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Effects of influenza A virus infection on migrating mallard ducks

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The natural reservoir of influenza A virus is waterfowl, particularly dabbling ducks (genus *Anas*). Although it has long been assumed that waterfowl are asymptomatic carriers of the virus, a recent study found that low-pathogenic avian influenza (LPAI) infection in Bewick's swans (*Cygnus columbianus bewickii*) negatively affected stopover time, body mass and feeding behaviour. In the present study, we investigated whether LPAI infection incurred ecological or physiological costs to migratory mallards (*Anas platyrhynchos*) in terms of body mass loss and staging time, and whether such costs could influence the likelihood for long-distance dispersal of the avian influenza virus by individual ducks. During the autumn migrations of 2002–2007, we collected faecal samples ($n = 10\,918$) and biometric data from mallards captured and banded at Ottenby, a major staging site in a flyway connecting breeding and wintering areas of European waterfowl. Body mass was significantly lower in infected ducks than in uninfected ducks (mean difference almost 20 g over all groups), and the amount of virus shed by infected juveniles was negatively correlated with body mass. There was no general effect of infection on staging time, except for juveniles in September, in which birds that shed fewer viruses stayed shorter than birds that shed more viruses. LPAI infection did not affect speed or distance of subsequent migration. The data from recaptured individuals showed that the maximum duration of infection was on average 8.3 days (s.e. 0.5), with a mean minimum duration of virus shedding of only 3.1 days (s.e. 0.1). Shedding time decreased during the season, suggesting that mallards acquire transient immunity for LPAI infection. In conclusion, deteriorated body mass following infection was detected, but it remains to be seen whether this has more long-term fitness effects. The short virus shedding time suggests that individual mallards are less likely to spread the virus at continental or intercontinental scales.

Keywords: influenza A virus; disease ecology; migration

1. INTRODUCTION

Influenza A viruses (IAV) have a broad host range, causing disease in mammalian hosts (including humans; Webster *et al.* 1992; Olsen *et al.* 2006) and epizootics in poultry (Capua & Alexander 2006). The natural reservoirs are birds associated with wetlands, especially those in the order Anseriformes, and, in particular, dabbling ducks (genus *Anas*; Olsen *et al.* 2006). The virus is genetically diverse and typed according to the combination of two surface proteins: the haemagglutinin (HA) and the neuraminidase (NA). Nearly all possible combinations of the 16 HA and 9 NA antigenic subtypes have been found in wild dabbling ducks (Krauss *et al.* 2004; Olsen *et al.* 2006; Munster *et al.* 2007; Wallensten *et al.* 2007), and several studies show that strains causing disease in humans, poultry and other mammals, including the H5 and H7 highly pathogenic avian influenza strains, have their precursors in wild birds (Munster *et al.* 2005; Capua & Alexander 2006).

In wild ducks, the virus has historically not been associated with advert disease and normally only low-pathogenic avian influenza (LPAI) strains circulate in this reservoir (Olsen *et al.* 2006; Munster *et al.* 2007). Most studies regarding epizootiology and pathogenesis have focused on either IAV strains isolated from moribund poultry or zoonotic transmission to mammals. Much less is known about LPAI virus pathogenicity and its putative effects at the individual and population levels in wild birds.

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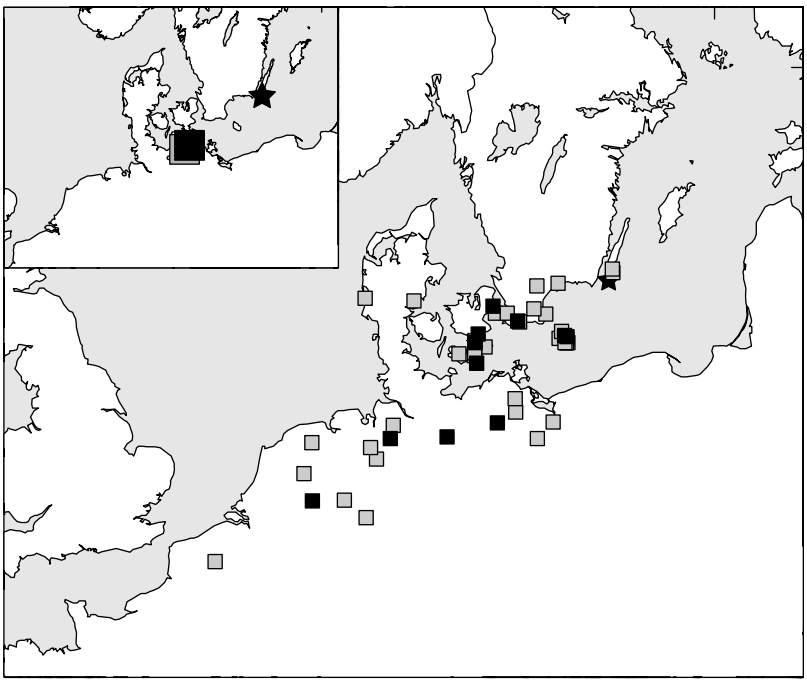

Figure 1. Recovery position of mallards *Anas platyrhynchos* banded at Ottenby (denoted by a star) and recovered elsewhere during the same autumn and winter. Grey squares show recoveries of Influenza A virus (IAV) real time-(RT) PCR-negative ducks, while black squares show RT-PCR-positive ducks. The inset shows the mean recovery position for each group.

All experimental work with LPAI viruses has been carried out in laboratory settings, most often using domestic varieties of the mallard (*Anas platyrhynchos*). In nearly all studies, no apparent disease signs or pathological lesions have been observed, even when the virus was shed in the faeces of inoculated ducks (Homme & Easterday 1970; Alexander *et al.* 1978; Slemons & Easterday 1978; Hinshaw *et al.* 1980; Kida *et al.* 1980; Kawaoka *et al.* 1988; Kishida *et al.* 2005; Sturm-Ramirez *et al.* 2005). However, in one study, LPAI strains were linked to pathological pneumonia-like lesions in the lungs of infected ducks, which otherwise appeared to be healthy (Cooley *et al.* 1989).

For a number of reasons, the results from laboratory studies may not be applicable to conditions in nature, where birds experience ecological and physiological effects of sub-lethal infections. Recently, it has been shown that wild Bewick's swans *Cygnus columbianus bewickii* fed less efficiently and had a much delayed spring migration departure when naturally infected with a LPAI virus (van Gils *et al.* 2007).

Large-scale studies of the effects of LPAI viruses on physiology, behaviour and fitness-related traits in wild birds are still lacking and much needed. Here, we present the first study of effects of naturally occurring IAV on free-living migratory mallards, a near-cosmopolitan species frequently alleged to be a significant vector. Based on samples from a major staging site in a flyway connecting breeding and wintering areas in Europe, we related the infection status of individual mallards to body mass and stopover duration during autumn migration. We predicted that infected ducks would have lower body mass (corrected for body size, i.e. a measure of body condition) and stage for a longer period compared with uninfected ducks. We also hypothesized that (i) body mass would be negatively related to the amount of viruses shed in faeces, and (ii) infection would affect migration speed and distance negatively.

2. MATERIAL AND METHODS

(a) *Sampling, detection and virus cultivation*

Wild mallards ($n=4539$) on autumn migration (defined as 15 July to the end of December) were caught in a duck trap at Ottenby Bird Observatory, Sweden (56°12′ N 16°24′ E; figure 1) from 2002 to 2007. The trap was emptied daily and all birds were taken indoors for sampling by swabbing fresh faeces or the cloaca. Each bird was banded, sexed and aged. Most of the birds in the full dataset were positively sexed: 2647 were males and 1870 females. Accurate ageing was possible in 2586 juveniles and 902 adults (i.e. birds fledged the previous year or earlier). Measurements were taken of body mass and wing length (left wing only). In addition, tarsus length (right tarsus) and distance from bill-tip to the back of the skull ('bill-head') were measured in 2004–2007. Approximately one-third of the birds were retrapped at Ottenby the same season ($n=1539$, most of which were positively sexed and aged; $n_{\text{males}}=966$, $n_{\text{females}}=568$, $n_{\text{juveniles}}=1048$ and $n_{\text{adults}}=259$; 1–42 retrappings per bird). The latter data were used to estimate the minimum number of days these birds stayed in the area (i.e. staging time; see §2*b*). Details on location, trapping, sampling techniques and materials are found in a previous publication (Wallensten *et al.* 2007).

A total of 10 918 samples for IAV detection were taken, and infection status was assessed by real-time PCR assays targeted at the IAV matrix gene (Karlsson *et al.* 2007; Wallensten *et al.* 2007). The synthesis of amplicons was measured over the course of the PCR, and a crossing point (threshold cycle) value (*Ct*-value; see §2*b*) was expressed when amplification started. PCR-positive samples from 2002 to 2004 were propagated in embryonated hens' eggs using standard methods (Munster *et al.* 2005, 2007). The HA subtype of virus isolates was characterized using haemagglutination inhibition (HI) and the NA subtype was characterized by PCR and sequencing (Munster *et al.* 2005, 2007).

(b) *Generalized linear models*

Generalized linear models (GLM) were used to explore the effect of IAV infection on two dependent variables: body mass and minimum length of the staging period. The latter is the number of days between the first and the last trapping occasions within a season. To avoid pseudoreplication, we used only the data from the first available season for individuals caught in more than one year. Age was included in all GLMs, using two categories: juvenile and adult (see §2*a*).

In the first analyses, we investigated the effect of IAV infection on body mass at first capture, thus ignoring all data from resampled individuals. In different GLMs, two measures of IAV infection were used: (i) real time-(RT) PCR positive or RT-PCR negative, and (ii) viral shedding for positive mallards, expressed as the *Ct*-value. The latter is inversely proportional to the number of initial copies of the RNA template. Therefore, we argue that a low *Ct*-value is an indication of high shedding of viruses, whereas a high value reflects a lower number of shed viruses. Analyses involving the *Ct*-value were restricted to birds caught in 2004–2007, i.e. the years for which such data were available ($n=2820$ individuals). Because large birds are generally heavier than smaller birds, it is necessary to control for structural body size. A principal component analysis showed that the three body size traits correlated with each other; the correlation with the first principal component was 0.91 for bill-head, 0.88 for wing and 0.85 for tarsus ($n=2820$ as for the *Ct*-values above). Wing length was the only body size trait with data available from all years (2002–2007), and since it turned out to be a good predictor of body size, we were able to use data from all years for the analysis including data on positive versus negative birds. In addition to using wing length as a covariate, sex and age of each bird were included as controlling factors in the GLMs, as were year and month of capture (but see below).

In another set of GLMs, we explored the effect of infection status (positive or negative, and *Ct*-value) at first capture on individual staging time, controlling for sex, age, year and month. Again, an individual occurred only once in the analysis if it was retrapped in subsequent seasons. Power transformation was used ($y=x^p$, $p=0.29$; see Tan *et al.* 2004) for the dependent variable 'length of stay' to fulfil the assumptions for parametric testing.

We included all biologically relevant two-way interactions in all GLMs, and we used a backward selection procedure in which non-significant interactions were excluded stepwise to yield a simplified final model. The fit of the most general model was acceptable for all GLMs (lack of fit test: $p>0.05$), except the one exploring the effect of LPAI infection on body mass ($F=1.10$, $p=0.03$). However, this was circumvented by excluding month as a controlling factor in the GLM (lack of fit test: $F=1.077$, $p=0.09$).

Hypothesis testing was performed with the statistical software SPSS v. 15.0.

(c) *Banding recoveries*

We used recovery data from mallards sampled at Ottenby and later retrapped or found dead elsewhere to determine wintering areas and speed of migration. Ducks that were infected when initially banded were compared with those that were uninfected. We included only birds sampled in autumn and recovered during the same autumn or winter (up to and including February). Mean positions of recoveries of infected and uninfected mallards were calculated using formula 3 in Perdeck (1977) and compared in a randomization test (Lokki & Saurola 1985).

Table 1. GLM test results of the impact of LPAI on body mass of mallards ($n=3389$). (Included are also other main effects as well as significant interactions.)

	d.f.	*F*	*p*-value
wing length	1	489.33	<0.001
LPAI	1	6.37	0.01
sex	1	19.19	0.06
year	5	2.49	0.17
age	1	0.74	0.55
sex×year	5	5.04	<0.001
sex×age	1	8.93	<0.01

(d) *Duration of infection*

We used the data from mallards that were RT-PCR positive on at least two consecutive trapping occasions (range 2–4 times) to estimate for how long individuals shed IAV. We calculated the minimum length of the shedding period in two ways, depending on the certainty of data. The high-certainty category ('certain cases') comprised individuals for which infection status was known on at least four consecutive sampling occasions, and in which the RT-PCR-result over time was negative–positive and positive–negative. The lower certainty category ('uncertain cases') included birds that were RT-PCR positive on at least two consecutive sampling occasions (range 2–6 times), but for which we lacked data on infection status before and/or after this period. We defined the minimum shedding period as the interval between the first and the last dates of detected infection. Furthermore, we estimated the maximum duration of infection for cases in which the full infection cycle was covered, from the time prior to infection to infection clearance (RT-PCR results negative–positive–positive–negative) and where the time between individual sampling occasions was less than 10 days.

3. RESULTS

(a) *Prevalence rates*

IAV was detected by RT-PCR in 13.9 per cent of all samples, with some variation among years: 2002 (12.6%); 2003 (14.4%); 2004 (17.4%); 2005 (23.4%); 2006 (10.2%); and 2007 (13.3%). Prevalence increased during the autumn months as previously reported (Munster *et al.* 2007; Wallensten *et al.* 2007). Among the 1539 recaptured mallards, 629 (40.1%) were RT-PCR positive on at least one resampling occasion.

(b) *Infection and body mass*

Since body mass may depend not only on health status, but also on year, age, sex and body size, all these variables were included in the GLMs, evaluating the impact of virus infection on body mass. Controlling variables, indeed, turned out to be important, and explained much of the variation in body mass, but mallards were nevertheless negatively affected by infection (table 1). Body mass was lower in the positive ducks compared with the negative ducks when other variation in body mass was controlled for ($\beta=-12.5$, s.e. 5.0). Ignoring other significant variables, the overall mean body mass was 1082.9 g (95% CI 1078.2–1087.5) for the IAV-negative and 1064.8 g (95% CI 1055.0–1074.6) for the IAV-positive ducks.

Table 2. GLM results including the effect of the *Ct*-value on body mass of mallards ($n=260$). (Other main effects as well as significant interactions are also included.)

	d.f.	*F*	*p*-value
wing length	1	44.47	<0.001
sex	1	9.31	<0.01
Ct-value	1	4.62	0.03
age	1	4.48	0.04
year	3	0.56	0.66
month	5	0.56	0.73
year×month	9	5.68	<0.001
age×year	3	4.16	<0.01
Ct-value×age	1	3.83	0.05

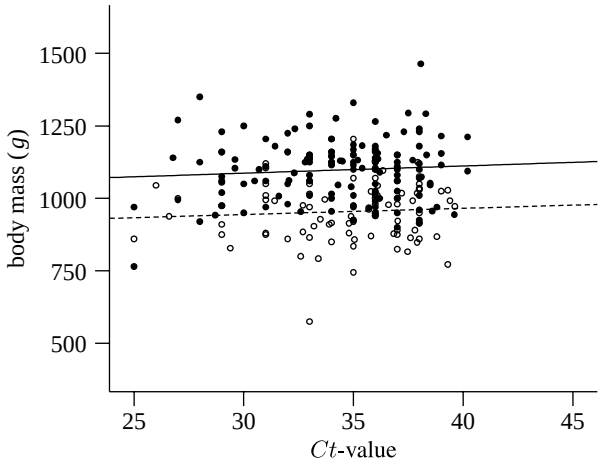

Figure 2. Body mass of juvenile female (open circles) and juvenile male (filled circles) mallards correlated with the corresponding *Ct*-values, i.e. a measure of the amount of shed LPAI virus. Regression lines for the two groups of birds are given.

The effects on body mass may potentially differ depending on the stage of the infection cycle. The *Ct*-values from the RT-PCR for influenza-positive mallards ($n=277$) were indeed related to body mass (table 2). However, a significant interaction between the *Ct*-value and age revealed that only juveniles were positively affected, i.e. the birds shedding less viruses (high *Ct*-values) had higher body mass than those shedding more viruses (low *Ct*-values) ($\beta=9.7$, s.e. 4.9; figure 2).

(c) *Infection and length of stay*

Length of stay averaged 17.1 days (s.e. 0.5) in all recaptured mallards. The factors such as age and sex affected length of stay, but infection status did not (table 3). However, when analysing data for those mallards infected at first capture and later recaptured the same season ($n=129$), there was a correlation between the *Ct*-value and the number of staging days at Ottenby. The effect was complex though, since the *Ct*-value variable was significant only when interacting with month of capture, and not as an independent main effect (table 4). Further investigation of this interaction showed that there was a negative effect of the *Ct*-value in September only ($\beta=-0.3$, s.e. 0.1), i.e. the less virus the mallards shed, the shorter their staging period (figure 3). All of these mallards were juveniles (six males and three females).

Table 3. GLM of the effects of LPAI infection and controlling variables on the staging time of mallards ($n=1302$). (Main effects as well as significant interactions are reported.)

	d.f.	*F*	*p*-value
age	1	6.14	0.04
sex	1	4.18	0.04
month	5	2.13	0.13
year	5	1.51	0.22
LPAI	1	0.18	0.67
year×month	19	5.35	<0.001
age×month	5	2.45	0.03

Table 4. GLM of the *Ct*-value along with controlling variables and their impact on the staging time of LPAI-positive mallards ($n=129$). (Main effects as well as significant interactions are reported.)

	d.f.	*F*	*p*-value
month	4	4.69	<0.01
year	3	2.97	0.04
sex	1	2.62	0.11
Ct-value	1	1.83	0.18
age	1	1.42	0.24
Ct-value×month	4	4.17	<0.01

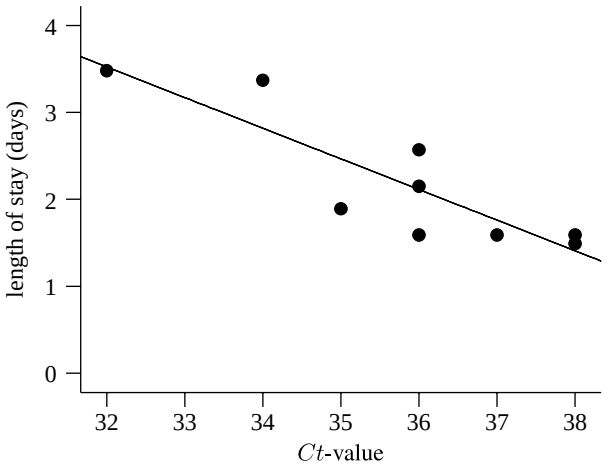

Figure 3. Juvenile mallards trapped at Ottenby in September stayed longer when shedding increasing amounts of IAV. Length of stay in days is power transformed ($p=0.29$).

(d) *Infection, recapture frequency and recoveries*

Food was provided in our duck trap and we were able to explore whether feeding patterns depended on infection status by studying the frequency of recapture. The overall mean number of trapping occasions for all recaptured mallards was 4.9 (s.e. 0.1), and there was no statistically significant difference between infected and uninfected ducks (status at first capture), even when controlling for the effects of sex and age ($p>0.05$ for all cases; Mann–Whitney tests were used since no transformation successfully normalized the data). Likewise, there was no correlation between the number of recaptures and the *Ct*-value in the infected ducks (Spearman's correlation, $p>0.05$).

Banding recovery data showed that sampled ducks were reported in areas southwest of Ottenby (figure 1), which is also the general autumn migration direction for mallards banded in southern Sweden (Fransson & Pettersson 2001). The mean position of recoveries

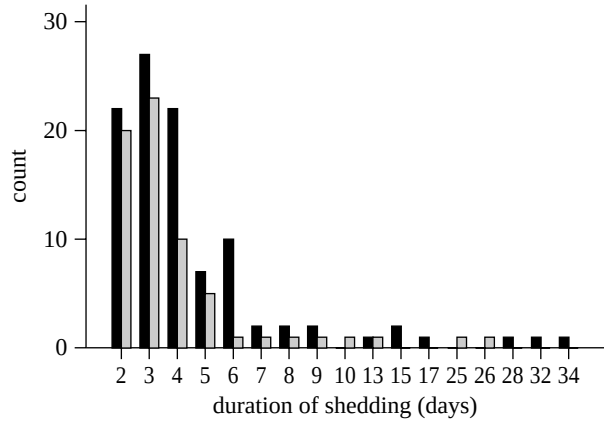

Figure 4. Minimum duration of IAV shedding in mallards *Anas platyrhynchos* as detected by RT-PCR. For definitions of certain (grey bars) and uncertain (black bars) cases, see text.

of infected ducks did not differ from that of uninfected ducks (figure 1), nor was there any difference in (i) time lapsed between banding and recovery (infected ducks = 64.4 days and uninfected ducks = 47.7 days, $Z = 1.93$, $p > 0.05$, $n = 12$ versus 36), (ii) distance to recovery place (363.4 km versus 350.8 km, $Z = 0.44$, $p > 0.05$, $n = 12$ versus 36), and (iii) speed of migration (6.7 km d^{-1} versus 13.8 km d^{-1}, $Z = -0.62$, $p > 0.05$, $n = 12$ versus 36).

(e) *Duration of infection*

Initial calculations showed that the mean minimum length of the virus shedding period lasted 4.2 ($n = 66$, s.e. 0.5) and 5.2 days ($n = 102$, s.e. 0.5) for the certain and uncertain cases, respectively (no significant differences with respect to sex and age classes; Mann–Whitney: $p > 0.05$ for all cases). However, the range in the number of shedding days was large (2–34), and we could not determine whether the most extreme cases were caused by the same or two different and subsequent infections because virus isolation was not possible for all samples. Therefore, we recalculated the minimum duration of shedding excluding the 10 per cent most extreme cases (shedding period >6 days for the certain cases and >8 days for the uncertain cases; figure 4). After this restriction, the mean duration of minimum shedding was 3.1 days ($n = 59$, s.e. 0.1) and 3.7 days ($n = 92$, s.e. 0.2) for the certain and uncertain cases, respectively (no significant difference between sex and age classes; Mann–Whitney: $p > 0.05$ for all case). The estimated mean value for the maximum infection duration was 8.3 days ($n = 55$, s.e. 0.5).

IAV subtyping yielded 10 cases in which the HA–NA combination was the same on two or three consecutive sampling occasions (table 5). The minimum duration of shedding of viral particles averaged 3.0 days (s.e. 0.3) in these birds, which were all juveniles, and in three of the cases shedding was intermittent (RT-PCR-negative results in between positive isolations). In another 10 birds, we detected two different virus subtypes within a season, and in the extreme case one individual had three different subtypes. The recorded time between infections by different subtypes ranged from 1 to 32 days (mean 9.3 days, s.e. 2.9).

In order to determine whether the immunological status changed over the season, i.e. if there was a temporal trend in the duration of shedding, we performed Spearman's

Table 5. Shedding patterns of 10 individual mallards on 3–14 occasions (0, RT-PCR negative; 1, RT-PCR positive; X, not sampled).

	sampling												
bird	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	0	0	0	H7N7	1	H7N7	0	0	0	X	x	X	x	x
2	0	0	0	1	H7N7	0	H7N7	0	X	X	x	X	x	x
3	0	0	0	0	0	1	0	H7N7	0	H7N7	0	0	0	x
4	H7N7	H7N7	0	0	X	X	x	x	X	X	x	X	x	x
5	H6N8	H6N8	0	0	X	X	x	x	x	X	x	x	x	x
6	0	H6N2	0	H6N2	1	0	x	x	x	X	x	x	x	x
7	1	H6N2	H6N2	0	X	X	x	x	x	X	x	x	x	x
8	0	H6N2	H6N2	x	X	X	x	x	x	X	x	x	x	x
9	0	0	H1N1	H1N1	x	x	x	x	x	X	x	x	x	x
10	0	0	1	0	H6N2	H6N2	H6N2	0	0	0	1	0	0	0

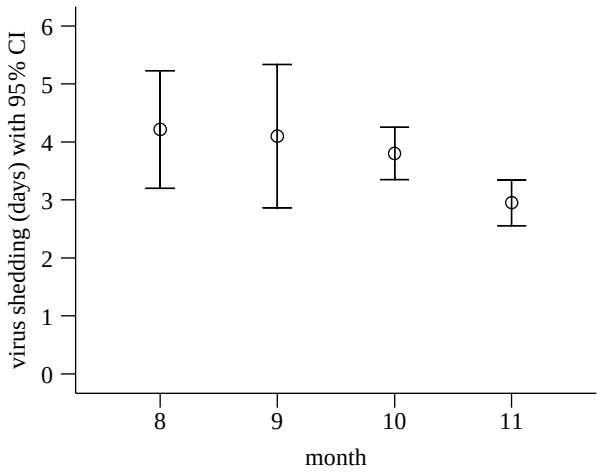

Figure 5. Days of virus shedding (uncertain cases; see text) decrease during the autumn (August–November).

correlations (owing to skewed data distributions) with duration of shedding (in days) and season (in months; data from July and December were sparse ($n<5$) and we therefore considered only August–November). There was indeed such a negative relationship for minimum shedding for the uncertain cases (Spearman's correlation: $r_s=-0.28$, $n=92$, $p<0.01$; figure 5), but not for the certain cases ($r_s=-0.17$, $n=58$, $p=0.20$) or for maximum shedding ($r_s=-0.25$, $n=54$, $p=0.07$).

4. DISCUSSION

The proportion of mallards infected by IAV varied in a seasonally consistent way at our sampling site, being lower (less than 10%) during the spring and summer and higher (10–25%) during autumn migration and early winter (Wallensten *et al.* 2007). A similar annual temporal pattern occurs in North America (Krauss *et al.* 2004), and seems to be a general feature of IAV ecology (Olsen *et al.* 2006). IAV prevalence in mallards appears to be important for the perpetuation of the virus, as it is the most abundant species of dabbling duck over much of Eurasia and North America (Krauss *et al.* 2004; Olsen *et al.* 2006; Munster *et al.* 2007; Wallensten *et al.* 2007).

If LPAI infection is costly for wild mallards, we would anticipate the effects on body mass and length of stay at stopover sites, especially because migration *per se* reduces energy stores (Lindström & Piersma 1993), which may lead to immunosuppression (Gylfe *et al.* 2000; Weber & Stilianakis 2007). Mallards with LPAI infection did indeed lose more weight than healthy birds. This finding seems very robust since infection status (infected or uninfected) as well as the *Ct*-value (proportional to the amount of shed virus) was related to body mass. The latter relationship is particularly valid for juveniles since they are more susceptible to infection than adults (Wallensten *et al.* 2007). We therefore argue that it is not unlikely that young birds are also more affected in terms of body mass loss. This view is supported by our finding that the amount of shed viruses was positively correlated with length of stay, albeit only in the limited September sample, in which all were juveniles. That we did not find a more general effect of IAV infection on length of stay may be due to complex interactions between staging time, age and season. Besides, the effects of LPAI infection *per se* on staging time and body condition were also affected by sex, age, month and year, just as in many other large migratory birds (Lindström 1995; Berthold 2001). Moreover, the ornithological literature abounds with examples of how body condition affects individual migration strategies and subsequent population patterns (Berthold 2001; Arzel *et al.* 2006). Yet, the LPAI infection status of mallards while at Ottenby did not affect subsequent migration as analysed here (speed of migration and recovery distance). We acknowledge, though, that data for this analysis were sparse and not of the same power as for the other analyses.

Infectious diseases such as avian cholera, duck plague, aspergillosis and toxins causing mycotoxicosis or avian botulism are known to affect waterfowl dramatically (Wobeser 1997; Thomas *et al.* 2007), but this is the first large-scale study showing that wild mallard ducks with non-overt disease are negatively affected by LPAI infection. Our results run counter to the findings from experimental infections with LPAI virus (Alexander *et al.* 1978; Kida *et al.* 1980; Cooley *et al.* 1989; Webster *et al.* 1992; Kishida *et al.* 2005; Sturm-Ramirez *et al.* 2005), but to some extent still corroborate the negative effects reported for wild Bewick's swans naturally infected with LPAI H6N2 and H6N8 (van Gils *et al.* 2007). Although sample sizes differ greatly, and despite the fact that there are natural temporal variations in nutrition, body condition and possibly immunological status that may bias a comparison between different seasons, both studies conclude that common reservoir species can be negatively affected by LPAI infection. It is a question of fundamental importance to IAV ecology and epidemiology alike to explore whether such effects also translate into fitness costs in terms of reduced survival and/or breeding success.

During 6 years of mallard surveillance, we detected HA subtypes H1–H12 and all NA subtypes in 55 different combinations (Wallensten *et al.* 2007; N. Latorre-Margalef, J. Waldenström & B. Olsen unpublished data 2003–2007). The short-term and long-term infection 'costs' in mallards could potentially be strain-, lineage- or subtype-specific, but owing to pending virus characterization, including sequencing of internal genes, this cannot yet be fully analysed and interpreted.

The period of minimum duration of virus shedding was surprisingly short (mean 3–4 days) and individual birds regularly lost and picked up infections while staging at our study site. The measure of maximum length of infection was considerably larger, 8.3 days, compared with the values based on dates with detected viral RNA shedding. Interestingly, the maximum length estimate is more similar to the values obtained in infection experiments (7–17 days shedding of viral RNA; Hulse-Post *et al.* 2005).

Mallards that recovered from an infection have an antibody-specific response (Hulse-Post *et al.* 2005; Kishida *et al.* 2005), and they acquire at least a transient immunity to future homologous influenza infections. When all individuals positive on two consecutive trapping occasions were analysed (the so-called uncertain cases), we found a negative trend in virus shedding duration in infected birds with the progress of autumn. However, there was no such trend using only those birds for which we had a complete infection history (certain cases) or for the estimate of 'maximum shedding'. At present, very few of our samples have been subtyped, but a testable hypothesis would be that a build-up of immunity in the population results in

shorter shedding times, if reinfected individuals, in turn, acquire a shorter infection clearance time.

The observed short shedding time implies that the spatial dynamics of LPAI is mainly characterized by 'travelling within bird flocks' or, at the most, by transport to adjacent staging areas where many ducks congregate, thus corroborating one of the main conclusions in Weber & Stilianakis (2007). If a short shedding time is a general pattern in the infected ducks, then single birds are less likely to carry the virus at the continental and intercontinental scales, which would be of profound importance to LPAI ecology and zoonotic surveillance alike. The circulation of several subtypes in a staging population of hosts, together with the reinfection of single hosts, sets a scene where the recombination of co-occurring infecting viruses is likely to occur. This also has profound implications for LPAI spread and subtype perpetuation.

Although LPAI infection affected the body mass of Ottenby mallards, we argue that the effects were probably rather mild for individuals. Mallards are relatively heavy birds and do probably tolerate some loss in body mass before consequences become severe, as is also indicated by the lack of effects on migration speed and distance. The question of whether or not the mallard is 'an ideal reservoir species' for the IAV virus, in the sense that the latter does not affect the former too much, remains to be resolved. We conclude that this is a host–virus system where more is known about 'the two players', respectively, than about their interactions. Discerning such interactive patterns and pinning down their causality remain top priorities for avian ecology as well as for zoonotic research. The present study offers one intriguing point of entry with testable predictions for experiments as well as for field studies; it remains to be found out whether the body mass change due to LPAI infection is large enough to also affect the body condition in a biologically significant fashion, i.e. whether the virus is a true pathogen or not. If so, medium- to long-term fitness traits such as survival and breeding success may be affected, and possibly also physiological functions, for instance the immune response to other pathogens. The effects on staging time were not clear-cut in our study and need more attention. If infection generally leads to prolonged stopover time, we hypothesize that this facilitates virus transmission because infected ducks may need to forage more intensively to maintain body condition and fight the infection when compared with uninfected ducks. By doing so, they are likely to remain on sites with many ducks and interact with them while foraging. In an evolutionary arms race, this might be a core interaction, scaling up the effects of infection from individuals to the population level and even to ecological guilds.

All trapping, handling and sampling of ducks were done in accordance with national legislation under permit from the National Ringing Centre and ethical approval from Linköping's animal research ethical board.

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REFERENCES

Alexander, D. J., Allan, W. H., Parsons, D. G. & Parsons, G. 1978 The pathogenicity of four avian influenza viruses for fowls, turkeys and ducks. *Res. Vet. Sci.* **24**, 242–247.

Arzel, C., Elmberg, J. & Guillemain, M. 2006 Ecology of spring-migrating Anatidae: a review. *J. Ornithol.* **147**, 167–184. (doi:10.1007/s10336-006-0054-8)

Berthold, P. 2001 *Bird migration. A general survey*, 2nd edn. Oxford, UK: Oxford University Press.

Capua, I. & Alexander, D. J. 2006 Avian influenza infections in birds—a moving target. *Influenza* **1**, 11–18. (doi:10.1111/j.1750-2659.2006.00004.x)

Cooley, A. J., Van, C. H., Philpott, M. S., Easterday, B. C. & Hinshaw, V. S. 1989 Pathological lesions in the lungs of ducks infected with influenza A viruses. *Vet. Pathol.* **26**, 1–5.

Fransson, T. & Pettersson, J. 2001 *Swedish bird ringing atlas*. Stockholm, Sweden: Swedish Museum of Natural History & Swedish Ornitological Society.

Gylfe, A., Bergstrom, S., Lundstrom, J. & Olsen, B. 2000 Reactivation of Borrelia infection in birds. *Nature* **403**, 724–725. (doi:10.1038/35001663)

Hinshaw, V. S., Webster, R. G., Bean, W. J. & Sriram, G. 1980 The ecology of influenza viruses in ducks and analysis of influenza viruses with monoclonal antibodies. *Comp. Immunol. Microbiol. Infect. Dis.* **3**, 155–164. (doi:10.1016/0147-9571(80)90051-X)

Homme, P. J. & Easterday, B. C. 1970 Avian influenza virus infections. IV. Response of pheasants, ducks, and geese to influenza A-turkey-Wisconsin-1966 virus. *Avian Dis.* **14**, 285–290. (doi:10.2307/1588473)

Hulse-Post, D. J. *et al.* 2005 Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. *Proc. Natl Acad. Sci. USA* **102**, 10 682–10 687. (doi:10.1073/pnas.0504662102)

Karlsson, M., Wallensten, A., Lundkvist, A., Olsen, B. & Brytting, M. 2007 A real-time PCR assay for the monitoring of influenza A virus in wild birds. *J. Virol. Methods* **144**, 27–31. (doi:10.1016/j.jviromet.2007.03.013)

Kawaoka, Y., Chambers, T. M., Sladen, W. L. & Webster, R. G. 1988 Is the gene pool of influenza viruses in shorebirds and gulls different from that in wild ducks? *Virology* **163**, 247–250. (doi:10.1016/0042-6822(88)90260-7)

Kida, H., Yanagawa, R. & Matsuoka, Y. 1980 Duck influenza lacking evidence of disease signs and immune response. *Infect. Immun.* **30**, 547–553.

Kishida, N., Sakoda, Y., Isoda, N., Matsuda, K., Eto, M., Sunaga, Y., Umemura, T. & Kida, H. 2005 Pathogenicity of H5 influenza viruses for ducks. *Arch. Virol.* **150**, 1383–1392. (doi:10.1007/s00705-004-0473-x)

Krauss, S., Walker, D., Pryor, S. P., Niles, L., Chenghong, L., Hinshaw, V. S. & Webster, R. G. 2004 Influenza A viruses of migrating wild aquatic birds in North America. *Vector Borne Zoonotic Dis.* **4**, 177–189. (doi:10.1089/vbz.2004.4.177)

Lindström, Å. 1995 Stopover ecology of migrating birds: some unsolved questions. *Israel J. Zool.* **41**, 407–416.

Lindström, Å. & Piersma, T. 1993 Mass changes in migrating birds: the evidence for fat and protein storage re-examined. *Ibis* **135**, 70–78. (doi:10.1111/j.1474-919X.1993.tb02811.x)

Lokki, H. & Saurola, P. 1985 Bootstrap methods for two-sample location and scatter problems. *Acta Ornithologica* **23**, 133–147.

Munster, V. J., Wallensten, A., Baas, C., Rimmelzwaan, G. F., Schutten, M., Olsen, B., Osterhaus, A. D. & Fouchier, R. A. 2005 Mallards and highly pathogenic avian influenza ancestral viruses, northern Europe. *Emerg. Infect. Dis.* **11**, 1545–1551.

Munster, V. J. *et al.* 2007 Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. *PLoS Pathog.* **3**, e61. (doi:10.1371/journal.ppat.0030061)

Olsen, B., Munster, V. J., Wallensten, A., Waldenstrom, J., Osterhaus, A. D. & Fouchier, R. A. 2006 Global patterns of influenza a virus in wild birds. *Science* **312**, 384–388. (doi:10.1126/science.1122438)

Perdeck, A. C. 1977 The analysis of ringing data: pitfalls and prospects. *Vogelwarte* **29**, 33–44.

Slemons, R. D. & Easterday, B. C. 1978 Virus replication in the digestive tract of ducks exposed by aerosol to type-A influenza. *Avian Dis.* **22**, 367–377. (doi:10.2307/1589291)

Sturm-Ramirez, K. M. *et al.* 2005 Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia? *J. Virol.* **79**, 11 269–11 279. (doi:10.1128/JVI.79.17.11269-11279.2005)

Tan, W. D., Gan, F. F. & Chang, T. C. 2004 Using normal quantile plot to select an approriate transformation to achieve normality. *Comput. Stat. Data Anal.* **45**, 609–619. (doi:10.1016/S0167-9473(03)00009-4)

Thomas, N. J., Hunter, D. B. & Atkinson, C. T. 2007 *Infectious diseases of wild birds*. Oxford, UK: Blackwell Publishing.

van Gils, J. A., Munster, V. J., Radersma, R., Liefhebber, D., Fouchier, R. A. & Klaassen, M. 2007 Hampered foraging and migratory performance in swans infected with low-pathogenic avian influenza A virus. *PLoS ONE* **2**, e184. (doi:10.1371/journal.pone.0000184)

Wallensten, A. *et al.* 2007 Surveillance of influenza A virus in migratory waterfowl in northern Europe. *Emerg. Infect. Dis.* **13**, 404–411.

Weber, T. P. & Stilianakis, N. I. 2007 Ecologic immunology of avian influenza (H5N1) in migratory birds. *Emerg. Infect. Dis.* **13**, 1139–1143.

Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M. & Kawaoka, Y. 1992 Evolution and ecology of influenza A viruses. *Microbiol. Rev.* **56**, 152–179.

Wobeser, G. A. 1997 *Diseases of wild waterfowl*. New York, NY: Plenum Press.