 0.5). A difference between the Ahrens et al. (2022) study and ours is that while they found that all contact ducks became infected when exposed to low viral doses in water, we found that higher doses (>1.4 10^3 EID_{50} equivalents/mL of pre-peak viral concentration) were necessary for all contact ducks to become infected. This discrepancy could be due in part to differences among the viral strains (we examined an H6N2 virus, whereas they examined an HP H5N8). However, a larger part of the discrepancy is likely due to the methodological differences in Ahrens et al.'s (2022) experiment, where some initially infected contacts increased the viral loads in the water, thus allowing additional contacts to become infected (26). In our experiment, each group of four contact ducks was only exposed to the viral loads in water for 17 hours and then housed separately (the minimum time between infection and the onset of shedding for our H6N2 virus was 19 hours; Susan Shriner, unpublished data). Thus, each contact duck only could be infected by the viral loads we measured in water. However, in Ahrens et al.'s (2022) experiment, each group of four contact ducks was housed together for 13 consecutive days, making it challenging to disentangle how many ducks became infected from the initial low viral concentration in water or additional viral excretion by infected contacts.

Another strength of our experimental design is that it enabled us to quantify the dose-response curve from water-borne infection during a relatively short exposure period. We changed the water after each contact group's exposure so that each group was only exposed to the current levels of viral shedding from focal ducks. This allowed us to link transmission probability directly to the virus that was shed during different phases of the focal duck's infection. Also, we were able to relate the infectiousness of the focal duck throughout its infectious period to transmission probability in contact ducks because each contact cohort was only exposed to the focal duck for 17 hours and then housed individually to avoid cross-contamination among contact ducks.

Our finding that higher viral concentrations are needed to reach the same transmission probability in late relative to early phases of a focal duck's infection is similar to SARS-CoV-2 in humans (37) (although the cited study used a measure of infectiousness instead of transmission probability). A likely explanation for these patterns is that more of the viral particles detectable by RT-qPCR are viable early during infection when viral populations are growing exponentially rather than later on when viral replication is declining. Our results suggested that only ~1/10 of the viral particles detected by RT-qPCR after the peak viral load in focal ducks are viable for infecting contact ducks.

Quantifying variation in transmission rates among hosts due to social behavior or other factors is crucial for accurately predicting epidemiological parameters such as R_0 and the timing of outbreaks (38). In the same way, variations in infection dynamics

such as viral loads shed by hosts and the kinetics of shedding over the course of infection in combination with different transmission mechanisms or other processes such as the host immune response could be other important drivers of heterogeneity in transmission rates among hosts (39–42). For example, low-dose infections could lead to more low-dose infections or lower transmission rates in the population because it takes longer for a low-dose infection to reach peak viral loads such that the infectious period is characterized by a longer period of low viral loads (41). Furthermore, transmission rates are generally modeled as constant across the infectious period (e.g., 43) despite the well-described pattern of varying viral load over the course of infection. Data directly linking patterns of viral shedding with the probability of transmission have the potential to improve models of disease spread (e.g., 44).

Due to the logistical constraints of the experiment, our measures of transmission probability were coarse because each cohort only had four contact ducks. Larger numbers of contact ducks in each cohort would help to better resolve transmission probability but this was not possible as we needed to house each individual from each cohort separately to avoid cross-contamination. Although our measure of transmission probability was coarse, we were able to capture a distinct increase in peak transmission probability followed by a decline in transmission probability suggesting this limitation did not affect our main conclusions. Second, the pools we used to examine water-borne transmission were smaller than many water bodies used by wild birds where environmental transmission was found to be important (45). However, wild birds are known to use and be attracted to puddle-size water suggesting that small water pools are relevant in IAV epidemiology in wild birds (35). Finally, although our study investigated a single IAV strain in a single host species, we focused on one of the most common hosts of IAVs and a widely distributed endemic strain of IAV—H6N2 (29). H6Nx strains show frequent reassortment including intercontinental gene exchange, increased burden to poultry, and can infect and transmit in mammals (30, 33, 34).

The currently circulating HP 2.3.4.4.b viruses are showing signs of adaption and persistence in wild waterfowl and are spreading similarly to LP IAVs (46), suggesting that studies of highly prevalent LPIAVs with wide host range and frequent co-infection ability [such as H6Nx viruses (29, 32)] are a good model of the epidemiological processes driving the circulation of these HP viruses. Also, environmental transmission has historically been associated with LPIAV in wild birds while direct transmission has been associated with HPIAVs in captive birds. Circulation, persistence, and virulence of current 2.3.4.4.b viruses in wild birds suggest that these strains have adapted to some species of wild birds and are behaving like LPIA viruses in mallards (46, 47). Thus, it is likely that environmental transmission may be a dominant transmission mechanism for these HPIA 2.3.4.4.b viruses as well as the LPIA virus we studied, highlighting the importance of better characterization of the environmental transmission process.

Conclusions

Our work provides robust quantification of transmission processes to support risk assessments of viral emergence in different ecosystems, design risk-based surveillance, and develop realistic models of epidemiological dynamics that can be used to identify optimal control strategies. Transmission through water was overwhelmingly the most important transmission mechanism and occurred at very low viral concentrations in water highlighting an important role for environmental surveillance in risk assessment.

MATERIALS AND METHODS

Viral strains and hosts

We used a North American endemic IAV [A/wild bird/IL/183983–24/06(H6N2)] propagated from a wild bird fecal sample collected during national avian IAV wild bird surveillance (48). The virus was propagated in the allantoic cavities of 9- to 11-day-old-specific

pathogen-free embryonated hen eggs using standard methods (49) and the Reed and Muench method to calculate viral titers (50).

Flying mallards were obtained at 2 days of age from a commercial avian hatchery (Murray McMurray Hatchery, Inc. Webster City, IA, USA) and were housed in quarantine facilities for 2 weeks and later maintained in a large outdoor flight pen (18.5 m × 38.0 m) prior to testing when the mallards were approximately 10 months old. Oral, cloacal, fecal, and blood samples were collected from all ducks prior to study initiation to detect any current or previous IAV infection. In addition, sentinel mallards were co-housed in the flight pen and sampled regularly to ensure any natural exposures would be detected. All procedures were approved by our Institutional Animal Care and Use Committee (Protocol 1912). Food and water were provided *ad libitum*, indoor pens were equipped with an artificial pond, and the outdoor flight pen was equipped with artificial ponds, wooden shelters, and heat lamps during cold weather.

Experimental design

We inoculated a focal mallard orochoanally with 10^4 EID_{50}/mL of the H6N2 IAV. Post-inoculation, the focal duck was housed in a pen surrounded by a floor-to-ceiling plastic Zipwall around the perimeter of the pen in an indoor BSL-2 aviary. We sequentially introduced eight independent cohorts of four naïve mallards into the focal duck pen (Fig. 1). In a pilot study, we determined that the minimum time between inoculation of a focal duck and detection of shed viral RNA from an infected contact was 19 hours (unpublished data). Thus, we cohoused each cohort with the focal duck for a 17-hour interval and then removed the cohort from the pen before introducing the next cohort to ensure that contact infections stemmed only from the focal duck's routes of shedding. Upon removal from the focal duck pen, each contact mallard was individually housed and sampled at the time intervals as shown in Table 2. The eight cohorts were introduced at 19-hour intervals relative to inoculation of the focal duck (hours: 0, 19, 38, 57, 76, 101, 142, and 191; Fig. 1). We cleaned the focal duck pen in between the addition of naïve contact cohorts by hosing pen floors and sanitizing and refilling food bowls, water bowls, and water pools.

We replicated this procedure six times, i.e., six focal ducks and associated cohorts (N = 6 focal mallards × 8 cohorts/focal mallard × 4 mallards/cohort = 192 total ducks). One of the focal ducks did not have an eighth cohort, and the sample data from the sixth cohort of another focal duck were lost.

Sample collection

We collected oral, cloacal, and fecal swabs at regular intervals from both the focal duck and contact ducks (see Table 2 for sampling times). Each focal or contact duck was placed in an individual sample collection box until a fecal sample was available. Collection boxes were cleaned and sanitized between sample collection periods. We also collected water samples from the focal duck pen prior to cleaning the pen and before the addition of a new cohort of naïve contact ducks.

TABLE 2 Time points that samples were collected from focal ducks and contact ducks (cohorts)[a]

Cohort(s)	The hour samples were collected post-inoculation (focal ducks) or post-exposure (contact ducks)								
1, 2, 3		36	55	74	93		112	131	
4		36	55	74	93		112	134	
5		36	55	74	93		115	132	
6		30	55	77	96		114	138	
7		35	55	73	93		123	144	
8		30	48	74	95		120		
Focal	17	36	55	74	93	101	118	142	159, 167, 208, 221, 239

[a]Eight cohorts (labeled 1–8 in the first column) of contact ducks were introduced into a focal duck pen at hours 0, 19, 38, 57, 76, 101, 142, and 191 post-inoculation, respectively. Samples were collected post-exposure from each cohort at the hours shown in each row. The bottom row shows the post-inoculation hour that samples were collected in focal ducks.

Laboratory testing

We determined viral RNA loads for each sample type using RT-qPCR. Viral RNA was extracted using a MagMAX-96 AI/ND Viral RNA Isolation Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA), and RT-qPCR was performed as described previously (11). Of note, all samples were run in duplicate and we quantified viral RNA using a 4-point calibration curve developed from titered stock virus (10^2, 10^3, 10^4, and 10^5 EID_{50}/mL).

Estimating transmission probability from different exposure mechanisms

For each focal duck ***i*** and each group of contacts ***j***, we observed the number of those contact ducks that become infected, denoted y_{ij}. We defined infection for contact ducks as individuals that had a minimum of two positive swabs (greater than 10^2 EID_{50} equivalents) at two different sampling times. We modeled these data using logistic regression to predict transmission probability as follows.

$$y_{ij} \sim \text{Binom}(4, p_{ij})$$
$$\text{logit}(p_{ij}) = \beta_0 + x`_{ij}\beta + \gamma_i$$
$$\gamma_{ij} \sim \text{Normal}(0, \sigma^2)$$

Here, $x`_{ij}$ denotes the vector of viral loads measured for focal duck *i* at the end of the exposure period for group *j*. For one of the $x`_{ij}$, we also included a two-level factor ("PrePost") indicating whether the data point occurred before (and including) the peak or after the peak viral load in the focal duck. We identified the time point for the viral peak as the highest concentration of virus measured across all four sample types (i.e., we summed the viral concentration in oral, cloacal, fecal, and water at each time point and identified the maximum in the trajectory; then we assigned all values after the maximum 1, and 0 otherwise). We included a random effect for the focal duck (γ_i) to account for repeated measures on the focal ducks because each focal duck had a unique viral curve. We used viral loads within focal duck samples as linear predictors of transmission probability. The logit transformation naturally induces the expected saturating relationship between viral exposure and transmission probability. Considering each of the four sample types (water, fecal, oral, and cloacal) separately, we found that log-transforming the predictors (after adding 1 to account for cases where the measured viral load was exactly zero) performed better by Akaike information criterion (AIC) than either using the predictors untransformed or using a saturating transformation (which was only explored for water because viral loads in water were cumulative over each 17-hour exposure period).

All candidate models were coded in Matlab (9.9.0.1538559, R2020b, The Mathworks Inc., Natick, MA, USA) using the Statistics and Machine Learning Toolbox. We used the "Laplace" fit method in the fitglme function for estimating fits of the binomial models and the perfcurve function to estimate AUC. We compared model performance using AIC.

All data used in the analyses, including infection status and qPCR data (oral, cloacal, fecal, and water concentrations of influenza A viral RNA) are included as supplemental information.

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Kim M. Pepin, Conceptualization, Data curation, Formal analysis, Investigation, Writing – original draft | Clinton B. Leach, Conceptualization, Formal analysis, Methodology, Writing – review and editing | Nicole L. Barrett, Investigation, Methodology, Writing – review and editing | Jeremy W. Ellis, Investigation, Methodology, Writing – review and editing | Kaci K. VanDalen, Conceptualization, Investigation, Methodology, Writing – review and editing | Colleen T. Webb, Conceptualization, Formal analysis, Methodology, Writing – review and editing | Susan A. Shriner, Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing – original draft, Writing – review and editing

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental material (mBio00862-23-s0001.pdf). Table S1, Figures S1 and S2, and raw data.

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