Experimental infection of ostriches with H7N1 low pathogenic and H5N8 clade 2.3.4.4B highly pathogenic influenza A viruses

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Highlights

- Ostriches infected with H7N1 LPAIV or H5N8 HPAIV shed similar amounts of virus from the tracheas
- Tracheal shedding duration was at least 14 days for H7N1 LPAIV and just beyond 14 days for H5N8 HPAIV
- Influenza A viruses in wing and/or tail feather pulp were evaluated for the first time in ratites
- Only ostrich tracheal swabs with RT-qPCR Ct values < 30 yielded virus isolates in eggs
- RDE treatment of sera prior to HI tests was required to avoid false positive results in H5-infected ostriches

Abstract

Infection dynamics data for influenza A virus in a species is important for understanding host-pathogen interactions and developing effective control strategies. Seven-week-old ostriches challenged with H7N1 low pathogenic viruses (LPAIV) or clade 2.3.4.4B H5N8 high pathogenic viruses (HPAIV) were co- housed with non-challenged contacts. Clinical signs, virus shed in the trachea, cloaca, and feather pulp, and antibody responses were quantified over 14 days. H7N1 LPAIV-infected ostriches remained generally healthy with some showing signs of mild conjunctivitis and rhinitis attributed to *Mycoplasma* co-infection. Mean tracheal virus shedding titres in contact birds peaked 3 days ($10^{6.2}$ EID_{50} equivalents / ml) and 9 days ($10^{5.28}$ EID_{50} equivalents / ml) after introduction, lasting for at least 13 days post infection. Cloacal shedding was substantially lower and ceased within 10 days of onset, and low virus levels were detected in wing feather pulp up until day 14. H5N8 HPAIV - infected ostriches showed various degrees of morbidity, with 2/3 mortalities in the in-contact group. Mean tracheal shedding in contact birds peaked 8 days after introduction ($10^{6.32}$ EID_{50} equivalents/ ml) and lasted beyond 14 days in survivors. Cloacal shedding and virus in feather pulp was generally higher and more consistently positive compared to H7N1 LPAIV, and was also detectable at least until days post infection in survivors. Antibodies against H5N8 HPAIV and H7N1 LPAIV only appeared after day 7 post exposure, with higher titres induced by the HPAIV compared to the LPAIV, and neuraminidase treatment was essential to remove non-specific inhibitors from the H5N8-positive antisera.

Key words: high pathogenic avian influenza, low pathogenic avian influenza, ostriches

1. Introduction

Avian influenza is a highly contagious disease of poultry caused primarily by influenza A viruses (AIV) of the H5 or H7 subtypes. Gallinaceous birds are highly susceptible to infection with mortalities reaching 100 % depending on factors such as the virus strain, host type, age, and immunological status (OIE, 2019; Lee et al., 2021). The highly pathogenic avian influenza (HPAI) virus (HPAIV) subtypes cause epidemics with severe economic losses and some have zoonotic potential, therefore the detection of any H5 or H7 strain is notifiable to the World Animal Health Organization (OIE) and, where this occurs in domestic poultry, causes the immediate suspension of international trade in birds or their products (OIE, 2019). HPAIVs emerge from low pathogenic avian influenza (LPAI) virus (LPAIV) precursors through mutation in terrestrial birds only (Lee et al., 2021), but in recent years, lineages of Asian-origin Goose/Guangdong H5Nx HPAIVs from the northern hemisphere have repeatedly been carried by wild migratory waterfowl to their overwintering sites in the southern hemisphere, posing a recurring threat to poultry production and human health in regions that were previously free of these viruses (Zhang et al., 2020).

Ostriches farmed commercially for meat, leather and feathers fall within the OIE definition of poultry (OIE, 2019). In South Africa, which is the lead global export producer, ostriches are raised in extensive systems in semi-desert regions that are ideally suited to their physiology. Ostriches contract AIVs through direct or indirect contact with wild waterfowl and bridge species. However, they normally show no signs of disease, even when infected with HPAI strains including clade 2.3.4.4B H5N8 HPAIVs that caused outbreaks in South African poultry and wild birds from 2017 to 2018 (Abolnik et al., 2019). In field infections, clinical signs tend only to manifest with inclement weather, concomitant infections with other respiratory pathogens, or poor management. Green urine syndrome (bilirubinuria) is a classical historic indicator of AIV infection in ostriches (Olivier, 2006; Mutinelli et al, 2002;

Manvell et al., 2003), but conjunctivitis, reduced appetite, respiratory and neurological signs may also be present. Mortalities are more common in chicks under 6 months of age, although this seems to be strain dependent (Olivier, 2006; Abolnik et al., 2016; 2019). Similarly, experimental infections of ostriches with LPAI or HPAI viruses mostly produced very mild or no clinical signs (Manvell et al., 1998, Manvell et al 2003; Clavijo et al., 2003; Toffan et al., 2010).

Commercial ostriches are rigorously screened to comply with strict international trade requirements and to prove freedom from AIV infection. Serological testing forms the basis for South Africa's official AIV surveillance and ostrich farms are tested bi-annually at a minimum, but also before and after ostrich movements, and before slaughter. Sampling to identify the agent by real-time quantitative RT-PCR (RT-qPCR) usually initiates only after antibodies are detected (DAFF, 2012) and only rarely after observation of clinical signs or increased mortality rate. Serologic surveillance indicates that AIV infection with multiple subtypes are common in ostriches and occur seasonally (Abolnik et al., 2016), but when the flock is revisited for sampling, the agent is often not detected, or present in too low quantities for identification using molecular methods and/ or virus isolation (Pieterse et al., 2021). This has led to speculation that AIVs circulate at very low levels in ostriches (below the limit of current detection targets), or for short periods of time and/ or that transmission within the flock is poor; but this may not necessarily be the case.

There are substantial differences in the innate capacities of species to replicate and transmit AIVs. Pigeons and doves for example are poor propagators and disseminators of AIVs, but waterfowl and gallinaceous birds are highly efficient and can spread vast quantities of virus through their oral and fecal secretions (Abolnik et al., 2014; Germeraad et al., 2019). Experimental infection studies with AIV in ostriches are few in number but provide some useful data, for example it was previously determined that ostrich tracheal swabs are the

sample of choice for RT-qPCR because AIVs were isolated more frequently and for a longer period from ostrich tracheas than cloacal swabs (Manvell et al., 2003). However, all prior studies in ostriches were conducted pre-2006 and therefore did not include the Asian clade 2.3.4.4 HPAI H5 viruses that emerged later, and none used molecular detection methods to quantify the viral shedding (Manvell et al., 1998, Manvell et al 2003; Clavijo et al., 2003; Toffan et al., 2010).

Data on the dynamics of AIV infection in a particular species such as the site, duration and magnitude of viral shedding is important for understanding host-pathogen interactions and developing effective control strategies (Germeraad et al., 2019). The aims of this study were therefore to conduct two independent challenge experiments in ostriches to investigate the infection dynamics and transmissibility of an ostrich-origin H7N1 LPAIV and a clade 2.3.4.4B H5N8 HPAIV. In addition to quantifying relative tracheal and cloacal shedding using RT-qPCR, we investigated virus in the feather pulp for the first time in ratites. The data will assist in improving AIV surveillance, risk assessment and control measures in ostriches.

2. Materials and Methods

2.1.Challenge viruses

A/ostrich/South Africa/ORD/2012 (H7N1), an LPAIV (Abolnik et al., 2016), and A/Speckled pigeon/South Africa/08-004B/2017 (H5N8), a clade 2.3.4.4B HPAIV (Abolnik et al., 2018) were selected as the challenge strains. A prior study established that a 10^6 EID_{50} dose of A/Speckled pigeon/South Africa/08-004B/2017 (H5N8) administered by the intra-ocular or intra-nasal route to specific pathogen free (SPF) chickens produced 100% mortality within 3 to 4 days (Abolnik et al., 2018). Second passage stocks of both viruses were propagated in 9-

to-11-day old SPF embryonated chicken eggs (ECEs) and the egg infectious dose 50 (EID_{50}) titres were determined according to the Reed and Muench method (1938). Challenge doses of 10^8 EID_{50} per 3 ml were diluted in OculoNasal diluent (Intervet). The selection of this challenge dose was based on prior studies in ostriches where doses ranged from $10^{7.2}$ EID_{50} (Clavijo et al., 2003) to $10^{9.0}$ EID_{50} (Toffan et al., 2010).

2.2.Ostriches and housing

Ostriches were sourced from Buffelskom Boerdery (Pty) Ltd., a commercial farm in the Limpopo province with no history of AIV infections. Chicks aged 5 weeks (n=6) and 7 weeks (n=6) of mixed sexes were kept in an outdoor camp until their enrolment in the experiments. Ostrich starter crumbs (Obaro, Pretoria) and water were provided *ad libitum*. Challenge experiments were conducted in the Poultry Biosafety Level 3 (BSL3) facility at the University of Pretoria's Faculty of Veterinary Science campus. The six birds in each study were kept in a raised pen with a grid floor and dimensions of 2 m x 2.84 m x 0.94 m. The isolation room had natural light and the temperature was maintained at 24°C. Waste was rinsed out daily. Drinking water was de-chlorinated with AviBlue (Lohman Animal Health, Germany) according to the recommended procedure and provided in a 50-litre raised plastic trough that was topped up once or twice daily.

2.3.Experimental design

The studies were designed as an artificial challenge group (n=3) co-housed with a non-challenged contact group (n=3). Challenge trials with the H7N1 LPAI and H5N8 HPAI viruses were run consecutively for 14 days each, separated by a 2-day interval for cleaning and decontamination. The 7-week-old chicks were used in the first challenge trial with H7N1 LPAIV, and the 5-week old chicks were used in the second challenge trial with the

H5N8 HPAIV, so that both experiments used birds of approximately the same age. Both trials followed the same experimental design: at day 0, three chicks from the 7-week age group were randomly selected and moved into the BSL3 facility where each bird was inoculated with 3 ml of the challenge virus via the ocular, nasal, and intra-tracheal routes. On day 2, the drinking trough was thoroughly cleaned and disinfected before three new 7-week-old ostrich chicks (non-challenged contacts) were placed into the pen.

Ostriches were observed twice daily for any clinical signs of disease and body temperatures were measured daily in the early morning with a rectal thermometer. Normal body temperatures of adult ostriches are in the range of 38.2- 40.2 °C (Louw et al., 1969), therefore rectal temperatures ≥ 40.3 °C were interpreted as indicating pyrexia. Sterile plastic applicator rayon-tipped swabs (Copan) were used to sample the trachea and cloaca of each ostrich daily; swabs were stored individually in 2 mL of viral transport media (VTM; brain–heart broth, 0.1 mg/mL doxycycline, 0.1 mg/mL enrofloxacin, 1 mg/mL penicillin–streptomycin and 10% glycerol). At days 3, 5, 7, 9, 11 and 14 a primary wing feather (H7N1 and H5N8 trial) and a primary tail feather (H5N8 trial only) was plucked from each chick and 1 cm of the end of the follicle was cut into sample tubes containing 2 ml of VTM. 5 ml blood samples were collected from the jugular veins at days 0, 7 and 14. Drinking water samples were collected daily into sterile 50 ml containers prior to refilling of the water trough. On day 14, all ostriches were humanely euthanized by intramuscular injection with medetomidine (1 mg/ kg) and after 8 minutes with midazolam (0.15 mg/ kg) and ketamine (2.5- 5 mg/ kg) intravenously to effect, followed by severing of the spinal cord at the base of the neck. Post-mortems were conducted where birds died or showed clinical signs prior to death. All samples were stored at 4 °C until testing. All animal procedures were approved by the University of Pretoria's Research and Animal Ethics committees.

2.4.Antibody detection

Serum was collected from blood left to clot at room temperature for one hour and centrifuged at 5,000 x *g* for 10 min. Sera were tested with the AI Multispecies antibody ELISA (IDEXX Laboratories) kit where a sample to negative (S/N) ratio < 0.50 is considered as positive. This kit detects anti-nucleoprotein (NP) antibodies. Hemagglutination inhibition (HI) tests were performed at Assurecloud (Pty) Ltd veterinary laboratory in Oudtshoorn, according to the OIE-recommended methods for non-chicken species (OIE, 2019), with the modification of incubation at 56°C for 30 minutes prior to chicken red blood cell (CRBC) pre-absorption, as per the official South African laboratory protocol for ostrich sera. Alternatively, ostrich sera pre-absorbed to CRBCs (with no pre-heat treatment) were treated with receptor destroying enzyme (RDE) by incubating 0.5 ml of serum with 0.5 µl of neuraminidase from *Vibrio cholera* (Sigma-Aldrich) overnight at 37°C, and heat inactivating at 56°C for 30 minutes prior to HI tests with the homologous antigen. HI titres were expressed as $\log_2$ of the reciprocal of the highest dilution causing complete inhibition of 4 hemagglutinin units of antigen with 4 $\log_2$ considered as positive.

2.5.Pathogen detection

Swabs and feather shafts stored in VTM were vortexed for at least 30 seconds. Total nucleic acid was extracted from 200 µl of the swab fluids, feather shaft fluids and drinking water with IndiMag Pathogen Kits according to the recommended procedure in an IndiMag® 48 instrument (Indical BioSciences). Influenza A virus matrix and nucleoprotein gene RNAs were detected by RT-qPCR using VetMax™-Gold AIV Detection Kits (ThermoFisher Scientific) in a StepOnePlus™ instrument (Life Technologies, Thermo Fisher Scientific). Reaction mixes comprising 6.25 µl 2 X multiplex RT-PCR buffer, 1.25 µl multiplex RT enzyme mix, 0.5 µl influenza virus primer probe mix, 0.5 µl nuclease-free water were mixed

with 4 µl of RNA. Reactions were incubated at 48°C for ten minutes followed by 40 cycles of 95°C for 15 seconds and 65°C for 45 seconds. Standard curves were produced from 10-fold serial dilutions of RNA extracted from 200 µl allantoic fluid of the titrated H5N8 challenge virus. The H5N8 standard curve was used for both the H7N1 and H5N8 clinical samples. PCR cycle threshold (Ct) values <40 were considered positive, and all RT-qPCR results were expressed as EID_{50} equivalents/ ml. The limit of detection of the VetMax™-Gold AIV RT-rtPCR assay was 0.39 EID_{50} equivalents / ml.

Swabs were frozen at -80°C immediately after processing for total nucleic acid extraction, and were only defrosted once, just prior to virus isolation. Virus isolation in SPF ECEs from H7N1- and H5N8-positive tracheal swabs were performed at Assurecloud (Pty) Ltd laboratory in Centurion using the standard method (OIE, 2019), with up to three passages and/ or filtration through 0.8/0.2 µm Acrodisc syringe filters as required (Pall Corporation, Separations).

Ostriches harbour novel species of Mycoplasma that are commonly associated with upper respiratory tract infections (Botes et al., 2005), therefore total nucleic acid extracts from tracheal swabs were tested for the presence of *Mycoplasma* DNA with a *Mycoplasma* genus assay which amplifies a 280-bp fragment of the 16S rRNA gene (van Kuppeveld et al., 1992), in a Veriti thermal cycler (Applied Biosystems). PCR products were visualized under UV light after electrophoresis in 2% agarose gels stained with ethidium bromide.

2.6. Statistical analysis

Results were interpreted using descriptive statistics and generally at an individual bird level. Positive RT-qPCR results were graphically depicted for each experimental group (including water samples) and subset by the sample location. Boxplots were used where all three animals per group tested positive; otherwise, individual bird results were plotted daily over the experimental period. Sample sizes per animal experimental group were small (n=3).

While formal statistical analysis of differences between groups is likely to be underpowered, the dataset provided an opportunity to establish hypotheses which may be useful in surveillance. To account for incubation periods in groups, particularly in the contact groups, the maximum Ct value for each animal was established for unique sample location. Kruskal-Wallis rank sum tests were performed to establish: (a) whether there were differences in maximum viral titres between HPAI and LPAI in tracheal and cloacal sample locations respectively and (b) whether there were differences in maximum viral titres between sample locations for HPAI and LPAI, respectively. To account for potentially skewed titre values in birds artificially inoculated with virus (the experimental group) a second evaluation was performed on contact birds only. All analytics were performed in R including the tidyr, dplyr and ggplot2 packages for data manipulation and graphics (R Core Team, 2019; Wickham & Henry, 2020; Wickham et al., 2020).

3. Results

3.1. Morbidity and mortality in ostriches was evident with H5N8 HPAI infection, but not H7N1 LPAI infection

In separate experiments, three non-challenged ostriches were placed as contacts with three birds challenged two days prior with high doses of H7N1 HPAI or H5N8 HPAI viruses. All ostriches that were challenged with the H7N1 LPAIV or that were placed contacts remained generally healthy throughout the 14-day trial. Of the three challenged birds, #11 developed mild unilateral conjunctivitis on days 10 and 11 and diarrhoea was noted in bird #15 on day 7, correlating with a peak rectal temperature of 40.4 °C (Supplemental tables 1(a) and (b)). Only one other bird in this group, #13 had mild pyrexia on day 8. In the in-contact group, only #12 showed clinical signs of mild conjunctivitis, rhinitis and laryngitis from day 4 onwards, but the bird recovered by day 13. The clinical signs in bird #12 corresponded with

an onset in fever at day 4 that peaked on day 7 at 40.5 °C. Birds #6 and #16 showed no clinical signs but had intermittent pyrexia starting at day 5 and peaking at 40.7°C on day 14 in bird #6; and from days 8 to 10 in bird #16. An unidentified *Mycoplasma* sp. was detected by PCR in the tracheal swabs of all ostriches (Fig. S1, Supplemental table 2(a)), but there was no correlation between the degree of PCR positivity with the clinical signs of conjunctivitis or rhinitis. No major *post mortem* changes were observed apart from the hyperaemic trachea, larynx and conjunctiva. No signs of bilirubinuria were observed with H7N1 LPAIV infection.

Ostriches infected with H5N8 HPAIV showed various degrees of morbidity throughout the trial with two mortalities in the in-contact group (Supplemental tables 3(a) and (b)). In the challenged group, bird #17 appeared clinically normal for the entire 14-day period, with fever of 40.6 °C recorded only on day 7. Bird #19 was healthy up until day 13, when early signs of weakness, incoordination and torticollis were noted. A fever response in this bird was recorded on days 4 to 6. Bird #18 displayed signs of mild conjunctivitis that started on day 5 and lasted until day 14, with haemorrhages on the third eyelid from days 9 to 11. On day 10, haemorrhages were also visible on the tongue and larynx, with tracheitis on day 14. The clinical signs corresponded with a peak body temperature of 40.8 °C on days 6 and 7, with lower fever responses recorded on most other days until the trial terminated on day 14. In-contact ostrich #20 developed laryngitis and tracheitis from days 4 to 7 that corresponded with fever peaking at 41.1°C on day 10. On day 11 haemorrhages were visible on the inner eyelids and nostrils, and swabs were visible stained with blood. The bird died later that day. Bird #21 appeared clinically healthy up until day 12, but the pyrexia, that had been moderate since onset at day 8 peaked on day 12 at 41.1 °C. On the following day the bird presented with signs of severe conjunctivitis, rhinitis with nasal haemorrhages, laryngitis and tracheitis and it died later that day. Bird #22 survived until 14 days, with only mild clinical signs of

tracheitis from day 7, as well as mild unilateral conjunctivitis on days 10 and 11, which had resolved by day 12. Pyrexia in this bird started at day 8, but did not exceed 40.6 °C. All six ostriches in the H5N8 trial were also infected with a *Mycoplasma* sp. (Supplemental table 2(b)) but there was no correlation with the incidence of conjunctivitis. The degree of strong positivity, however, was 2X more in the H7N1 challenged birds compared to the H5N8 challenged ones, and 4X more in the in-contact H7N1 birds (Supplemental tables 2 (a) and (b)). Bilirubinuria was evident on days from day 5 onward but prominent on days 10, 11 and 14. On *post mortem* examination of birds that died acutely from H5N8 HPAI infection, generalised macroscopic changes included petechiae and ecchymosis of mucous membranes, subcutaneous tissue and serosal surfaces of most organs including the lungs, liver, kidneys, heart, spleen and intestines. Specific changes included hydropericardium, hepatomegaly, haemorrhagic conjunctivitis, severe haemorrhagic tracheitis, laryngitis, pericarditis, encephalitis and meningitis (Fig 1).

3.2. Ostriches seroconverted after 7 days post infection but pre-treatment with neuraminidase was required to remove non-specific inhibitors in sera

AIV-specific antibodies were tested to verify that infection with had occurred, and two methods for pre-treatment of ostrich sera to remove non-specific inhibitors (NSIs) were compared: the official method used in South Africa for ostriches which includes a 56°C heat-treatment step, and RDE-treatment recommended by the WHO for non-poultry species (WHO, 2002). All day 0 blood samples tested negative for the presence of AIV NP-specific antibodies. On day 7, no NP-specific antibodies were detectable in any of the birds infected with H7N1 LPAI or H5N8 HPAI viruses, but HA-specific antibodies detected by HI assay in the H5N8 contact ostriches were only present in heat-treated sera, and not those treated with RDE, indicating that these early titres were non-specific reactors (Table 1). By day 14, all ostriches infected with either H7N1 LPAI or H5N8 HPAI viruses had seroconverted with

NP-specific and HA-specific antibodies. On day 14 in the H7N1-infected birds, a higher HI geometric mean titre (GMT) of 7.7 Log_2 (range 6 -10 Log_2) was obtained with neuraminidase-treated antisera compared to 5.3 Log_2 (range 4-6 Log_2) with the standard treatment. In H5N8-infected birds the GMT at day 14 in the survivors was more similar between the standard and RDE-treatments at 11 Log_2 and 10.8 Log_2, respectively. Overall, HI as well as ELISA GMT's induced by the HPAI virus were ≥ 3 logs higher than those of the LPAI virus after 14 days, irrespective of the antiserum pre-treatment method.

3.3. Tracheal shedding was higher than cloacal shedding and lasted for at least two weeks

Tracheal and cloacal swabs collected daily for 14 days were tested with RT-qPCR to determine the duration, magnitude and patterns of LPAI or HPAI virus shedding in ostriches. An initial peak with a mean titre of $10^{6.15}$ EID_{50} equivalents / ml in the three H7N1 LPAIV-challenged ostriches (tracheal samples) was likely caused by residual challenge inoculum (Fig 2, Supplemental Table 1(a)) Tracheal shedding in these birds peaked again at day 7 with a mean titre of $10^{3.96}$ EID_{50} equivalents/ ml. By day 14, only #15 was still shedding detectable virus from the trachea, but the titre was very low at $10^{0.92}$ EID_{50} equivalents/ ml.

H7N1 LPAIV was detected in the tracheal swabs of 2/3 in-contact ostriches as early as day 3 (1 day after placement), and peak tracheal shedding was reached at day 5 (i.e., 3 days post placement), with a mean of $10^{6.2}$ EID_{50} equivalents/ ml, which was substantially higher than the levels in the ostriches directly challenged with LPAIV. The levels declined thereafter, with a secondary peak at day 9 ($10^{5.28}$$EID_{50}$ equivalents/ ml), but by day 14, 2/3 in-contact ostriches were still shedding virus from the trachea of $10^{1.49}$ and $10^{2.58}$ EID_{50} equivalents/ ml respectively.

Low-level H7N1 LPAIV shedding from the cloacae of all three challenged ostriches was detected at day 1, with a mean of $10^{1.04}$ EID_{50} equivalents/ ml, but could also be the residual of inoculum swallowed during the high-titre challenge (Fig 2, Supplemental table 1(a)). Shedding in both the challenged and contacts groups was erratic, with all three birds per group showing positive results on a single day occurring only on three other occasions – day 10 (both groups) and day 11 (the contact group). No cloacal shedding was detected in any challenged bird after day 12. In the contact group, cloacal shedding was detectable the day after placement, at day 3 in 2/3 birds, with the mean peaking a day later at $10^{2.07}$ EID_{50} equivalents/ ml. The virus was detected intermittently in birds over the 14-day period, but by day 14 low levels of with a mean of $10^{0.81}$ EID_{50} equivalents/ ml were still detectable. Unlike tracheal shedding, the levels in the cloaca were more alike between the challenged and in-contact birds.

As with H7N1, a high quantity of virus (mean $10^{6.30}$ EID_{50} equivalents/ ml) was detected at day 1 in the tracheas of the three ostriches challenged with H5N8 HPAIV, that was likely to be residual challenge inoculum from the previous day because the titres in this group declined slightly before peaking at day 6 with a mean tire of $10^{5.96}$ EID_{50} equivalents/ ml (Fig 3, Supplemental table 3(a)). Unlike the H7N1 tracheal excretion in the challenged group, excretion remained consistent with all three birds testing positive throughout and only a slight decrease in mean titre across the 14 days. By day 14, titres with a mean value of $10^{3.4}$ EID_{50} equivalents/ ml were still being shed from the tracheas in this group. In the contact group, tracheal shedding commenced by day 5 in 2/3 ostriches (i.e. 3 days post placement), but increased rapidly thereafter with tracheal shedding peaking at day 10 (8 days post placement) at $10^{6.31}$ EID_{50} equivalents/ ml. Virus levels in the trachea of $10^{4.2}$ EID_{50} equivalents/ ml were still detected at day 14 in the surviving bird, #22.

Cloacal shedding of H5N8 HPAIV was detected in #17 from day 1, but this bird ceased all shedding from the cloaca after day 12 (Fig 3, supplemental table 3(b)). The onset of detectable cloacal shedding in all three challenged birds occurred on day 6 and peaked at day 8 with a mean of $10^{4.26}$ EID_{50} equivalents/ ml.

Cloacal shedding of the H5N8 HPAI virus was intermittent in the contact group in the first 8 days of the experiment, with a single bird, #20, shedding detectable virus at day 4 (day 2 post placement), but the titres in this bird reached $10^{6.63}$ EID_{50} equivalents / ml at day 9 (one week after placement), and were still high when the bird died at day 11. Cloacal shedding in #21 started to increase from day 11 ($10^{3.23}$ EID_{50} equivalents/ ml) and reached $10^{4.79}$ EID_{50} equivalents/ ml on day 12 before this bird died. Cloacal shedding in the survivor, #22, had reached $10^{4.8}$ EID_{50} equivalents/ ml on day 14.

3.4.Low titres of LPAI and high titres of LPAI viruses were detected in ostrich feather pulp

RT-qPCR was used to determine the levels of AIV that accumulated in ostrich feather pulp over the course of infection with H7N1 LPAI or H5N8 HPAI viruses. Accumulated virus in the wing feather pulp of the three ostriches directly challenged with H7N1 was evident in at least two of the three birds from day 5 with only day 9 resulting in all three birds testing positive with a mean value of $10^{1.46}$ EID_{50} equivalents/ ml (Fig 2; Supplemental table 4(a)). In-contact birds tested more consistently positive, with means around the 10^2 EID_{50}/ml level on days 5, 7, 9 and 11. Virus was still detectable at low levels at day 14 in 2/3 birds of both challenged and in-contact groups.

The wing feather pulp virus titre levels in both challenged and contact birds in the HPAI trial were consistently higher than those of the LPAI trial (Fig. 3, Supplemental table 4(b)). In the

challenged group particularly, there was a rise in titre means from day three through day 14 with all three birds testing positive in this period.

In the contact birds, virus was only detected from day 7 in 2/3 birds and on day 9 in 3/3 birds before peaking on day 11 with a mean level of $10^{3.70}$ EID_{50} equivalents/ ml. The level in the wing feather pulp of #20 was highest on day 11, and the bird died later that day. The higher level in bird #20 also correlates with the earlier peak in tracheal shedding in this individual (Supplemental table 3(b)). In the surviving bird, #22, virus levels of $10^{2.25}$ EID_{50} equivalents/ ml were detected at day 14 when the trial ended. Tail feathers were collected in addition to the wing feathers in the H5N8 trial (Fig. 3, Supplemental table 4(c)). The results were generally similar to the wing feathers except that the peak in virus accumulation in challenged ostriches was detected at day 9 instead of day 11. In the contact birds, it was interesting that the titre in the tail feathers of #21, which died on day 13, was somewhat higher at $10^{3.85}$ EID_{50} equivalents/ ml than what was detected in the wing feather collected at the same time ($10^{2.25}$ EID_{50} equivalents /ml). As with the wing feathers, the mean peak titre of $10^{3.4}$ EID_{50} equivalents/ ml in this group was reached at day 11, and by day 14 the titre in the surviving bird was only slightly higher in the tail feather at $10^{3.14}$ EID_{50} equivalents/ml.

3.5.Maximum levels of viral shedding in the ostrich trachea was comparable between HPAI and LPAI, but cloacal shedding was higher for HPAI

We assessed whether there were differences in maximum viral titres between viruses of different pathogenicity in tracheal and cloacal sample locations, respectively. Where samples were taken from the trachea, both LPAI and HPAI virus maximum levels were generally similar, and this when considering all birds (Kruskal-Wallis chi-squared = 0.92632, df = 1, p-value = 0.3358), and when only considering contact birds (Kruskal-Wallis chi-squared = 0.04902, df = 1, p-value = 0.8248). For the cloacal samples however, this was not the case.

When considering all birds (Kruskal-Wallis chi-squared = 7.4103, df = 1, p-value = 0.006485) and only contact birds (Kruskal-Wallis chi-squared = 3.8571, df = 1, p-value = 0.04953), maximum titre levels were higher for HPAI than for LPAI.

When assessing differences in maximum viral titres between sample locations (trachea, cloaca and wing feathers for LPAI; trachea, cloaca, wing and tail feathers for HPAI) for HPAI and LPAI, respectively. LPAIV was found to be at higher levels in the trachea when compared to the cloaca and wing feathers, both for all birds (Kruskal-Wallis chi-squared = 11.942, df = 2, p-value = 0.002552) and only contact birds (Kruskal-Wallis chi-squared = 5.4222, df = 2, p-value = 0.06646). When all birds were considered, it was unlikely that HPAIV would present at different maximum levels (Kruskal-Wallis chi-squared = 3.8083, df = 3, p-value = 0.2829). When only contact birds were considered however it was found that HPAI was more likely to be at higher levels in the tracheal and cloacal samples when compared to the two feather locations (Kruskal-Wallis chi-squared = 8.5684, df = 3, p-value = 0.03561). We then analysed a sub-set of these data to establish any difference between only the tracheal and cloacal locations – and it's unlikely there is any important difference between them (Kruskal-Wallis chi-squared = 0.44118, df = 1, p-value = 0.5066).

3.6.High levels of AIV accumulated in the drinking water

To investigate the potential role of drinking water as a source of AIV infection, the levels of virus in water samples collected daily were determined. 0.2 ml volumes of un-concentrated drinking water containing sediment particles in suspension were tested by RT-qPCR and the EID_{50} / ml were converted to litres in Fig. 4. Virus detected in the drinking water on days 1 and 2 was most likely residual challenge inoculum, but the trough was thoroughly cleaned and disinfected prior to the placement of the contacts. The trough was raised, thereby eliminating any possibility of faecal contamination. Any virus in the water from day 3

onwards would therefore have been deposited via oropharyngeal shedding from the challenged ostriches in the preceding 24 hours. Peak titres were reached of $10^{6.96}$ EID_{50} equivalents/ ℓ ($10^{3.96}$ EID_{50} equivalents/ ml) for H7N1 LPAIV on day 5 and H5N8 HPAIV on day 10 at $10^{6.99}$ EID_{50} equivalents/ ℓ, but the titres of both viruses never dropped below 10^{3} EID_{50} equivalents/ ℓ ($10^{0.93}$ EID_{50} equivalents/ ml) over either 14 day trial periods. There appeared to be a correlation between peak virus shedding in the trachea (Figs 2 and 3), and amount of virus present in the drinking water.

3.7. AIV could only be isolated from ostrich tracheal swabs with RT-qPCR Ct values below 30

The RT-qPCR Ct threshold value for successful ostrich-origin AIV isolation in ECEs was determined. Only the tracheal swabs were used for virus isolation because these are the standard sample collected in the field, but cost considerations precluded performing virus isolations on all RT-qPCR positive samples. Tracheal swab RT-qPCR results were tabulated and sorted according to descending Ct values from low to high positive (Supplemental tables 1(a) and (b) and 3 (a) and (b); sorted results not shown). Ct category bands were designated as 30.0-39.99; 29.0-29.99; 28.0-28.99; 27.0 to 27.99; 26.00 to 26.99; 25.0 to 25.99 and 24.0 to 24.99. At least two samples per Ct category were tested where possible, but all positive samples in the 30.0-39.99 low positive category were inoculated into eggs.

No H7N1 LPAIVs (0/14) could be isolated from tracheal swabs with Ct values >30 (supplemental table 1). H7N1virus was isolated from 1/ 2 samples in the 29.0-29.99 category (bird #11, day 5; Ct=29.51); 0/3 in the 28.0-28.99 category; 1/2 in the 27.0 to 27.99 category (bird #12, day 11; Ct=27.52), 0/1 in the 26.00 to 26.99 category, and 2/2 in the 24.0 to 24.99 category, namely bird #16, day 10 (Ct=24.76) and bird #6 (Ct= 24.91). No isolations (0/2) were successful in the 25.0-25.99 category.

No H5N8 HPAIVs (0/7) could be isolated from tracheal swabs with Ct values >30 (supplemental table 2). Virus was isolated from 1/2 samples in the 29.0-29.99 category (bird #20, day 9; Ct=29.45); 1/2 were positive in the 28.0-28.99 category (bird #22, day 8; Ct=28.8); 2/2 were positive in the 27.0 to 27.99 category (bird # 21, day 8; Ct=27.3 and bird #20, day 7; Ct=27.4); 2/2 were positive in the 26.00 to 26.99 category (bird #17, day 8; Ct=26.2 and bird #19, day 13; Ct=26.36), and1/2 were positive in the 25.0-25.99 category (bird #17, day 5; Ct=25.31). Overall, for samples with Ct values <30.0, virus isolation was more successful with the H5N8 HPAI virus (7/10, 70 %) compared to the H7N1 LPAI virus (4/12, 33.3 %).

4. Discussion

Clinical outcomes of influenza virus infection in different bird species vary from subclinical to severe with acute death within 48 hours, and depends on virus-specific traits like cellular receptor-binding ability and specific molecular determinants of virulence as well as host-specific factors such as the species' innate susceptibility, age and immune status (Perkins & Swayne, 2002; van Dijk et al., 2018). Experimental infection studies of specific hosts with AIVs of economic or zoonotic importance like the clade 2.3.4.4 H5Nx viruses have become indispensable to assessing risk and designing or refining surveillance and control strategies. A substantial number of such studies have focused on the pathobiology and transmissibility of AIVs in chickens, turkeys, quail, and wild and domestic waterfowl species (Lee et al., 2017; Germeraad et al., 2019), but this data for ratites is limited, even though they are farmed in many countries.

In this study, ostriches were challenged in separate experiments with LPAI H7N1V or clade 2.3.4.4 H5N8 HPAIV, but the group size was a major limitation. Ostrich chicks easily become stressed under an indoor high containment environment, so we were restricted by the

available space and their physical size (~ 15 kg) to a maximum group size of 6. Three birds in each study were challenged with high challenge doses of egg-cultured virus to ensure infection. Two days after the challenge, three non-challenged contact chicks were introduced. As expected, infection with the H7N1 LPAIV caused zero mortalities and only mild symptoms of conjunctivitis and rhinitis in one bird that was likely associated with *Mycoplasma* co-infection. Bilirubinuria has been observed in field infections with H7N1 LPAIV (Olivier et al., 2006) as well as experimental infections (Manvell et al., 2003), but was absent with the strain of ostrich-origin H7N1 LPAIV we used.

The death of two in-contact ostriches in the H5N8 HPAI trial was unexpected, because the near-homologous strains in the 2017-2018 outbreaks in ostriches caused sub-clinical infections on most farms, with only three farms recording mortalities of any significance: two breeder farms had 5 % mortality and one slaughter farm had 10 to 12 % mortality and only ostriches over 6 months were affected (Abolnik et al., 2019). One of the three challenged ostriches in the present study remained clinically healthy throughout the 14-day period, whereas the other two showed varying degrees of morbidity but recovered, but only one of the contact ostriches survived to 14 days. Ostriches use their open beaks to scoop water into their gular pouch, which rinses some food debris and mucous back into the water source. AIV accumulates in sediment from poultry drinking water (Leung et al., 2007; Munoz-Aguayo et al., 2019; Numberger et al., 2019), therefore de-chlorinated water containing the accumulated sediment was used to facilitate virus transmission to the non-challenged contacts here (although airborne transmission may also have occurred). Ostriches of the age associated with the trial but under farming conditions would typically drink approximately ~2 litres of water per day (A. Olivier, pers. comm.) but we observed the birds to be consuming at least 8 litres of water each per day, probably due to its unfettered access, which correlated with a daily viral intake of between $10^{4.8}$ and $10^{7.9}$ EID_{50} equivalents. The heavily contaminated

drinking water was the likely source of virus transmission to the non-challenged contacts but also likely contributed to the deaths of the two birds in the H5N8 HPAI trial. Dose-dependent effects of AIV exposure are better understood in mice and other mammalian species than birds, where it was shown that the host's immune response can contribute to the pathogenicity of influenza infection. In mice, an initial high virus load led to inappropriate CD8+ T cell responses and elevated production of pro-inflammatory cytokines, causing acute lung injury, respiratory distress syndrome and multi-organ failure (Marois et al., 2012). High fevers, neurological signs and haemorrhagic lesions in various organs are consistent with a similar mechanism in ostriches. The pathogenesis in poultry is well described and death due to multiple organ failure is the result of virus replication in cells, or mediators such as cytokines or ischemia due to vascular thrombosis or cardiovascular collapse as a result of coagulopathy or disseminated intravascular coagulation (Swayne, et al., 2020).

Determining the duration, magnitude and patterns of the viral shedding using RT-qPCR was a key objective of this study because all previous experimental infections of ostriches had relied upon virus isolation for agent detection (Manvell et al., 1998, Manvell et al 2003; Clavijo et al., 2003; Toffan et al., 2010) but RT-qPCR is the detection method used in practice. Prior studies with LPAI H5 or H7 viruses reported isolation from the tracheas until days 11 or 12 post infection and in the cloacae up until day 5 or 11 (Manvell et al., 1998; 2003). Our results generally concur with the previous findings for LPAI, but another limiting factor was that this study was not continued beyond 14 days. There was a distinct difference in tracheal shedding patterns detected in challenged vs. in-contact ostriches in the H7N1 LPAI trial, but the challenge method represented an artificial route of exposure, therefore the in-contact results in both the H7N1 and the H5N8 experimental studies were likely to be a truer reflection of the infection dynamics in ostriches, albeit at higher viral exposure due to water intake patterns as discussed above. The trajectory of H7N1 LPAIV tracheal shedding in

the contact birds suggests that virus is detectable for at least 13 and up to14 days post infection in ostriches. H7N1 LPAIV cloacal shedding was also consistent with previous findings for ostriches with much lower magnitude compared to tracheal shedding and cessation in the challenged birds within 10 days of onset. A meta-analysis of a large number of studies on experimental infections of other poultry species (chickens, ducks, goose, guinea fowl, pheasant, pigeon, quail and turkey; Germeraad et al., 2019) reported that chickens shed LPAIVs from the respiratory tract for an average of 6.2 days (0.8-17.8) and cloaca for 5.5 days (0.7 to 15.7); ducks from the respiratory route at an average 5.3 days (0.7 to 15.3) and cloaca 8.2 (1.0 – 23.3) and turkeys had the longest at a mean 10 days (1.3-28.7 from the respiratory tract) and 14.1 days (1.8 to 40.2) from the cloaca. From our and previous data, ostriches shed LPAIVs from the respiratory tract for longer and at higher levels than the cloaca, with shedding patterns more consistent with turkeys than other species.

Prior experimental infections of ostriches with H5 or H7 HPAIVs indicated shedding for up to 12 or 14 days in the tracheas and between 5 and 14 days from the cloacae (Manvell et al, 1998; 2003; Clavijo et al., 2003). The Asian-type HPAI H5N8 lineage however has unique characteristics compared to the strains used in previous trials, and our results showed shedding beyond 14 days that could, judging from the trajectory of our graphs, extend by a further two or three days. Cloacal shedding for this H5N8 HPAI strain was generally higher and more consistently positive when compared to the H7N1 LPAIV, and also lasted beyond 14 days in ostriches. These findings are consistent with those for certain H5 clade 2.3.4.4 strains in Pekin ducks where oropharyngeal shedding lasted an average 12 days but reached up to 18.5 days and cloacal shedding of 9.4 days up to 16.6 days; and Eurasian widgeons with average oropharyngeal shedding lasting 11.1 days up to 18.4 days and cloacal shedding of 9.0 up to 15.7 days. The peak magnitude in shedding in these ducks also reached ~10^6 EID_{50}/ ml (Beerens et al., 2021), therefore we find that the shedding dynamics of HPAI clade 2.3.4.4

H5 viruses in ostriches resemble that of certain wild waterfowl species. The analysis of differences between groups showed that sampling at the tracheal level is more likely to result in similar results across viral types than sampling from the cloaca and other routes. This route of sampling is employed in the industry and our results support this approach.

The use of feathers has been touted as an attractive diagnostic target for poultry because feathers offer many practical and logistical advantages for collection and storage before processing and could be less sensitive to degradation by bacteria compared to oropharyngeal, tracheal or cloacal swabs (Perkins & Swayne 2001; Yamamoto et al., 2008; Aiello et al., 2013; Bertran et al., 2013; Nuradji et al., 2015; Busquets et al., 2010; Gaide et al., 2021). Studies in chickens, ducks, and quail, show that during active infection HPAIV is detectable in the feather pulp as early as the first day post infection, reached a maximum peak at 2-3 days post infection and increases proportionally with viremia. HPAI viral load in these species' feathers are reported to reach significantly higher levels than oropharyngeal and cloacal swabs and is detectable for a longer period (Perkins & Swayne 2001; Yamamoto et al., 2008; Aiello et al., 2013; Bertran et al., 2013; Nuradji et al., 2015; Gaide et al., 2021). It is thought that since the feather pulp is highly vascularized, the elevated amounts of virus may result from the active infection in feather epidermal cells as well as viruses deposited there during viremia (Yamamoto et al., 2008; Busquets et al., 2010; Gaide et al., 2021). Our findings showed similarities to these patterns although more so in challenged animals compared to the contacts. Sampling feathers was not performed with the same frequency so a direct comparison cannot be accurately made and more work on this sampling route would need to be undertaken.

Studies with LPAIVs in feathers are less common because viral replication is normally restricted to the epithelium of the respiratory and gastrointestinal tracts, but in chicken infection studies, a range of LPAIV subtypes could be detected systemically, as well as in the

peripheral blood mononuclear cells (Post et al., 2013). Bertran et al (2013) investigated European quails challenged with H7N2 LPAIV but did not detect virus in the feather pulp even though oropharyngeal and cloacal shedding was detected in challenged and in-contact birds. Lebarchenon et al (2013) tested suspensions of whole wild duck feathers collected from the environment and found that 39% were positive compared to 28% of fresh faecal swabs collected at the sampling sites and they identified multiple LPAI subtypes, but it was undermined whether these were viruses deposited on the feather surface by preening or contaminated water or accumulated within the feather shaft through active infections. In this first data for ratites, H7N1 LPAIV was detected in wing feather pulp, but levels in contact birds' wing feathers were substantially lower than tracheal swabs at peak levels and at day 14 when the trial ended and it did not graphically appear that virus would persist in the feathers for longer than tracheal swab shedding.

The H7N1 trial was completed first, and only when the H5N8 trial commenced did we decide to collect tail feathers too for comparison with the wing feathers. Ostrich wing feathers have a high commercial value whereas the tail feathers are discarded, and wing feather shafts are also much larger in diameter than tail feather shafts and more consequently more challenging to collect. It was important to establish that there was no substantial difference in virus levels between these two sources as the tail feather would be a more pragmatic sample than a wing feather. More work is required on this topic as challenged birds showed consistently higher levels in both tail and wing locations and it would be important to ensure that findings in natural infection are likely to maintain high levels of detectable virus. We conclude that the diagnostic value of ostrich feathers for AIV surveillance during LPAI or HPAI infections is likely to be limited, and the tracheal swab currently remains the best sample for virus detection.

Virus isolation is still considered the "gold standard" for agent identification, where it is used primarily for diagnosis of the first clinical case and to obtain antigen for subsequent testing (OIE, 2019). Virus isolates from RT-qPCR positive ostrich field samples have been rare (Abolnik et al., 2016), but recent studies aimed at investigating the possible reasons for this determined that the standard PBS: glycerol transport medium (50 %, v/v, without antimicrobials) negatively affects AIV viability in ostrich tracheal swabs (Pieterse et al 2021) and that excluding antimicrobials from VTM encourages the growth of specific bacteria that produce metabolites with anti-viral properties (Abolnik et al., 2021). The ostrich tracheal swabs in the present study had been stored in an appropriate protein-rich VTM that included antimicrobials; therefore we used them to determine the limits of detection for virus isolation from ostrich tracheal swabs. We established that for ostrich tracheal swab viruses the limit of detection for isolation in SPF ECEs was ~ Ct 29.5 for both H7N1 LPAI and H5N8 HPAI viruses.

Finally, after removing non-specific inhibitors with RDE treatment, we established that in response to a high virus titre challenge, antibodies against H5N8 HPAIV as well as H7N1 LPAIV appeared somewhere between days 7 and 14 post infection. The appearance of the earliest detectable AIV-specific antibodies shortly after day 7 is consistent with the only two other studies that investigated sero-conversion in ostriches (Clavijo et al., 2003; Toffan et al., 2010). Our results also showed that antibody levels induced by the HPAI virus were higher than those for the LPAIV. In South Africa, sampling to detect the agent is only mobilized in response to the presence of specific antibodies in ostriches or in the event of suspicious clinical signs. With most ostrich farms located in remote areas, logistics and serology test turnaround times of 72 hours means that it would usually take up to a week before the state veterinarian revisits the farm to collect swabs for follow-up testing. Hypothetically, if the earliest detectable antibodies appear at day 8, the tracheal swabs would only be collected at

day 15 post infection. According to our results, clade 2.3.4.4B H5N8 HPAIVs could still be detectable in tracheal swabs at day 15, albeit at low levels, but LPAIV is very highly likely to be missed. A swab-based agent detection surveillance program in the investigation period that requires that known seropositive birds are re-sampled is likely to be less sensitive (for outcomes that only RT-qPCR or isolation can provide) than one where known seropositive birds are excluded from the sampling frame.

5. Conclusion

The infection dynamics of AIVs in ostriches were more similar to turkeys (for H7 LPAI) and wild waterfowl (for clade 2.3.4.4B H5 HPAI) (Germeraad et al., 2019) than other terrestrial avian species, with viruses being shed for at least 14 days from the trachea, and both LPAI and HPAI virus maximum levels were generally similar. Cloacal shedding was much lower and erratic, but shed levels of HPAI were substantively higher than that of LPAI. Tracheal swabs remain the sample of choice for detecting AIVs in ostriches compared to cloacal swabs and feathers, as we established here for the first time. Our findings show that LPAI and HPAI viruses are unlikely to circulate at low levels or for short periods within ostriches, and further work to confirm this should entail a longitudinal study of field infections, using RT-qPCR. Drinking water containing accumulated sediment contained high levels of AIV RNA; therefore the drinking trough could present an ideal environmental sampling point that conveniently represents large numbers of birds in epidemiological units, and could be especially useful in detecting inapparent infections such as ostrich influenza. This study also found that the current standardized pre-treatment method used for ostrich sera caused false-positive reactions in H5 HPAI diagnosis, therefore neuraminidase (RDE) treatment should be applied to remove non-specific inhibitors, and it is furthermore recommended that only RT-qPCR positive samples with Ct values <30 be inoculated into eggs for virus isolation. Overall, applying the findings of these results will aid in improving AIV surveillance, risk

assessment and control measures in ostriches. Industry has already applied some of these findings and basic biosecurity measures such as chlorination of water and cleaning troughs are standard practises. Early response by provincial veterinary services would allow ostriches to move within the window of the recommendation for sampling and detection of viral material.

Conflict of interest

None

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Figure Captions

Figure 1. Post mortem lesions in seven-week-old ostriches that died acutely of a clade 2.3.4.4B H5N8 HPAI infection, showing widespread haemorrhages of (a) the third eyelid (nictitating membrane), (b) the oropharyngeal mucosa, (c) tracheal and subcutaneous tissue, (d) the pericardium, epicardium and liver and (e) bilirubinuria manifested from day 5 post infection.

Figure 2. H7N1 LPAI viral titres as detected by RT-qPCR (Log_{10} EID_{50} equivalents /ml) by experiment day, grouped by the experimental (red) and contact (blue) groups and categorised by the sample location. Where all three ostriches per group per day tested positive box plots have been used to indicate mean and spread of titre values. Where not all ostriches tested positive per group per day actual titres are indicated by dots in the graphs.

Figure 3. H5N8 HPAI viral titres as detected by RT-qPCR (Log_{10} EID_{50} equivalents /ml) by experiment day, grouped by the experimental (red) and contact (blue) groups and categorised by the sample location. Where all three ostriches per group per day tested positive box plots have been used to indicate mean and spread of titre values. Where not all ostriches tested positive per group per day actual titres are indicated by dots in the graphs.

Figure 4. Influenza A virus titres as detected by RT-qPCR (Log_{10} EID_{50} equivalents/ l) in unconcentrated ostrich drinking water.

Figure S1. PCR detection of mycoplasma DNA in the tracheal swabs of bird #12 (a) and bird #16 (b) presented as examples. Complete PCR results are presented in Supplemental tables 2 and 4.

Figure 1

Figure 2

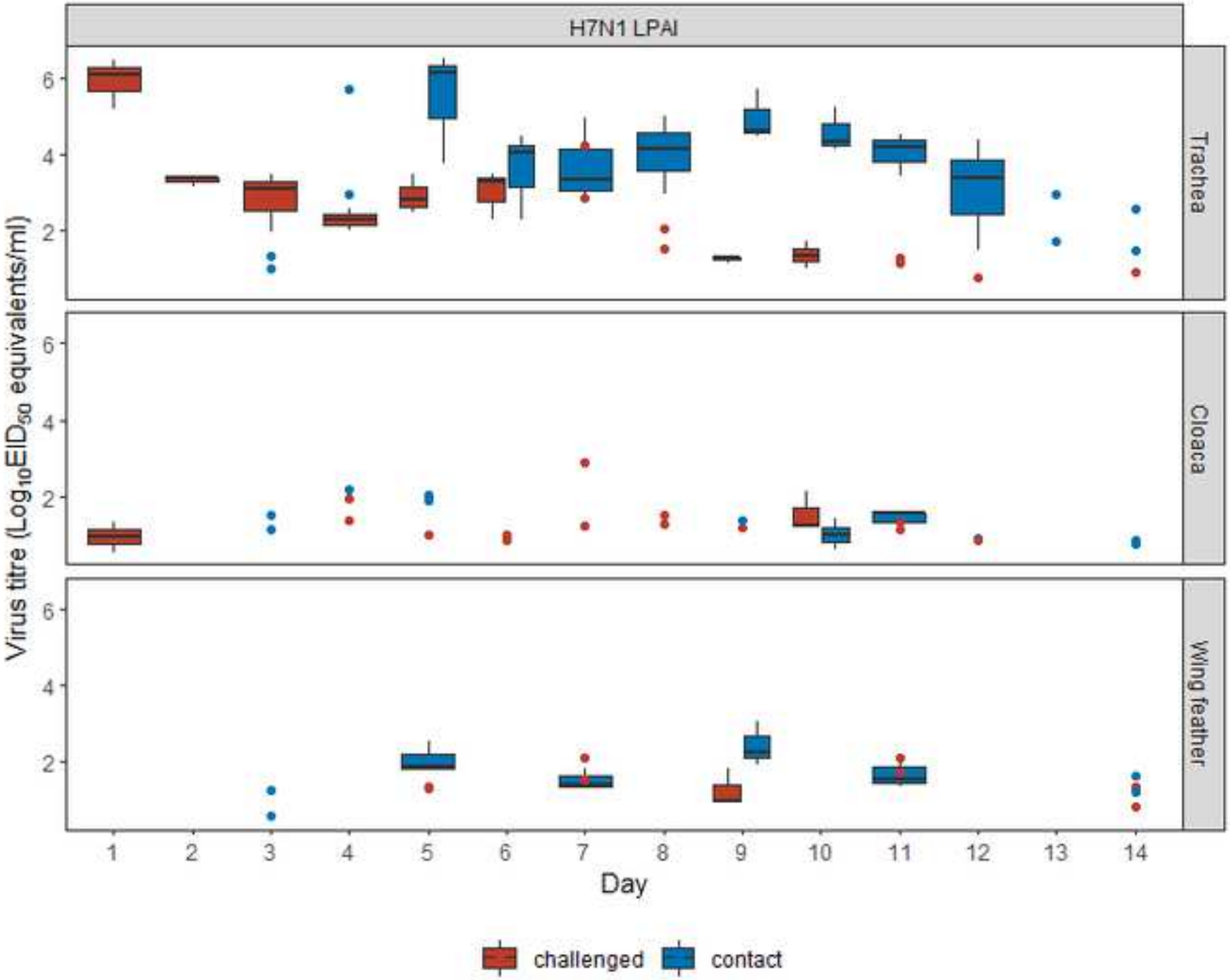
H7N1 LPAI
Trachea
Cloaca
Wing feather
Virus titre (Log10EID50 equivalents/ml)
Day
challenged
contact

Figure 3

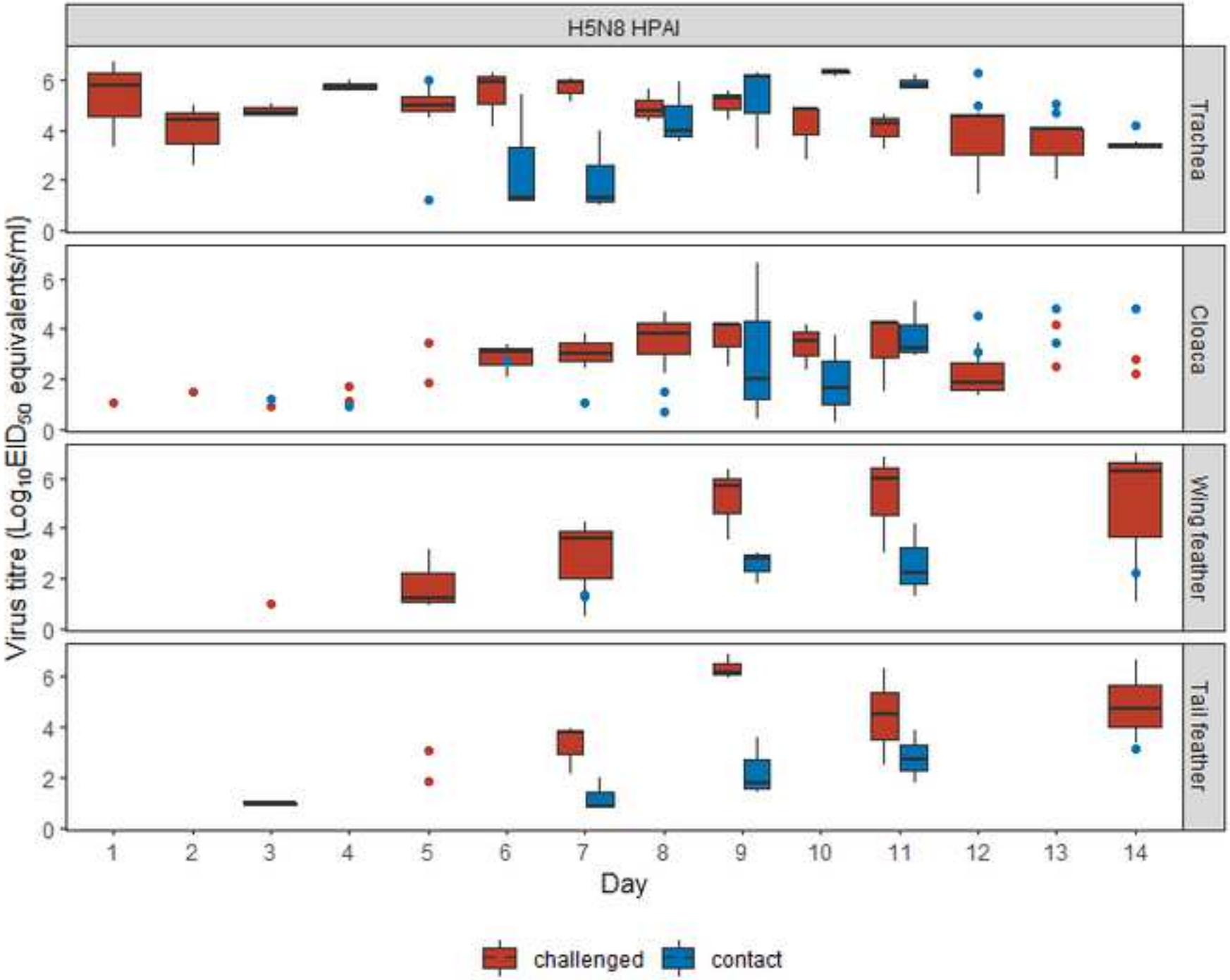
H5N8 HPAI
Trachea
Cloaca
Wing feather
Tail feather
Virus titre (Log10EID50 equivalents/ml)
0
2
4
6
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Day
challenged
contact

Figure 4

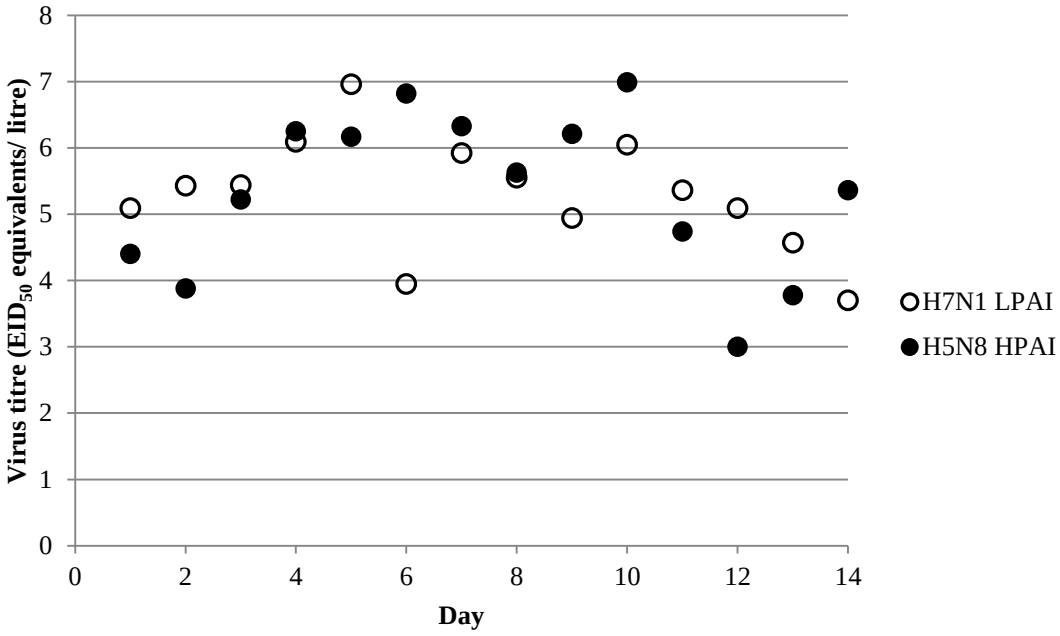
Virus titre (EID50 equivalents/ litre)
8
7
6
5
4
3
2
1
0
0
2
4
6
8
10
12
14
Day
H7N1 LPAI
H5N8 HPAI

Table 1. Antibody responses in ostriches

Virus	Bird no.	Day 7			Day 14		
		NP ELISA S/N	Log_2 HI titre		NP ELISA S/N	Log_2 HI titre	
			Standard treatment	RDE treatment		Standard treatment	RDE treatment
H7N1 LPAI	11 (challenged)	0.88	0	0	0.37	5	6
	13 (challenged)	0.81	0	0	0.47	6	7
	15 (challenged)	0.83	0	0	0.31	4	8
	6 (contact)	0.95	0	0	0.31	5	6
	12 (contact)	0.86	0	0	0.33	6	10
	16 (contact)	0.99	0	0	0.38	6	9
Geometric mean titre:		0.87	0	0	0.36	5.3	7.7

	17 (challenged)	0.80	0	0	0.17	10	11
	18 (challenged)	0.96	0	0	0.41	11	12
	19 (challenged)	0.55	0	0	0.27	11	11
H5N8 HPAI	20 (contact)	1.08	4	0	bird died		
	21 (contact)	0.81	7	0	bird died		
	22 (contact)	0.72	7	0	0.27	10	9
Geometric mean titre:		0.82	6	0	0.28	11	10.8

NP- nucleoprotein

S/N- sample to negative

RDE- receptor destroying enzyme

HI- hemagglutination inhibition