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Juveniles and migrants as drivers for seasonal epizootics of avian influenza virus

Jacintha G. B. van Dijk[1]*, Bethany J. Hoye[1,2], Josanne H. Verhagen[3], Bart A. Nolet[1], Ron A. M. Fouchier[3] and Marcel Klaassen[1,4]

[1]*Department of Animal Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, The Netherlands;* [2]*Department of Ecology and Evolutionary Biology, University of Colorado, Boulder, CO, USA;* [3]*Department of Virology, Erasmus Medical Center, Rotterdam, The Netherlands; and* [4]*Centre for Integrative Ecology, School of Life and Environmental Sciences, Deakin University, Geelong, Vic., Australia*

Summary

1. Similar to other infectious diseases, the prevalence of low pathogenic avian influenza viruses (LPAIV) has been seen to exhibit marked seasonal variation. However, mechanisms driving this variation in wild birds have yet to be tested. We investigated the validity of three previously suggested drivers for the seasonal dynamics in LPAIV infections in wild birds: (i) host density, (ii) immunologically naïve young and (iii) increased susceptibility in migrants.

2. To address these questions, we sampled a key LPAIV host species, the mallard *Anas platyrhynchos*, on a small spatial scale, comprehensively throughout a complete annual cycle, measuring both current and past infection (i.e. viral and seroprevalence, respectively).

3. We demonstrate a minor peak in LPAIV prevalence in summer, a dominant peak in autumn, during which half of the sampled population was infected, and no infections in spring. Seroprevalence of antibodies to a conserved gene segment of avian influenza virus (AIV) peaked in winter and again in spring.

4. The summer peak of LPAIV prevalence coincided with the entrance of unfledged naïve young in the population. Moreover, juveniles were more likely to be infected, shed higher quantities of virus and were less likely to have detectable antibodies to AIV than adult birds. The arrival of migratory birds, as identified by stable hydrogen isotope analysis, appeared to drive the autumn peak in LPAIV infection, with both temporal coincidence and higher infection prevalence in migrants. Remarkably, seroprevalence in migrants was substantially lower than viral prevalence throughout autumn migration, further indicating that each wave of migrants amplified local AIV circulation. Finally, while host abundance increased throughout autumn, it peaked in winter, showing no direct correspondence with either of the LPAIV infection peaks.

5. At an epidemiologically relevant spatial scale, we provide strong evidence for the role of migratory birds as key drivers for seasonal epizootics of LPAIV, regardless of their role as vectors of these viruses. This study exemplifies the importance of understanding host demography and migratory behaviour when examining seasonal drivers of infection in wildlife populations.

Key-words: age, C_T-value, infection intensity, infectious disease, nucleoprotein, origin, subtype, viral prevalence

Introduction

The prevalence of infectious diseases fluctuates over time, often showing clear seasonal patterns (Altizer *et al.* 2006). The incidence of malaria in humans, for instance, is affected by seasonal changes in temperature and precipitation (Parham & Michael 2010). In addition to abiotic conditions, many pathogens and parasites also show seasonal patterns as a result of host behaviour and population dynamics. Seasonal peaks in rabies are explained by the social behaviour of the host, such as breeding and

*Correspondence author. E-mail: j.vandijk@nioo.knaw.nl

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dispersal, and the addition of juveniles to the population (Rosatte 1984; Loveridge & Macdonald 2001). Similarly, seasonal mycoplasmal conjunctivitis epidemics are likely to be initiated by asymptomatic, recovered adults infecting naïve juveniles and by reintroduction of the bacterium into the population by dispersing or migrating individuals (Dhondt *et al.* 2012). Puumala virus epizootics, on the other hand, are mainly driven by host reproductive activity (Tersago, Verhagen & Leirs 2011). Hence, studying the mechanisms driving seasonal patterns in infectious diseases is important to understand their ecology, epidemiology and potential consequences for animal and human populations.

Avian influenza virus (AIV) has been studied increasingly over the last few decades, especially after the H5N1 highly pathogenic avian influenza virus (HPAIV) outbreaks in poultry (Hoye *et al.* 2010), for which low pathogenic avian influenza virus (LPAIV), which naturally circulates in wild bird populations, is thought to form the precursor (Alexander 2000). Surveillance studies of wild ducks in the northern hemisphere have shown an annual peak in late summer and early autumn, followed by low infection during the winter period and a small increase during spring (e.g. Hinshaw *et al.* 1985; Stallknecht *et al.* 1991; Krauss *et al.* 2004; Munster *et al.* 2007; Wallensten *et al.* 2007). Several mechanisms have been suggested to drive this seasonal variation in LPAIV prevalence in wild birds; however, these have yet to be explicitly tested. Host density, as well as the role of young and migratory birds has been considered potential drivers for these seasonal dynamics. An increase in local host density may enhance transmission rates and hence LPAIV prevalence (Gaidet *et al.* 2012). Young birds, because they are immunologically naïve and enter the population within a relatively narrow time window, have been suggested as a leading cause of the seasonal increase in LPAIV prevalence (Hinshaw *et al.* 1980). The potential contribution of migratory birds to the increase of LPAIV prevalence is less clearly defined, although they are frequently cited as playing an important role. Migrants may be more susceptible to infection because of (i) reduced immunocompetence, resulting from a trade-off between investment in immune defences and long-distance flight (Altizer, Bartel & Han 2011) and/or (ii) relatively low (specific) antibody levels to locally circulating strains.

Studies of disease dynamics rarely examine the distinctive roles played by migrant and resident host populations (Fenner, Godfrey & Bull 2011; Lachish *et al.* 2012; Leighton *et al.* 2012), despite the importance of movement behaviour to pathogen transmission (Altizer, Bartel & Han 2011; Galsworthy *et al.* 2011). The role of migratory populations in the transmission of AIV was recently examined at a macro-ecological scale, based on hosts wintering in California, USA (Hill *et al.* 2012). However, the dynamics of infectious diseases, such as AIVs, are likely to occur at small spatial scales probably leading to disease clusters of limited geographic size (Barlow 1991). We therefore consider that, in examining potential drivers of seasonal infection dynamics, studies should ideally be conducted at the relevant small scale at which these transmission interactions take place. Also, defining migratory status can be conducted with the least error at small spatial scales.

Monitoring an infectious disease throughout a full annual cycle of a host species enables assessment of host population size, demography and critical life-history events (i.e. breeding, moult, migration), which might be linked to the dynamics of the infectious disease. In the case of AIVs, most surveillance programmes are focused on a single season of the annual cycle, often sampling a wide range of duck species in an highly opportunistic manner (Hoye *et al.* 2010). As a result, our current understanding of seasonal variation of LPAIV is based on compilations of these separate studies (Stallknecht *et al.* 1991; Krauss *et al.* 2004; Munster *et al.* 2007). Furthermore, integration of pathogen incidence with seasonal variation in antibodies to AIV, which sheds light on past infection history, has received little attention, although this has the potential to significantly enhance understanding of the ecology and epidemiology of infectious diseases (Hoye *et al.* 2011).

The aim of this study is to investigate the potential drivers underlying the seasonal dynamics of LPAIV infections in wild birds. We intensively surveyed the full annual cycle of a single partially migratory bird species, the mallard *Anas platyrhynchos*, on a small spatial scale, connecting current LPAIV infection (i.e. viral prevalence) and antibodies to AIV (i.e. seroprevalence) to age and migratory strategy. We hypothesize that, if the density of hosts is driving increased LPAIV prevalence, the epizootic would start in spring when hatched juveniles enter the population and/or just after autumn migration when all migrants have arrived on the wintering grounds. The epizootic is also expected to start with the addition of hatchlings to the population if the increase in LPAIV prevalence is induced by the influx of young naïve birds, with juveniles more likely to be infected, shed higher quantities of virus (Hoye, Fouchier & Klaassen 2012) and exhibit lower seroprevalence than adults. If an increase in LPAIV prevalence is induced by migrant susceptibility to infection, we expected the epizootic to start with the arrival of these migrants in autumn, with migrants more likely to be infected and show lower seroprevalence than resident birds.

Materials and methods

STUDY SPECIES

Mallards are considered a main LPAIV reservoir, together with other birds of wetlands and aquatic environments (order *Anseriformes* and *Charadriiformes*) (Webster *et al.* 1992), and harbour all HA and NA subtypes discovered in birds to date frequently, with the exception of H13 to H16 (Olsen *et al.* 2006). Mallards are partially migratory: meaning that throughout Europe the

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population consists of both migratory and resident birds. Birds breeding in Western Europe (i.e. the Netherlands) are mainly sedentary, and northern breeding birds (i.e. Scandinavia, the Baltic, north-west Russia) migrate in autumn to winter from Denmark to northern France and Britain (Scott & Rose 1996).

DUCK SAMPLING AND COUNTS

Mallards were caught at a duck decoy (Payne-Gallwey 1886) (51°52′38″N, 4°43′26″E) located near Oud Alblas in the Alblasserwaard, the Netherlands, from March 2010 until February 2011. During the breeding season, when it was more difficult to catch birds on the decoy, females were also caught from nests in the woodland surroundings with a sweep net to enlarge the sample size. The duck decoy is part of the national AIV surveillance programme executed by the Virology Department of Erasmus MC in Rotterdam, the Netherlands, and mallards have been sampled for LPAIVs at this location from 2005 onwards. In 2005 and (most of) 2006, only cloacal samples were taken to detect current LPAIV infection, which was extended to include oropharyngeal samples from 2007 onwards.

On average, the duck decoy was visited six times per month capturing *c*. 15 individuals per visit (n = 1109; Appendix S1, Supporting information). Each captured mallard was marked with a metal ring, sexed and aged as juvenile (<1 year) or adult (>1 year), based on plumage characteristics following Boyd, Harrison & Allison (1975). In April, the month that the first chicks hatched, juveniles from the previous year were assigned as adults, being *c*. 1 year old. The age of unfledged chicks was assessed by a sex-specific regression model, using weekly head+bill measurements of unfledged mallard chicks raised in captivity (J.G.B. van Dijk, unpublished data). Primary moult was scored for adults in summer, with each primary feather given a score from 0 (old) to 5 (fully grown and new) (Newton 1966). Cloacal and oropharyngeal samples were taken using sterile cotton swabs and stored individually in transport medium (Hank's balanced salt solution with supplements; Munster *et al*. 2007) for detection of current LPAIV infection. Samples were preserved at 4 °C, transported to the Erasmus MC Department of Virology and analysed within 7 days of collection. Blood samples (0·5–1·0 ml, constituting up to 2% of the circulating blood volume) were collected from the brachial vein for detection of antibodies to AIV. Blood samples were allowed to clot for *c*. 6 h before being centrifuged in order to separate serum from red blood cells (Hoye 2012). Serum samples were stored at −20 °C until analysis. A small piece (1–2 cm) of the tip of the first primary feather (P1) of the right wing was collected, placed in a plastic zip-lock bag and stored at room temperature until stable hydrogen isotope analysis. When a bird was recaptured, a cloacal, oropharyngeal and a blood sample were collected, together with a piece of feather when feathers had been moulted between consecutive catches.

During the study, mallards were monthly counted (i) in the Alblasserwaard by the local bird group (only September 2010 until February 2011) (NVWA 2012), and (ii) across the Netherlands by the Dutch Centre for Field Ornithology (SOVON) (Hornman *et al*. 2013).

VIRUS AND ANTIBODY DETECTION

Cloacal and oropharyngeal samples were used to detect influenza A virus. For full details on RNA isolation, virus detection and isolation see Munster *et al*. (2007). In short, RNA was isolated using a MagnaPure LC system with the MagnaPure LC Total nucleic acid isolation kit (Roche Diagnostics, the Netherlands), and influenza A virus was detected using a generic real-time reverse transcriptase PCR assay targeting the matrix gene (M RRT-PCR). All M RRT-PCR positive samples were tested for the presence of H5 and H7 influenza A viruses by using haemagglutinin (HA)-specific RRT-PCR tests, and virus isolation was conducted in 11-day-old embryonated hens' eggs. The HA subtype of virus isolates were characterized with a haemagglutination-inhibition assay, and the neuraminidase (NA) subtype was determined by RT-PCR. The cycle threshold (C_T) value, which is the first real-time amplification cycle in which matrix gene amplification was detected, was used to assess the degree of viral shedding. The C_T-value is inversely proportional to the number of virus particles in a sample.

The presence of antibodies to the highly conserved nucleoprotein of AIV in serum was tested using a commercially available blocking enzyme-linked immunosorbent assay (bELISA MultiS-Screen Avian Influenza Virus Antibody Test Kit; IDEXX Laboratories, Hoofddorp, the Netherlands) following manufacturer's instructions. Samples were tested in duplicate, with each plate containing two positive and two negative controls. The absorbance was measured at 620 nm using an infinite M200 plate reader (Tecan Group Ltd, Männedorf, Switzerland). Signal-to-noise ratios (i.e. the absorbance of the samples divided by the mean absorbance of the negative control) <0·5 were considered positive for the presence of antibodies to AIV.

STABLE ISOTOPE ANALYSIS

To determine the origin (i.e. moulting location) of individuals, stable hydrogen isotope analysis was performed on the feathers of birds sampled from August until December. Stable isotope signatures in feathers (and other animal tissue) reflect those of local food webs (Peterson & Fry 1987). Local precipitation is incorporated into feathers during the period of growth (Hobson 1999) causing the stable hydrogen isotope ratio (δ^2H) in feathers to be correlated with δ^2H of local precipitation (Chamberlain *et al*. 1997), which exhibits a gradient across Europe (Bowen, Wassenaar & Hobson 2005, van Dijk, Meissner & Klaassen 2013). Birds were classified as either resident, local migrant (i.e. short distance) or distant migrant (i.e. long distance) based on feather δ^2H. Criteria used to allocate individuals to each group were time of capture, recapture rate and whether or not they were in moult (for full details see Appendix S2, Supporting Information).

Stable hydrogen isotope measurements were analysed on a Delta Plus XL isotope ratio mass spectrometry (IRMS) (via CONFLO II) equipped with a thermo-electron high-temperature conversion elemental analyzer (TC/EA) pyrolysis furnace at the Colorado Plateau Stable Isotope Laboratory (Northern Arizona University, Flagstaff, AZ, USA). Prior to analysis, feathers were cleaned with a 2 : 1 chloroform : methanol solvent mixture to remove surface contaminants and oils, and air-dried overnight under a fume hood. Feather samples (*c*. 0·33–0·37 mg) were placed into 5·0 × 3·5 mm silver capsules, folded into tiny balls and stored in 96-well trays. Stable hydrogen isotope measurements on feathers and keratin standards were performed on H_2 derived from high-temperature flash pyrolysis of feathers and continuous-flow IRMS. All feather δ^2H results are reported in units per mil (‰) relative to the Vienna Standard Mean Ocean

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Water-Standard Light Antarctic Precipitation (VSMOW-SLAP) standard scale and calibrated using the comparative equilibration technique with pre-calibrated keratin standards (Wassenaar & Hobson 2003). Repeated analyses of the standards indicated an external repeatability of 2‰.

STATISTICAL ANALYSIS

A bird was considered LPAIV positive when either the cloacal or the oropharyngeal sample was positive. The 12-month data set contained individuals that were sampled once, and those which were recaptured and sampled multiple times throughout the sampling period. For recaptured birds, only one measurement per month with a sampling interval of at least 30 days was (randomly) selected and used in the analysis. This was done to ensure that birds were not used twice within the same infectious period; *c.* 90% of viral particles are shed through cloacal and oral routes within 3–4 days, with a maximum shedding duration of 18 days (Henaux & Samuel 2011). For season, the northern hemisphere meteorological seasons were used: *spring*: March–May; *summer*: June–August; *autumn*: September–November; *winter*: December–February. Only measurements from August until December were used in the analysis of viral and seroprevalence between birds of different origin.

Seasonal differences in viral and seroprevalence were determined using generalized linear mixed models (GLMMs), with season, age and sex as fixed factors, interactions between all variables and individual bird as random factor to correct for repeated measures. GLMMs were also used to assess viral and seroprevalence differences between birds of different origin, with a birds' origin (i.e. resident, local migrant, distant migrant), age, sex and month as fixed factors, all interactions and individual as random factor. A Tukey's post hoc test was performed to detect differences in viral prevalence between birds of different origin.

Linear mixed models (LMMs) were performed to determine differences in the degree of viral shedding (C_T-value) in the cloaca and oropharynx with age, sex and season as fixed factors, interactions between all variables and individual bird as random factor. To assess differences in the C_T-value in cloaca and oropharynx between birds of different origin, we performed a linear model (LM) with the birds' origin, age, sex and month as fixed factors, together with all interaction effects. All analyses were conducted using R 2.14.1 (R Development Core Team 2012). Package lme4 was used to fit LMMs and GLMMs, and multcomp to perform a Tukey's *post hoc* test.

Results

Over the 12-month period, samples were collected from 679 individual mallards of which 259 individuals were recaptured yielding an additional 430 samples (Appendix S1, Supporting information). On average, individuals were recaptured (mean ± SE) 1·7 ± 0·1 times with 108 ± 3 days between captures.

VIRAL AND SEROPREVALENCE

Prevalence of LPAIV infection differed among seasons (GLMM, $X^2 = 182{\cdot}86$, $P < 0{\cdot}001$) with a dominant peak in the proportion of birds infected in the autumn months, a minor peak in summer, while in spring no birds were found infected (Fig. 1a). The temporal pattern in LPAIV prevalence found in this study is in accordance with the seasonal pattern measured between 2005 and 2010 at this location, with some variance in the precise timing of the peaks, but consistently showing a dominant peak in prevalence at the end of summer or autumn (Appendix S3, Supporting information). Prevalence of AIV antibodies also differed among seasons (GLMM, $X^2 = 40{\cdot}51$, $P < 0{\cdot}001$), with seroprevalence being notably higher in winter and spring (November until May), and lower in summer and autumn (June until October) (Fig. 2).

HOST DENSITY

In the Alblasserwaard and across the Netherlands, mallard numbers showed a clear seasonal fluctuation, with the highest numbers found in winter and the lowest in spring and summer (Fig. 1d). The LPAIV infection peak in summer and autumn did not correspond with the highest density of mallards (Fig. 1).

NAÏVE JUVENILES

The modest increase in viral prevalence in summer coincided with the entrance of progressively more naïve unfledged juveniles to the resident population (Fig. 1b). In June, juveniles were between 4·5 and 8 weeks old, whereas in July, juveniles were either 3–4 weeks old or had fledged. Based on the LPAIV sampled birds from June until August, 11·1% of unfledged juveniles ($n = 18$) were infected compared with just 4·1% of adults ($n = 244$). Overall, juveniles were more likely to be infected with LPAIV than adults (juveniles: 32%, adults: 13%; GLMM, $X^2 = 7{\cdot}03$, $P = 0{\cdot}008$; Fig. 3a), with no effect of sex (♂: 17%, ♀: 15%; $X^2 = 0{\cdot}10$, $P = 0{\cdot}758$), or an interaction effect between age and sex ($X^2 = 0{\cdot}02$, $P = 0{\cdot}891$), age and season ($X^2 = 1{\cdot}20$, $P = 0{\cdot}573$), and season and sex ($X^2 = 0{\cdot}10$, $P = 0{\cdot}992$).

The quantity of viral particles shed from the cloaca did not vary between juveniles and adults (LMM, $X^2 = 2{\cdot}17$, $P = 0{\cdot}141$; Fig. 4a), with no effect of sex ($X^2 = 1{\cdot}47$, $P = 0{\cdot}225$), season ($X^2 = 0{\cdot}71$, $P = 0{\cdot}399$), nor an interaction effect between age and sex ($X^2 = 3{\cdot}37$, $P = 0{\cdot}067$). There was an interaction effect between season and sex ($X^2 = 11{\cdot}30$, $P = 0{\cdot}004$), such that females shed more viral particles from the cloaca as the year proceeded, while males shed less viral particles. In summer, juveniles tended to shed higher quantities of virus, although there was no overall age difference in the degree of viral shedding from the oropharynx (LMM, $X^2 = 0{\cdot}99$, $P = 0{\cdot}320$; Fig. 4b), with no effect of sex ($X^2 = 0{\cdot}92$, $P = 0{\cdot}337$). There was a significant effect of season ($X^2 = 6{\cdot}71$, $P = 0{\cdot}035$), with the lowest number of viral particles shed from the oropharynx in winter, after infection prevalence had peaked. There was an interaction effect between season, sex and age in the degree of viral shedding in the oropharynx ($X^2 = 9{\cdot}88$, $P = 0{\cdot}007$).

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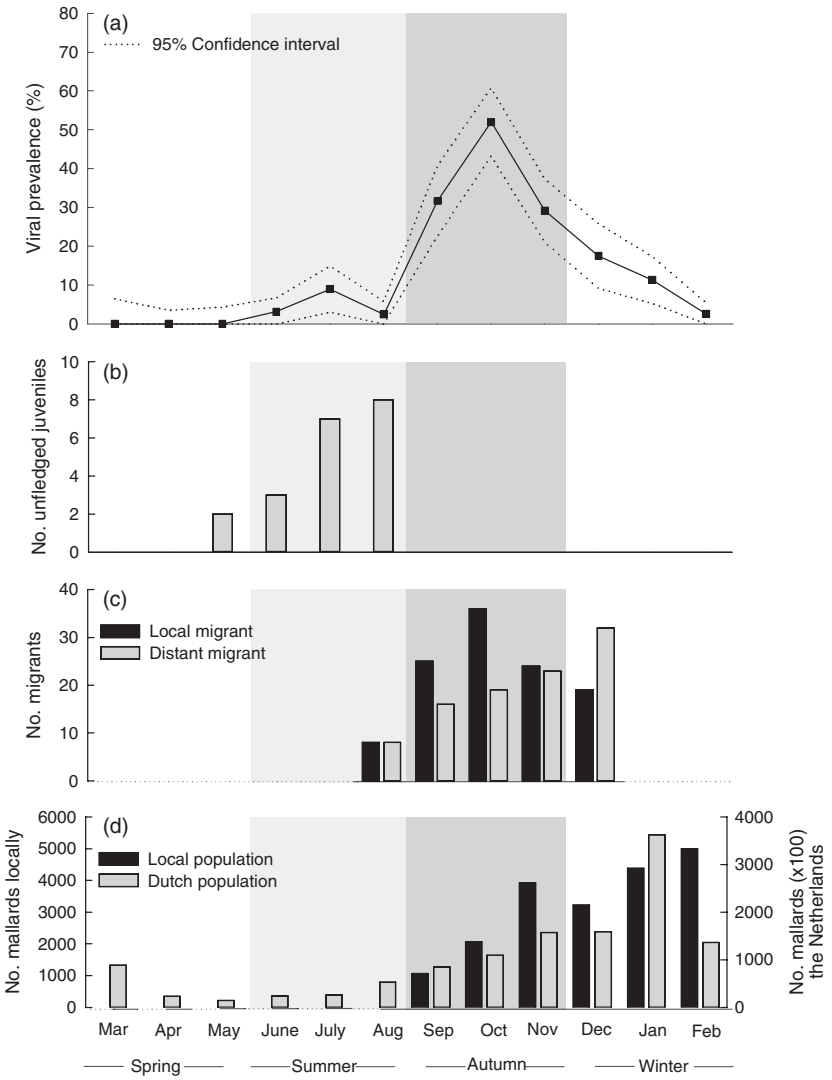

Fig. 1. Monthly prevalence (± 95% CI) of low pathogenic avian influenza virus (LPAIV) infection and population numbers of mallards, from March 2010 until February 2011. (a) Viral prevalence (i.e. current infection), (b) number of unfledged juveniles of the resident population, (c) number of local and distant migrants and (d) mallard counts in the Alblasserwaard (left *y*-axis) and across the Netherlands (right *y*-axis). The dotted line of the *x*-axis means that there is no data available.

There was no difference in seroprevalence between juveniles and adults (juveniles: 31%, adults: 38%; GLMM, $X^2 = 0{\cdot}21$, $P = 0{\cdot}644$; Fig. 3b). However, the juvenile age definition considered newly hatched individuals to belong to the same group as individuals up to 12 months of age. Importantly, there was a significant interaction between age and season ($X^2 = 10{\cdot}74$, $P = 0{\cdot}013$), with more adults being AIV seropositive in spring and summer, but more juveniles tending to have AIV antibodies in winter. There was a significant effect of sex ($X^2 = 7{\cdot}53$, $P = 0{\cdot}006$), with females more likely to be AIV seropositive than males (♂: 32%, ♀: 41%), and an interaction effect with season ($X^2 = 11{\cdot}84$, $P = 0{\cdot}008$), with the highest proportion of females having AIV antibodies in spring and winter. There was no interaction effect between age and sex ($X^2 = 0{\cdot}00$, $P = 0{\cdot}962$).

MIGRANTS

Peak prevalence of LPAIV infection in autumn corresponded with the arrival of migrants (Fig. 1c). Both local and distant migrants were more often infected with LPAIV than residents (respectively, 44%, 41% and 12%; GLMM, $X^2 = 37{\cdot}18$, $P < 0{\cdot}001$; Fig. 5a), with no effect of age ($X^2 = 0{\cdot}59$, $P = 0{\cdot}444$), sex ($X^2 = 0{\cdot}01$, $P = 0{\cdot}916$), but with a significant effect of month ($X^2 = 49{\cdot}10$, $P < 0{\cdot}001$). There was a significant interaction effect between a bird's origin and month ($X^2 = 16{\cdot}03$, $P = 0{\cdot}042$), with distant migrants most often infected with LPAIV in October. Other interactions were non-significant ($P > 0{\cdot}05$). Viral prevalence did not differ between local and distant migrants (Tukey, $P = 0{\cdot}711$).

Residents, local and distant migrants were shedding similar quantities of viral particles from the cloaca (LM, $F_{2,36} = 1{\cdot}63$, $P = 0{\cdot}210$), with no age ($F_{1,36} = 1{\cdot}30$, $P = 0{\cdot}262$), sex ($F_{1,36} = 2{\cdot}14$, $P = 0{\cdot}153$), month ($F_{2,36} = 0{\cdot}13$, $P = 0{\cdot}877$) or interaction effects (all $P > 0{\cdot}05$). Similarly, a birds' origin had no effect on the degree of viral shedding in the oropharynx (LM, $F_{2,85} = 1{\cdot}22$, $P = 0{\cdot}302$), with no age ($F_{1,85} = 0{\cdot}57$, $P = 0{\cdot}452$), sex ($F_{1,85} = 0{\cdot}09$, $P = 0{\cdot}770$), month ($F_{4,85} = 0{\cdot}77$, $P = 0{\cdot}547$) or interaction effects (all $P > 0{\cdot}05$).

Seroprevalence was similar among residents, local and distant migrants (respectively, 33%, 31% and 38%;

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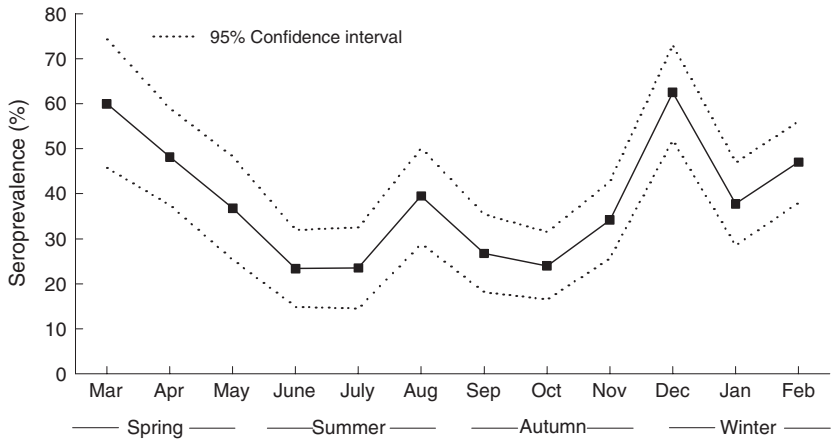

Fig. 2. Monthly prevalence (± 95% CI) of avian influenza virus (AIV) antibodies (i.e. past infection) in mallards from March 2010 until February 2011.

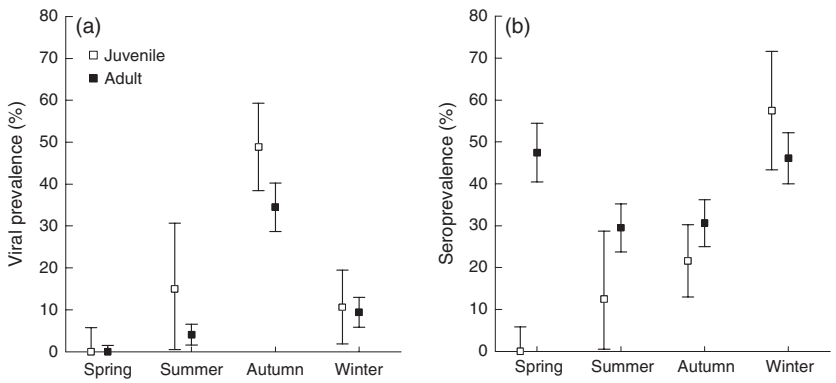

Fig. 3. Seasonal prevalence (± 95% CI) of low pathogenic avian influenza virus (LPAIV) infection in juvenile (<1 year) and adult (>1 year) mallards. (a) Viral prevalence and (b) seroprevalence.

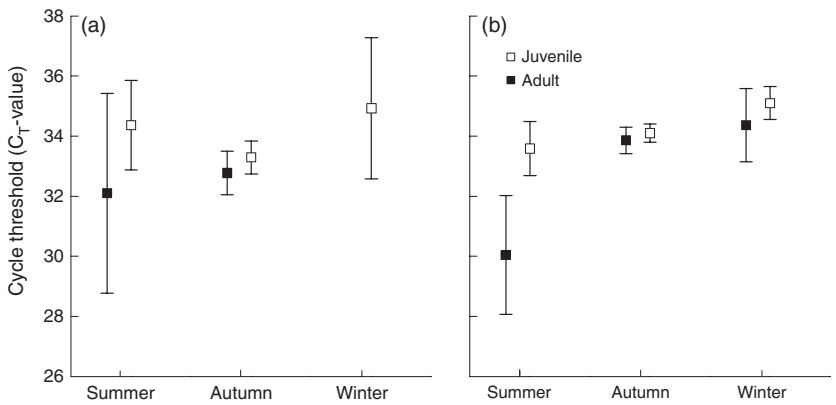

Fig. 4. Degree of viral shedding (i.e. C_T-value) (mean ± SE) of low pathogenic avian influenza virus (LPAIV) -positive juvenile (<1 year) and adult (>1 year) mallards detected in (a) cloacal and (b) oropharyngeal samples. The C_T-value is inversely proportional to the number of virus particles in a sample, with lower C_T-values indicating large quantities of virus.

GLMM, $X^2 = 0{\cdot}76$, $P = 0{\cdot}685$; Fig. 5b), with no effects of age ($X^2 = 1{\cdot}65$, $P = 0{\cdot}199$) and sex ($X^2 = 0{\cdot}00$, $P = 0{\cdot}960$), but with a significant month effect ($X^2 = 27{\cdot}18$, $P < 0{\cdot}001$). There was a significant interaction between sex and month ($X^2 = 11{\cdot}11$, $P = 0{\cdot}025$), with a high proportion of males being AIV seropositive in August and December, and most females having AIV antibodies in December. All other interactions were non-significant ($P > 0{\cdot}05$).

SUBTYPES

Subtypes were determined in 12·6% (22/174) of the LPAIV-positive birds with H7N3 ($n = 3$) in summer and H3N8 ($n = 16$), H4N6 ($n = 2$) and H10N7 ($n = 1$) in autumn. Of the H3N8 birds, 12 individuals were local migrants, and two were distant migrants (two individuals could not be assigned to either the resident or migratory group). Only distant migrants were infected with H4N6 and H10N7. No subtypes could be determined for viruses detected in resident birds.

Discussion

HOST DENSITY

The peak of LPAIV infection in autumn did not coincide with the peak in numbers of mallards at our study site and across the Netherlands, which occurred in winter (see also mean monthly numbers of mallards from 2005 to

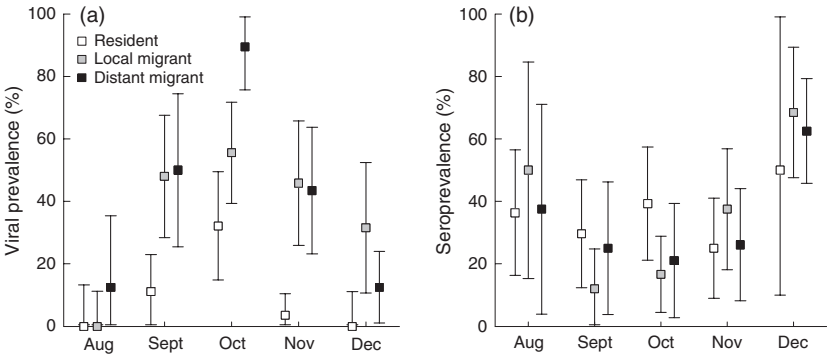

Fig. 5. Prevalence (± 95% CI) of low pathogenic avian influenza virus (LPAIV) infection in mallards for residents, local migrants and distant migrants from August until December. (a) Viral prevalence and (b) seroprevalence.

2009; Hornman *et al*. 2013). However, the increasing host densities in autumn may still have played a role in the increase of LPAIV prevalence. Gaidet *et al*. (2012) also failed to find a positive relation between AIV prevalence and host density at the species level, but did find a positive correlation at the wildfowl community level in Africa. Similarly, host density has been shown to play a role, though not necessarily always of overriding importance in other host-parasite systems (Johnson *et al*. 2009; Winternitz, Yabsley & Altizer 2012; Estep *et al*. 2013).

NAÏVE JUVENILES

The LPAIV infection peak in summer seemed to be driven by the entrance of unfledged naïve young in the population. In addition to temporal correspondence, juveniles were more likely to be infected with LPAIV, shed higher quantities of virus from the oropharynx and were less likely to have detectable antibodies to AIV. Similar patterns of higher AIV infection and lower seroprevalence in juveniles have been shown in Bewick's swans *Cygnus columbianus bewickii* (Hoye, Fouchier & Klaassen 2012). The importance of juveniles driving disease dynamics has also been shown in a wide range of other host-parasite systems (e.g. Young & VanderWerf 2008; Chylinski *et al*. 2009; Dhondt *et al*. 2012). For instance, pulses of infected juveniles have been shown to coincide with peak prevalence of Marburg virus in *Rousettus aegyptiacus* bats (Amman *et al*. 2012). Infected juvenile bats tended to be relatively old and had lost maternal antibody protection, which is likely also the case in our study with LPAIV-infected juveniles being at least older than 3·5 weeks. Maternal antibodies (notably immunoglobulin IgY), resulting from the mother's past exposure to pathogens and transferred via the egg to the offspring (Boulinier & Staszewski 2008), reach minimum levels 2 weeks after hatching in mallards (Liu & Higgins 1990). In our study, the proportion of juveniles having AIV antibodies seemed to increase with age, similar to the results found in bats (Amman *et al*. 2012). The high quantity of viral particles shed by young LPAIV-infected juveniles in summer is in accordance with the overall results of AIV experimental studies across juvenile mallards from 1 to 4 months of age (Costa *et al*. 2010), except that they found most viral particles in the cloaca, but in our study the highest quantity of viral particles were detected in the oropharynx. Similar to Costa *et al*. (2010), we found a decrease in viral shedding with age (i.e. high number of viral particles in summer and lower numbers in autumn and winter in juveniles).

MIGRANTS

The peak of LPAIV infection in autumn corresponded with the arrival of migrants, both local and distant. Passage of migrants was coincident with the timing of this peak, and migrants were more likely to be infected with LPAIV and tended to show lower seroprevalence for AIV, although the latter was not significantly different from resident birds. Migratory juveniles and adults were equally likely to be infected, suggesting their migratory status may be an overriding driver of LPAIV infection in autumn. Our results contradict the findings of Hill *et al*. (2012), who did not find a difference in AIV infection between residents and migrants. However, these authors defined residents as birds residing across a much larger area (i.e. CA, USA), whose movements may have been sufficient to have been classified as local migrants and possibly even distant migrants in our study.

Interestingly, peak infection seemingly does not start with the arrival of the very first migrants in August, implying that their numbers were too low to cause an infection peak. In mallards, the predominant influx of autumn migrants generally extends from late September until mid-December, peaking in October/November (Scott & Rose 1996; LWT/SOVON 2002). This influx coincided with LPAIV peak prevalence, which was mainly due to infected migrants. Remarkably, the temporal pattern in seroprevalence was very similar for migrants and residents, despite large differences in infection prevalence. Moreover, during the peak migratory period, migrants were found to have a substantially higher prevalence of active infections than antibodies to infection (Fig. 5). This highly unusual yet robust finding reflects the dynamic nature of infection in the sampled population. Migrants sampled during this period, particularly those classified as distant migrants, represent successive populations transiting through the study

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site rather than a longitudinal sample from a single population, as was the case for residents. As such, we would not expect seroprevalence in migrants to track infection prevalence, increasing as autumn progressed as it did in the resident population. Taken together, these results suggests that on arrival at the study site each wave of migrants had low seroprevalence, rendering them more susceptible to infection and resulting in local amplification of AIV circulation. Increased susceptibility to infection in migrants may reflect naivety to AIV, naivety to locally circulating strains, reduced immunocompetence or a combination of these processes.

It is possible that migratory birds, despite having similar prevalence of antibodies to AIV in general, were naïve to LPAIV strains circulating on the study site. In contrast, residents may have had a greater degree of immunity to these LPAIV strains conferred by previous infections and were therefore less susceptible. Indeed, laboratory infections of mallards have shown that heterosubtypic antibodies provide only partial protection against reinfection (Fereidouni *et al*. 2009). Ideally, the relative naivety of migrants and residents to circulating LPAIV strains could be assessed indirectly, by comparing the AIV subtypes to which these groups have specific antibodies to the virus subtypes detected in this study. Unfortunately, samples for which subtypes could be determined were all assigned to local and distant migrants, making it impossible to assess whether migrants were either infected by dominant subtypes that were already present in resident birds, or had brought a novel subtype to the wintering grounds infecting resident birds. Alternatively, migrants may have faced physiological trade-offs that resulted in a reduction of immune function (Altizer, Bartel & Han 2011). However, such a reduction in immunocompetence would be expected not only to increase the number of individuals infected, but also result in more intense infections. This appears not to have been the case in our study, as the degree of viral shedding was similar between migrants and residents. Finally, loss of immunity, through a period without exposure to AIV, could also enhance susceptibility in migrants and even potentially drive seasonal variation in LPAIV. Limited studies of natural AIV infection and immunity in wild birds suggest that antibodies to LPAIV have a relatively short life span (Kida, Yanagawa & Matsuoka 1980; Fereidouni *et al*. 2010; Hoye *et al*. 2011); however, the exact duration of the antibody response and any associated protective immunity against subsequent LPAIV infection is still largely unknown. Although migrants have often been implicated to play a role in the dispersal of AIVs (e.g. Hill *et al*. 2012), our study highlights their likely pivotal role in local amplification and raises the intriguing question of whether they are primarily a source or sink of infections when arriving at a new site.

In conclusion, by examining a single host species at an epidemiologically relevant spatial scale throughout a complete annual cycle, we identified two likely key drivers of LPAIV infection dynamics: the influx of immunologically naïve juveniles in summer and the arrival of susceptible migrants in autumn. For the role of migrants in infection dynamics, we suggest two non-mutually exclusive processes that await further testing: increased susceptibility to infection due to (i) reduced immunocompetence as a result of migration and (ii) absence of antibodies against locally circulating viral strains. Immunocompetence may be tested using immuno assays (i.e. leucocyte concentrations, haptoglobin, haemolysis–haemagglutination), while AIV subtypes of antibodies may be assessed using a haemagglutination-inhibition test. We found far less compelling evidence for a role of host density in driving seasonal epizootics; however, we cannot preclude this as an additional modulating influence on local LPAIV prevalence. With this study, we highlight the importance of host demography and migratory behaviour in explaining seasonal epizootics in wildlife diseases.

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Data accessibility

-Number of birds sampled: uploaded as online Supporting Information.
-Virus and antibody information, C_T-values and hydrogen stable isotope measurements are available from the Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.j855b.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Appendix S1. Number of samples collected from primary captures and recaptures of mallards, by age and sex.

Appendix S2. Assessing the origin of mallards using stable hydrogen isotope analysis in feathers.

Appendix S3. Monthly prevalence of LPAIV infection in mallards sampled at the duck decoy from January 2005 until February 2010.

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