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Hampered Foraging and Migratory Performance in Swans Infected with Low-Pathogenic Avian Influenza A Virus

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It is increasingly acknowledged that migratory birds, notably waterfowl, play a critical role in the maintenance and spread of influenza A viruses. In order to elucidate the epidemiology of influenza A viruses in their natural hosts, a better understanding of the pathological effects in these hosts is required. Here we report on the feeding and migratory performance of wild migratory Bewick's swans (*Cygnus columbianus bewickii* Yarrell) naturally infected with low-pathogenic avian influenza (LPAI) A viruses of subtypes H6N2 and H6N8. Using information on geolocation data collected from Global Positioning Systems fitted to neck-collars, we show that infected swans experienced delayed migration, leaving their wintering site more than a month after uninfected animals. This was correlated with infected birds travelling shorter distances and fuelling and feeding at reduced rates. The data suggest that LPAI virus infections in wild migratory birds may have higher clinical and ecological impacts than previously recognised.

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INTRODUCTION

Influenza A viruses have been isolated from many species including humans, pigs, horses, mink, felids, marine mammals and a wide range of domestic birds, but wild birds belonging to the orders Anseriformes (particularly ducks, geese, and swans) and Charadriiformes (particularly gulls, terns, and waders) are thought to form the virus reservoir in nature [1]. In wild birds and poultry throughout the world, influenza viruses representing 16 hemagglutinin (H) and 9 neuraminidase (N) antigenic subtypes have been detected [1,2]. In contrast to other virus subtypes, influenza viruses of subtypes H5 and H7 may become highly pathogenic following introduction in poultry and cause outbreaks of highly pathogenic avian influenza (HPAI). Although LPAI viruses may also cause mild symptoms in poultry, it is generally thought that they do not cause disease in wild birds [3]. However, this information is based primarily on experimental infection of captive birds rather than information on natural infections of wild migrating birds. Subclinical or mild diseases observed in the laboratory may have significant ecological fitness consequences in the field.

Upon infection, migratory birds may reallocate some of their resources to boost their immune system [4], at the expense of other demanding processes such as the accumulation of body stores, which are required to fuel their migratory flights and to overcome periods of adverse weather and foraging conditions. Lower fuelling rates will presumably lead to slower migrations with more frequent stopovers along the route [5]. In the context of our studies on migration and feeding patterns of Bewick's swans (*Cygnus columbianus bewickii* Yarrell, breeding in NW Russia and wintering in NW Europe), 25 swans were caught in The Netherlands at one of their main wintering sites, and 12 were fitted with a coded GPS-collar (Figure 1). From the same group of birds, cloacal swabs and blood samples were collected to test for the presence of influenza A virus as part of ongoing wild bird influenza A virus surveillance [6]. Three out of 25 swans were infected with influenza A virus at the time of capture, two of which we could track in great detail due to them carrying a GPS-collar. Here we compare their feeding and migratory performance with the performances of their healthy counterparts.

RESULTS

Influenza A virus isolates of subtypes H6N2 and H6N8 were obtained from two out of the three infected birds (the GPS-collared ones). Although no virus isolate could be obtained from the third infected swan, sequence analyses on PCR products obtained from the original sample revealed 100% nucleotide sequence identity of the HA and NA genes with the HA and NA of A/Bewick's swan/Netherlands/2/05 (H6N8). Phylogenetic analyses based on the H6 HA1 nucleotide sequences and all H6 HA1 sequences available from public databases revealed that the H6 HA1 genes clustered in four different groups as described by Spackman et al. [7]. Furthermore, the swan H6 gene segments were closely related to sequences obtained from an influenza A virus found in a free-ranging migrating duck in NW Europe (data not shown). Representative full-length H6 HA sequences from each of the 4 different lineages were used to construct a bootstrapped DNA maximum likelihood tree (Figure 2). BLAST analyses of the 7 other gene segments of the two swan viruses,

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Figure 1. Bewick's swan 923A infected with an H6 influenza A virus. This (adult) individual not only carried an active influenza infection, it also showed the highest antibody response among all 25 sampled birds. Photo by W. Tijsen.
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indicated a 95 to 99 percent nucleotide sequence identity with avian influenza A viruses of Eurasian origin. The phylogenetic and BLAST analyses indicate that the two influenza A viruses obtained from the infected swans are ordinary LPAI viruses circulating in the natural wild bird reservoir.

Serological analyses revealed that 5 out of 25 swans had detectable antibodies against the nucleoprotein (NP) of influenza A viruses (3 adults, 1 yearling and 1 juvenile out of 20 adults, 2 yearlings and 3 juveniles). The highest antibody response was found in the two swans from which influenza A viruses were isolated (an adult bird and a yearling). The sera of the 5 swans with influenza A virus NP antibodies were further analysed using the hemagglutination inhibition assay (HI). The HI was performed with virus strains representing all currently known influenza A virus subtypes (H1–H16). No specific HI antibody titres were detected in the 5 sera.

Not knowing the infection status of the swans during processing immediately after capture, two of the three influenza A virus infected swans were fitted with a GPS collar (the ones from which we were able to obtain virus isolates). The 3 infected and 22 uninfected swans did not differ in structural body size ($F_{1,21} = 0.62$, $p>0.4$; GLM with age [$F_{2,21} = 0.10$, $p>0.9$] as covariate; body size was calculated as the first principal component [PC1] from a PCA including wing length and total head size, a method often used in avian studies [8,9], e.g. see [10,11] for applications in swans), nor in body mass right after catch ($F_{1,20} = 0.17$, $p>0.6$; GLM with age [$F_{2,20} = 1.60$, $p>0.2$] and body size [$F_{1,20} = 23.03$, $p<0.0005$] as covariates). This result was maintained when restricting the analyses to the 12 GPS-collared birds ($F_{1,8} = 0.81$, $p>0.3$ for body size; $F_{1,7} = 0.80$, $p>0.4$ for size-corrected body mass).

While the biometric parameters of the GPS-collared birds did not differ at the time of capture, infected and uninfected birds differed in all ecological parameters collected after release. Date of departure from Wieringermeer, the agricultural area in NW Netherlands where the birds were caught, differed by more than a month, with the infected birds leaving the area later than the uninfected birds (Figure 3A; $F_{1,7} = 10.53$, $p<0.05$; GLM with age [$F_{2,7} = 0.29$, $p>0.7$] and body size [$F_{1,7} = 0.61$, $p>0.4$] as covariates). Compared to the phenology of the population as a whole (Figure 3B), the healthy birds were among the first to leave the study area, while the infected birds were among the last. Furthermore, the next (spring stopover) site visited was nearer by Wieringermeer for infected individuals than for uninfected individuals (Figure 3A; $F_{1,6} = 11.03$, $p<0.05$; GLM with age [$F_{2,6} = 0.43$, $p>0.6$] and body size [$F_{1,6} = 0.58$, $p>0.4$] as covariates; note that we have one degree of freedom less since

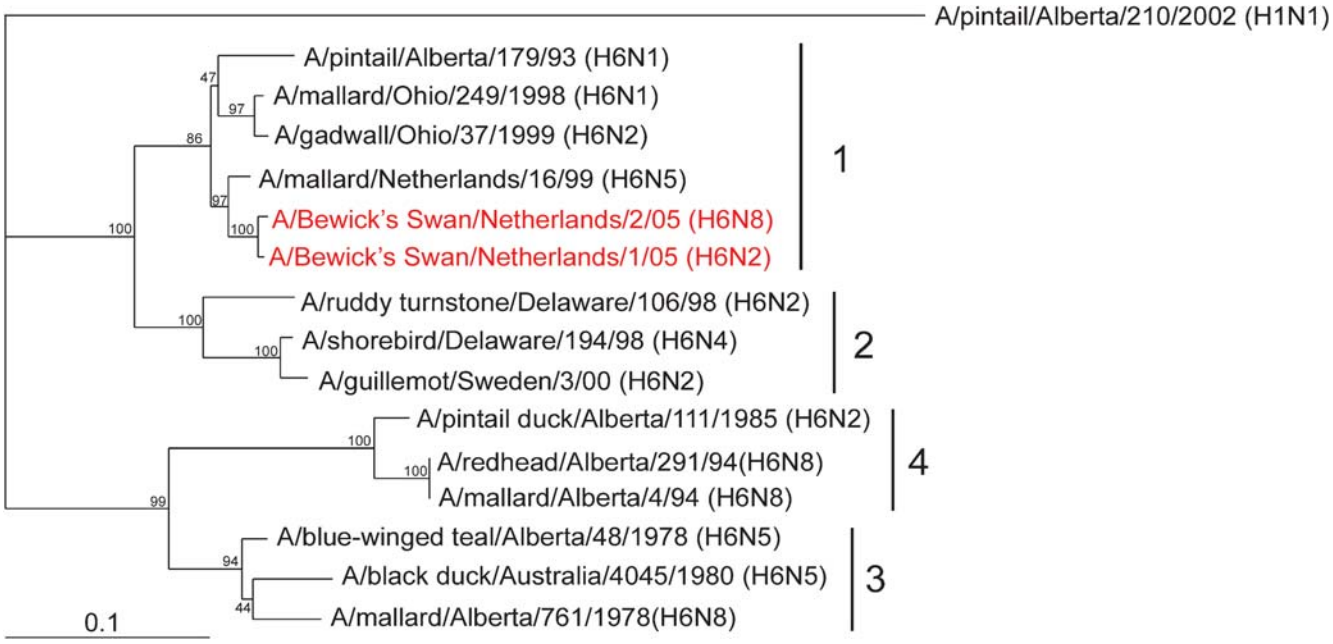

Figure 2. Phylogenetic tree for H6 influenza A viruses isolated from swans. The DNA maximum likelihood tree was constructed using A/pintail/Alberta/210/2002 (H1N1) as out-group and includes sequences from public databases. Genes clustered into four different groups as described by Spackman et al. [7]. The scale bar represents ~10% of nucleotide changes between close relatives. Small numbers in the tree represent the bootstrap values.
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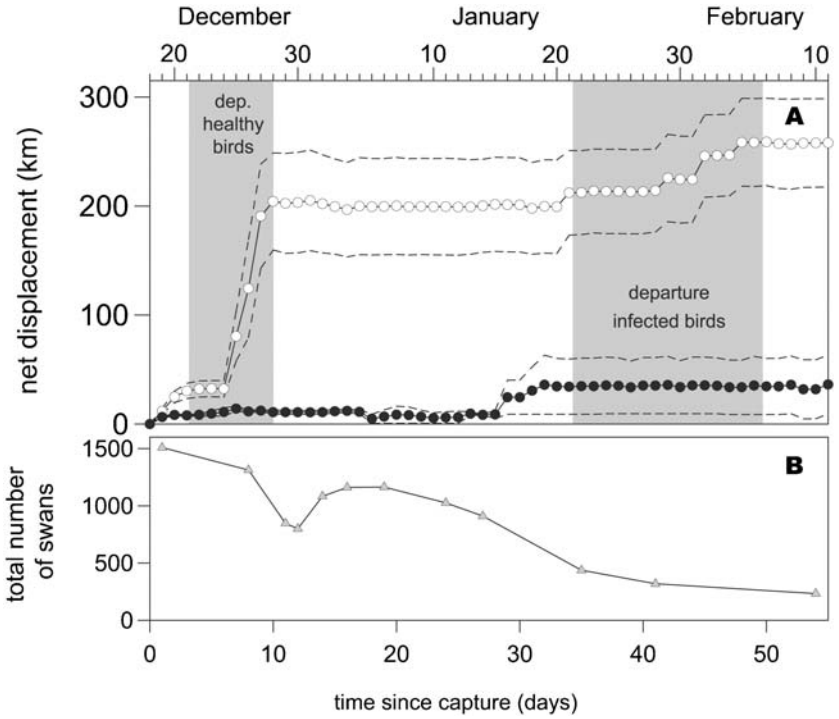

Figure 3. Timing of migration in healthy and infected swans. (**A**) Infected swans left the study area more than a month after the uninfected birds, indicated by broad vertical grey bars ranging from mean-SE to mean+SE. Moreover, their mean net displacement after departure (closed dots±dashed SE-lines) was shorter than for the uninfected birds (open dots±dashed SE-lines). Sample sizes of displacements estimates: $n = 2$ for infected birds (whole time span); sample size declined for uninfected birds due to birds that flew out of range: $n = 10$ (days 0–41), $n = 9$ (42–47), and $n = 8$ (48–55). (**B**) Total number of swans in our study area declined more or less gradually over time (W. Tijsen et al. unpubl. data), suggesting a continuous departure of about 30 birds per day (apart from the short dip around day 12 during a snowy cold spell).
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one bird was never seen after it left the study site). In spite of these differences in distance flown, a possible trigger to move on to the next site is the absolute amount of accumulated body stores [5,12]. Indeed, rate of fuelling, expressed as the rate of change in the visually scored abdominal-profile index (API) [13–15], was lower in infected birds than in uninfected birds (Figure 4A; $F_{1,2} = 22.96$, $p<0.05$; GLM with age [$F_{1,2} = 4.95$, $p>0.1$] and body size [$F_{1,2} = 0.42$, $p>0.5$] as covariates), whereas APIs upon departure did not differ ($F_{1,2} = 1.90$, $p>0.3$; GLM with age [$F_{1,2} = 0.15$, $p>0.7$] and body size [$F_{1,2} = 2.09$, $p>0.2$] as covariates).The observations on fuelling rates are consistent with the lower feeding or bite rates observed in the infected birds compared with the uninfected animals (Figure 4B; $F_{1,2} = 47.55$, $p<0.05$; GLM with age [$F_{1,2} = 0.94$, $p>0.4$] and body size [$F_{1,2} = 23.34$, $p<0.05$] as covariates). Possibly, infected swans cannot achieve high bite rates because they have impaired digestive functions as compared to their healthy counterparts (bite rates in herbivorous waterfowl are generally considered to be digestion- rather than encounter-limited [16]). This is supported by the finding that infected swans took fewer bites per produced faecal dropping than uninfected swans (Figure 4C; $F_{1,2} = 19.28$, $p<0.05$; GLM with age [$F_{1,2} = 19.19$, $p<0.05$] and body size [$F_{1,2} = 29.35$, $p<0.05$] as covariates).

DISCUSSION

Influenza A virus infected Bewick's swans thus showed reduced feeding and migratory performances. Fuelling in avian migrants is thought to be enhanced by the flexible enlargement of digestive organs [17], and indeed fuelling Bewick's swans increase the length of their intestine by 50% throughout winter [18]. In the infected birds the ability to make adjustments to the digestive system may have been impaired (as revealed by the lower bite-to-dropping ratio), leading to reduced bite rates, reduced fuel storage rates and a delayed migration schedule. That the infected birds ended up in a negative feedback loop *because* they traded off resources allocated towards "fighting the infection" with resources allocated towards fuelling and developing the digestive machinery can of course only be firmly established by experimental manipulation of infections in otherwise similar wild birds. An obvious alternative explanation is that the infected swans were poor foragers with such reduced energy allocation towards "infection prevention" that they could not *prohibit the control* of the infection. However, the previously-infected-but-still-seropositive birds were feeding ($F_{1,1} = 0.01$, $p>0.9$), fuelling ($F_{1,1} = 1.87$, $p>0.4$) and migrating ($F_{1,5} = 1.56$, $p>0.2$) at the same pace as the fully healthy, seronegative birds (all tests are GLMs with body size as covariate), which makes the former point of view more likely since it suggests virus-recovered birds to behave normally again. Moreover, the fact that at capture there was no difference in body condition between infected birds and uninfected birds may be another support for LPAI infection being the cause rather than the consequence. Presumably the two swans with active infections got infected shortly before capture, which resulted in reduced migratory and foraging performance. As migratory (re)fuelling in Bewick's swans takes place from late October onwards [19], the "poor-forager idea" would predict the infected birds to have had lower body conditions already at the time of capture (mid December), which was not the case. Whatever cause and effect may be in this matter, the epidemiology of at least one LPAI strain

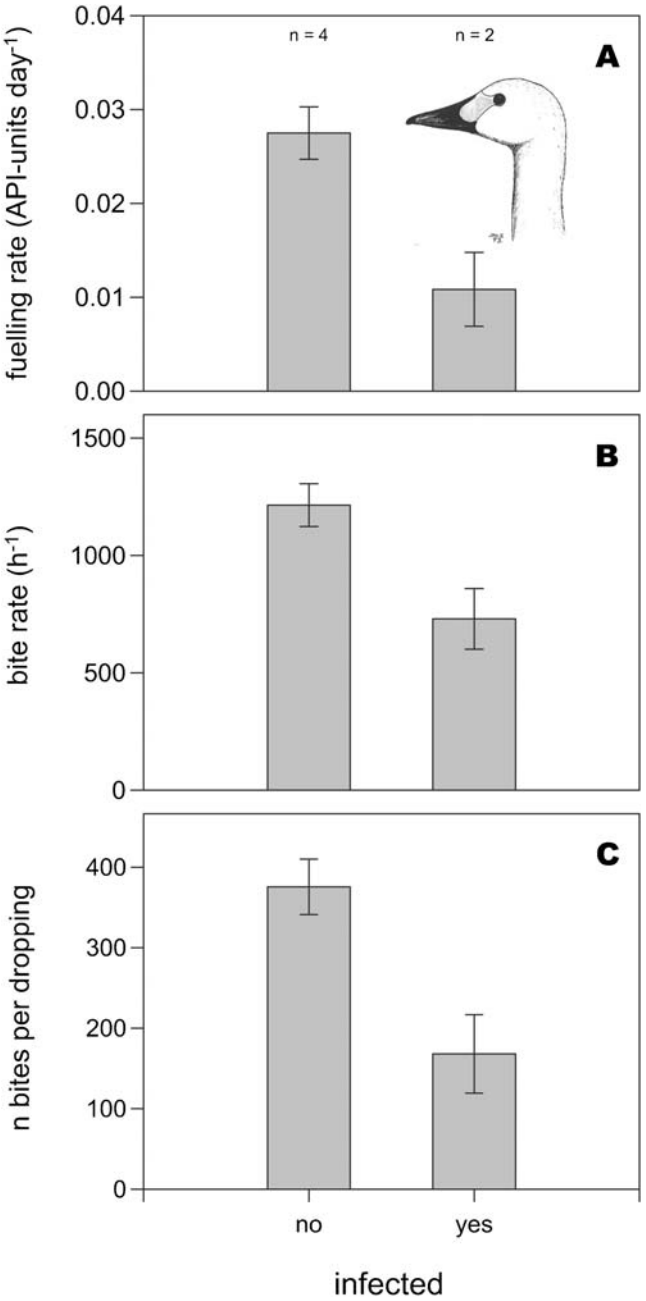

Figure 4. Feeding parameters as a function of health status. Compared to healthy birds, infected birds (**A**) fuelled slower, (**B**) took fewer bites per hour, and (**C**) took fewer bites per produced dropping. Because of a seasonal increase observed in bite rate ($p<0.05$ when all observations pooled), bite rate plotted in (**B**) were standardized to 1 February. In all graphs, bars give least-square means, error bars are SE.
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appears affected by the fact that birds with an active infection are migrating slower.

Tentatively accepting an LPAI virus infection to be the cause rather than the consequence, it is tempting to speculate about the knock-on effects of an infection throughout the rest of the annual cycle. Although seropostive birds without an active infection were performing just as well as seronegative birds, a delayed departure by the still infected birds may have cascading effects, even if those birds would eventually overcome their disease during further migration. Namely, a delayed departure from one site may lead to delayed departures from all sites en route (strengthened by the fact that food stocks are largely depleted by the first birds using a stopover site [20]), thereby possibly delaying arrival on their Arctic breeding grounds. If so, reproductive output may be reduced as the best territories may have been occupied [21] by the time infected birds arrive and eventual broods may hatch too late to take full advantage of the food peak [22]. If, on the other hand, they did manage to reach the breeding grounds in time, reproduction may still be affected because of the trade-off between reproductive effort and immunocompetence [23,24]. Having said this, survival rate does not seem affected, since, at the time of writing, approximately one year after catching the birds, 3 out of the 4 banded seropositive birds (75%) and 5 out of 8 banded seronegative birds (63%) have been seen back at their wintering grounds.

In domestic birds, the clinical signs and disease observed following infection with LPAI viruses vary with host species, age, the presence of other micro-organisms and environmental factors. In these hosts, LPAI viruses can cause a mild disease consisting primarily of respiratory symptoms, depression and egg production problems in laying birds [25]. Upon experimental infection of ducks, LPAI viruses replicate in the epithelial cells of the intestine of birds and virus may be shed in high concentrations in the faeces, without inducing apparent signs of disease [26,27]. It is generally believed that LPAI viruses are also non-pathogenic upon natural infection of wild birds, although data are scarce [3]. Influenza A viruses of the H6 subtype are among the most commonly isolated viruses in wild ducks in North America and Europe and have been isolated frequently from other wild birds, including geese, gulls, waders, and auks [7,28,29]. Our observations that infection of swans with such "ordinary" LPAI H6 viruses can result in altered migratory and feeding patterns may indicate that LPAI virus infections in wild birds have a higher clinical, epidemiological, and ecological impact than previously recognised. Based on these observations, the implications of influenza virus infections, including those caused by HPAI H5N1 viruses, for bird health and ecology and virus epidemiology may require re-evaluation.

MATERIALS AND METHODS

Swan catching and processing

By means of canon netting, we caught 25 Bewick's swans on 18 December 2005 on a sugar beet field in Wieringerwerf, Wieringermeer, The Netherlands (52°48′ N, 05°05′ E). The birds were aged and sexed on the basis of plumage coloration and cloacal examination, respectively. We determined wing length (to nearest mm), total head size (to nearest mm) and body mass (to nearest 0.1 kg). Twelve GPS-collars were fitted to 9 adults (presumably 1 male and 8 females; 4 of which were paired of which 2 were accompanied by young), 2 yearlings (sex unknown; of which one still accompanied its parents) and 1 juvenile male (accompanying its parents).

GPS-collars

GPS-collars were manufactured by Microtes Wildlife Engineering (Arnhem, The Netherlands). Each collar was fitted with a miniature GPS-receiver and antenna, a Bluetooth transceiver and antenna, a flash storage device, and a time-scheduled microprocessor controlling data collection and transmission. Total weight of the collar devices was 80 g. The GPS automatically collected geographical positions during 2–4 prescheduled times a day (accuracy<50 m). When within close range of the bird (<300–400 m), GPS-data were downloaded via Bluetooth at prescheduled times. Each collar had an individual code engraved (readable

up to 600 m; Figure 1), which enabled us, through the help of many volunteer ring-readers, to trace the birds for data collection. Until our last day in the field (22 February 2006), we were able to collect GPS-data on 8 birds; the whereabouts of the other 4 birds were reconstructed using resightings.

Visual observations

Throughout winter, we were able to regularly observe six of the GPS-collared swans, the two virus-infected birds included. We performed so-called focal scans [30], which meant that we carefully observed one of the collared swans for a one-hour period, or less when disturbed. Through a 20–60×spotting scope (Kowa Company Ltd., Chuo-Ku Tokyo, Japan) and a 60×optolite (Meade Instruments Corporation, Irvine, USA), we counted the number of bites taken (when feeding on grass) and, when possible, the number of droppings produced (mean±SE number of scans per bird = 9±2, of which during 5±2 scans the bird was near enough to enable dropping scans). At constant bite and dropping mass, the bite/dropping ratio is a measure of a bird's digestive performance (high at a high ratio). At the end of each scan we estimated the bird's abdominal profile index (API). This is a measure frequently used in waterfowl ecology to estimate a bird's abdominal fat storage [13,15] (on a scale of 1–6, in steps of ¼ unit). The change in API over time can be used to estimate a bird's rate of body store accumulation [14]; here we used the first and last API-estimate for this purpose (mean±SE of time interval between first and last score per bird = 31±6 days). All observations were performed by DL and RR, who were unaware of the identity of the infected birds, and therefore all measurements can be considered as blind with respect to infection-status. The design of the measurements was such that each observer observed all six birds, with no difference between observers in the number of observations per bird ($F_{1,10} = 1.61$, $p > 0.2$; GLM). Moreover, observations were equally spread in time for infected (range = 22 Dec–21 Feb; mean±SE = 27 Jan±3.4 days) and uninfected birds (29 Dec–22 Feb; 24 Jan±2.5 days).

Statistics

Generalized Linear Models were used for statistical comparison of infected and uninfected birds, with age and body size as covariates (using the GLM package in SYSTAT 10 [31]). The number of birds varied between comparisons; 25 swans were caught for analysis of biometric data at the start of the study (3 infected, 22 uninfected), 12 of these were GPS collar-banded (2 infected, 10 uninfected), and 6 of these were regularly observed to perform focal scans (2 infected, 4 uninfected).

Specimens

Cloacal swabs were collected using sterile cotton swabs, stored in transport media (Hanks balanced salt solution containing 0.5% Lactalbumin, 10% glycerol, 200 U/ml penicillin, 200 µg/ml streptomycin, 100 U/ml polymyxin B sulfate, 250 µg/ml gentamycin [ICN, The Netherlands]), and shipped to the laboratory where they were stored at −70°C. Blood samples were obtained by using manual restraint from the ulnar vein, centrifugation for 10 minutes at 14,000 rpm and separation of serum.

RNA isolation and virus detection

RNA isolation and RT-PCR was performed as described previously [32]. In short, RNA was isolated using a MagnaPure LC system with the MagnaPure LC Total nucleic acid isolation kit (Roche Diagnostics, Almere, The Netherlands) and influenza A virus was detected using a real-time RT-PCR assay. Amplification and detection was performed on an ABI7700 with the TaqMan EZ RT-PCR Core Reagents kit (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands). The samples were prepared and processed in parallel with several negative and positive control samples in each run.

Virus isolation and characterization

For influenza A virus RT-PCR positive samples, 200 µl of the original material was inoculated into the allantoic cavity of 11-day-old embryonated hens' eggs. The allantoic fluid was harvested two days after inoculation and influenza A virus was detected using hemagglutination assays with turkey erythrocytes. When no influenza A virus was detected upon the initial virus isolation attempt, the allantoic fluid was passaged once more in embryonated chicken eggs. Virus isolates were characterized using the hemagglutination inhibition (HI) assay with turkey erythrocytes and subtype-specific hyperimmune rabbit antisera raised against all HA subtypes [2].

Sequence analysis and phylogeny

All eight gene segments of the influenza A virus isolates were characterized by RT-PCR and sequencing, essentially as described by others [33]. PCR products were purified from agarose gels using the Qiaquick Gel Extraction kit (Qiagen, Leusden, The Netherlands) and sequenced using the Big Dye terminator sequencing kit version 3.0 (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) and an ABI PRISM 3100 genetic analyzer (Applied BioSystems, Nieuwerkerk a/d IJssel, The Netherlands), according to the instructions of the manufacturer. All primer sequences are available upon request. Trees were generated using full-length HA nucleotide sequences, including sequences from public databases. Sequences were aligned using the Clustal W program running within the BioEdit software package, version 5.0.9 [34]. Alignments were bootstrapped 100 times using the Seqboot package of Phylip version 3.6 and trees were constructed with the Dnaml package, using 3 jumbles. The consensus tree was calculated using the Consense package of Phylip 3.6 and this tree was used as usertree in Dnaml to recalculate the branchlengths from the nucleotide sequences. Trees were visualized with the Treeview 1.6.6 program distributed with Bioedit version 5.0.9. All nucleotide sequences presented in this manuscript are available from GenBank under accession numbers DQ822187 - DQ822202.

Serology

Swan sera were analysed using a commercially available influenza A virus antibody ELISA kit (European Veterinary Laboratory, Woerden, The Netherlands) as previously described [35]. For the detection of specific anti-NP antibodies, we used horseradish peroxidase-conjugated goat anti-wild bird immunoglobulin (Ig) G (Bethyl Laboratories, Inc., TX, USA) instead of the supplied anti-bird conjugate. Positive sera were analysed using the HI assay against all currently known HA subtypes, as previously described [2].

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Author Contributions

Conceived and designed the experiments: Jv. Performed the experiments: Jv RR DL. Analyzed the data: Jv VM. Contributed reagents/materials/analysis tools: Jv VM. Wrote the paper: RF Jv MK.

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