Pathogen dynamics in a partial migrant

Interactions between mallards (*Anas platyrhynchos*) and avian influenza viruses

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Pathogen dynamics in a partial migrant

Interactions between mallards (*Anas platyrhynchos*) and avian influenza viruses

De dynamiek van ziekteverwekkers in een deelmigrant

Interacties tussen wilde eenden (*Anas platyrhynchos*) en vogelgriep

(met een samenvatting in het Nederlands)

Proefschrift

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Contents

1 Introduction: zoonotic pathogens and their interaction with wildlife

A WORLD FREE OF PARASITES AND VECTORS:
WOULD IT BE HEAVEN,
OR WOULD IT BE HELL?
Holt (2010)

Nearly two-thirds of the world's infectious diseases in man are caused by zoonotic pathogens (pathogens from a non-human animal source; Taylor et al. 2001, Jones et al. 2008). The majority of these diseases originate in wildlife (72%). The number of events caused by pathogens that originate from wildlife has increased with time (King et al. 2006, Jones et al. 2008). Well-known examples of emerging infectious diseases are HIV/AIDS, severe acute respiratory syndrome (SARS), Nipah virus, Ebola haemorrhagic fever, H5N1 influenza virus, all of which emerged from wild animals (chimpanzees, bats, birds) and jumped directly or indirectly via domestic animals to humans (Morens and Fauci 2013). Recent emerging infectious diseases are the Middle East respiratory syndrome (MERS-CoV) and H7N9 influenza virus, both causing death in one third of the infected people. Many of these infectious diseases cause severe illness in humans and animals, an exception being H7N9 influenza virus. This disease can be seriously pathogenic for humans, while animals (wild birds, poultry) show no clear symptoms of disease (Kreijtz et al. 2013). Emerging zoonoses may pose a serious threat for humans, especially when they are able to spread silently in the animal reservoir, like the H7N9 virus. Infectious diseases are currently the greatest burden on human health and livelihoods around the world, with a billion people getting sick and millions of them dying every year (Karesh et al. 2012). Human actions are likely a major determinant for these pathogens to jump the species barrier, such as land modification for extractive industry (e.g. deforestation, habitat fragmentation, encroachment into wildlife habitat), modern agricultural practices (e.g. intensification of livestock farming) and international trade of livestock and wildlife (Daszak et al. 2000, Karesh et al. 2012). It has been argued that, besides focussing on livestock and humans, a better understanding of the ecology and transmission of emerging zoonoses in wildlife is required in order to manage and control these pathogens in the future (Kuiken et al. 2005, Coker et al. 2011).

Pathogen dynamics in wildlife

Most studies on pathogen population biology focussed on infections in humans and livestock (Anderson and May 1991, Scott and Smith 1994). However, over the last two decades, the importance of pathogens in the dynamics of wild animal populations is increasingly recognized (McCallum and Dobson 1995). Studying dynamics of zoonotic pathogens in

wildlife can potentially assist in predicting outbreaks in livestock and humans, and ultimately reduce the number of disease cases (Karesh et al. 2012). Notably, unravelling the ecological processes underlying pathogen dynamics in wildlife may be important to develop adequate control measures to prevent disease emergence and spread to other hosts. Improved disease prevention as a result of a more thorough understanding of the processes underlying malaria dynamics may serve as an example here. High temperatures and rainfall are used as an early warning system for malaria incidence in humans, since these factors result in increased population sizes of the *Anopheles spp*. mosquito vector. During these periods appropriate control measures can be taken, such as house spraying and chemoprophylaxis for pregnant women and school children (Thomson et al. 2005). Pathogen dynamics in wildlife frequently show seasonal fluctuations, which are generated by changes in host and pathogen biology that occur annually, approximately around the same time. Suggested mechanisms driving these seasonal infection dynamics are changes in weather conditions, as is the case with malaria, and changes in host factors, such as aggregation, behaviour, immune defences and changes in host demography (e.g. births, deaths) (Altizer et al. 2006). Seasonal patterns of rabies in jackals (*Canis mesomelas and C. adustus*) for instance, are likely explained by breeding and dispersal of animals, and the addition of juveniles to the population (Rosatte 1984, Loveridge and Macdonald 2001). Understanding these drivers enables to control rabies by vaccinating jackals before the mating season and after the whelping season (Loveridge and Macdonald 2001). Control measures are used to stop the disease from being transmitted and spread to other areas, potentially infecting (new) hosts. Thus in order to undertake adequate control measures, a better understanding of the effects that pathogens have on their host, and how pathogen and host dynamics interact, is required.

Effects of pathogens on wildlife hosts

Zoonotic pathogens frequently cause mild disease in wildlife. However, history shows that pathogens can also be responsible for massive declines of animal population numbers. Rinderpest morbillivirus for instance, killed around 90% of Kenya's buffalo (*Syncerus caffer*) population in ten years' time after it was first introduced in Africa at the end of the 20th century (Plowright 1982). Phocine and canine distemper epidemics caused mass mortality in several species of seals in 1988 and early 2000 (Härkönen et al. 2006, Kuiken et al. 2006). Pathogens may also drive threatened species to the verge of extinction, as is the case for canine distemper virus in Ethiopian wolves (*Canis simensis*) and black-footed ferrets (*Mustela nigripes*) (Thorne and Williams 1988, Laurenson et al. 1998). However, not always have pathogens such detrimental effects on wildlife populations. By inducing a nutritionally demanding immune response ('resistance') and/or by extracting resources to cope with

infection ('tolerance') (Roy and Kirchner 2000), pathogens may have subtle pathogenic effects shaping host population dynamics (Anderson and May 1978). Pathogens are recognized to impair host reproduction and survival (de Crespigny and Wedell 2006, Burthe et al. 2008, Mayack and Naug 2009). Such effects may be mediated by pathogen induced changes in host foraging behaviour, in which less food is obtained due to reduced foraging efficiency (Venesky et al. 2009), or by changes in the host's migratory behaviour, in which the capacity for locomotion is reduced (Bradley and Altizer 2005). The energetic status and the immunological condition of the host are correlated with disease susceptibility. Hosts in poor body condition and with low antibody levels are more susceptible to infection (Whiteman et al. 2006, Beldomenico et al. 2009). It is thus important to include host characteristics when studying pathogen dynamics and its effects on population numbers.

Effects of host characteristics in pathogen dynamics

Young animals are often more susceptible to pathogen infection than adults, since they are immunologically naïve with a less developed acquired immune system (Hudson and Dobson 1997). Males and females may also vary in susceptibility, due to differences in hormones affecting immunity and differential exposure to pathogens due to sex-specific behaviour (Zuk and McKean 1996). Animals that vary in migratory status are also likely to differ in their response to pathogen infection. Migratory birds often migrate long distances between breeding and wintering grounds in order to exploit seasonal changes in resource availability (Dingle 1996). The energetic demands of migration likely compromises a bird´s immune function, resulting from a trade-off between investment in immune defences and long-distance flight, increasing susceptibility to infection (Altizer et al. 2011). Resident (sedentary) species on the other hand, stay close to their breeding grounds year round, undertaking short-distance movements only. They likely differ in susceptibility from migrants, since their immune system is not impaired by migration. An example of such differences between migratory and resident birds was found in several thrush species by Owen and Moore (2006), where migratory individuals were found to have a lower energetic condition and were immunocompromised compared to non-migrating individuals.

Hosts that differ in disease susceptibility likely contribute differently to pathogen dynamics. The births of young animals that are naïve to locally circulating pathogens may thus drive seasonal variation in wildlife pathogen dynamics (Altizer et al. 2006). During the mating season, the role of males in disease dynamics could be more profound since immunosuppressive effects of testosterone, produced to maximize competitive ability and sexual attractiveness, increase susceptibility to pathogen infection (Zuk and McKean 1996). Hosts differing in migratory behaviour, and hence differing in susceptibility, are also likely to fulfil

different roles in pathogen dynamics. Migratory animals may amplify pathogen prevalence at breeding, wintering or staging sites due to naivety to locally circulating pathogens (Leighton 2002) or due to reduced immunocompetence caused by migration (Altizer et al. 2011). Migrants may also increase disease prevalence at sites by infecting other hosts with novel pathogens that they transported from areas they previously visited. In this way, migrants are suggested to importantly contribute to the global spread of infectious diseases (Altizer et al. 2011), such as *Borrelia burgdorferi* (spirochaetes that cause Lyme disease), West Nile virus and H5N1 influenza virus (Gylfe et al. 2000, Rappole and Hubálek 2003, Gilbert et al. 2006). However, this importantly relies on the assumption that migratory hosts are not dramatically affected by the pathogen itself and are still able to migrate successfully, remain infectious until arrival at a site and transmit the pathogen successfully to susceptible hosts (Bauer and Hoye 2014). On the other hand, the migratory process is suggested to reduce disease prevalence in populations of migratory animals through animals abandoning contaminated habitats (′migratory escape′) and the effect of reduced survival among infected migrants (′migratory culling′; Altizer et al. 2011). Conversely, resident animals may also influence disease prevalence at sites frequented by migrants. Being immunologically naïve to pathogens introduced by migratory hosts, residents may act as local amplifiers of disease prevalence. Residents are often suggested to play a key role in permanently maintaining pathogens as so-called reservoirs and transmitting these pathogens to other hosts (Haydon et al. 2002, Waldenström et al. 2002). Although the importance of including migratory status of animals in studies on pathogen dynamics is often acknowledged, the role of migratory and resident hosts within a species in infectious disease dynamics has largely remained unstudied.

Partial migration in pathogen dynamics

Migratory strategies may vary within animal species, in which individuals may differ in disease susceptibility and in their contribution to disease dynamics. An extreme case of such intra-species variation in migration strategy is partial migration: some individuals migrate between habitats whilst others remain resident in a single habitat (Lack 1943, Dingle 1996). Partial migration is a widespread phenomenon in nature, ranging from insects to higher vertebrates (Lundberg 1988). It is expected that similar assumptions for disease susceptibility between migratory and resident species also apply for migratory and resident hosts within a species. However, migrants and residents of the same species may also differ in morphology and behaviour, such as body size, competition and fasting endurance (Chapman et al. 2011). As a consequence, resident and migratory individuals may respond differently to infection, hereby contributing differently to pathogen dynamics. From the above it is evident that in host-pathogen systems involving partial migrants, a distinction between

migrant and resident hosts within species should be made in order to elucidate disease dynamics in wild animal populations.

Aim of this study

Avian influenza represents one of the greatest concerns for public and animal health that has emerged from the animal reservoir. Recently, numbers of outbreaks of highly pathogenic avian influenza virus (HPAIV) in poultry have been alarmingly high, with more than 150 outbreaks of HPAIV subtypes reported in various countries around the world in the last 10 years, with 13 outbreaks in 2014 alone (up to 31 July 2014; World Organisation for Animal Health 2014). This is largely due to HPAIV H5N1, which since its emergence in 1996 has led to the culling of hundreds of millions of poultry and also caused almost 400 human deaths (up to January 2014; World Health Organization 2014a). Reasons for these HPAIV outbreaks to occur are the densely populated areas with poultry, which emerged as a result of the intensification of the poultry industry, making disease outbreaks more likely to occur and more difficult to control. As an early warning system for the global spread of HPAIV H5N1 and other notifiable avian influenza viruses (AIVs), there are many surveillance schemes around the world that sample wild birds for both HPAIV and low pathogenic avian influenza virus (LPAIV). LPAIV circulates naturally in wild birds of which subtypes H5 and H7 form the precursor of HPAIV. The classification between LPAIV and HPAIV is based on the ability to cause respectively mild and systemic infections in poultry (Suarez 2008). However, just recently with the LPAIV H7N9 epidemic it became clear that not only HPAIV H5 and H7 subtypes, but also LPAIV H7 can be highly pathogenic for humans (Kreijtz et al. 2013). Thus not only HPAIVs, but also LPAIVs may pose a serious threat to man. The role of wild birds in the global spread of HPAIV and LPAIV is largely unclear, although their contribution to the spread of HPAIV H5N1 has often been suggested (Gilbert et al. 2006, Kilpatrick et al. 2006, Si et al. 2009). Hence, it is of great societal interest to clarify the role of wild birds in AIV infection dynamics.

The aim of my study was to advance our knowledge of the ecological processes underlying the epidemiology of LPAIVs in wild birds, by studying the interactive ecology of avian hosts with LPAIV in a natural setting. In this thesis, I studied the host-pathogen interaction between a free-living key LPAIV host species, mallard ducks *(Anas platyrhynchos),* and LPAIVs at a local scale in the Netherlands. By sampling mallards at a duck decoy (i.e. swim-in traps connected to a large pond) throughout a complete annual cycle, I investigated the underlying mechanisms of seasonal dynamics of LPAIV infections in wild birds, the role of migratory and resident hosts in LPAIV epizootics, and associations between LPAIV infection and host condition, immune status and behaviour. I focussed on

the role of resident and migratory hosts of a single species (i.e. partial migrant) in LPAIV infection dynamics, since these roles are still poorly understood. Thus far only one study, Hill et al. (2012), investigated the role of resident and migratory hosts in dynamics of LPAIV infections in the same species. However this study was conducted at a macro-ecological scale while LPAIV infection dynamics, and other infectious diseases, likely occur at small spatial scales. Since LPAIV infections are generally short (i.e. up to a week; Latorre-Margalef et al. 2009), and most virus particles are shed within the first few days (Hénaux and Samuel 2011), it is unlikely that LPAIV will be spread over long distances, even during migration (Lebarbenchon et al. 2009). Therefore, studying the dynamics of LPAIV infections at an epidemiologically relevant small spatial scale is important when elucidating the ecological processes underlying this infectious disease in wild birds.

Study system

Low pathogenic avian influenza virus

LPAIV (Box 1.1) is a common pathogen that circulates naturally in wild birds. Predominantly birds inhabiting wetlands and aquatic environments, particularly species of the orders *Anseriformes* (ducks, swans, geese) and *Charadriiformes* (gulls, terns, waders), are infected with LPAIV (Webster et al. 1992). Therefore they are considered the major natural LPAIV reservoir. Dabbling ducks of the *Anas* genus, and in particular mallards, are infected more frequently with LPAIV than any other species. Nearly all possible combinations of the 16 HA and 9 NA antigenic subtypes are found in wild dabbling ducks (Olsen et al. 2006). This is likely due to their foraging behaviour, since they feed primarily on food in surface waters (del Hoyo et al. 1992). LPAIV may thrive and survive in lake water from several days to weeks, and even months if the temperature remains below 0°C (Stallknecht et al. 1990a, Nazir et al. 2010). Dabbling ducks display the propensity of abmigration (i.e. switching of breeding grounds between years; del Hoyo et al. 1992), which may increase the chance of transmission of LPAIV to other host populations (Olsen et al. 2006).

Extensive surveillance studies of wild ducks in the northern hemisphere showed that prevalence of LPAIV exhibits marked seasonal variation. In North America viral prevalence (i.e. current infection) in wild ducks varies from 22-60% during autumn migration, to 0.4-2% in winter time and less than 1% during spring migration (Sharp et al. 1993, Krauss et al. 2004, Olsen et al. 2006). In summer, around 25% of ducks is infected with LPAIV in Alaska (Runstadler et al. 2007). In northern Europe similar trends in viral prevalence for wild ducks are observed: an annual peak in late summer and early autumn, followed by low infection during the winter period and a small increase during spring (Olsen

et al. 2006, Munster et al. 2007, Latorre-Margalef et al. 2014). However, it should be noted that our current understanding of seasonal variation of LPAIV is primarily based on compilations of data from separate studies that were often focussed on a single season of the annual cycle, by sampling a wide range of duck species in a highly opportunistic manner (Hoye et al. 2010). Mechanisms suggested to drive this seasonal variation in LPAIV prevalence in wild birds are host density, immunologically naïve juveniles and migratory birds (Hinshaw et al. 1980, Klaassen et al. 2011, Gaidet et al. 2012).

LPAIV infection in waterfowl and notably dabbling ducks causes only mild signs of disease (Daoust et al. 2011, Kuiken 2013). Protection from antibodies against AIV is expected to be short-lived (less than a year) in wild birds (Hoye et al. 2011). Only few studies focused on the relationships between LPAIV infection and waterfowl condition and behaviour (Latorre-Margalef et al. 2009, Hoye 2011, Hoye et al. 2012). LPAIV infected birds likely have a lower body mass than non-infected individuals (van Gils et al. 2007, Latorre-Margalef et al. 2009, Kleijn et al. 2010). Although based on a very small sample size, van Gils et al. (2007) showed that wild migratory Bewick's swans (*Cygnus columbianus bewickii*) that were naturally infected with LPAIV left one month later on spring migration than non-infected individuals. In the same species, Hoye et al. (2012) showed that individuals that were preferentially foraging in aquatic habitats experienced a higher risk of LPAIV infection than when foraging in terrestrial habitats.

The mallard

Mallards are one of the most numerous and well-known waterfowl species in the world. The global population is estimated at approximately 19 million individuals (Delany and Scott 2006), with population numbers in north-west Europe estimated at around 5 million (Scott and Rose 1996). Mallards are an important game species in many parts of the world (Hirschfeld and Heyd 2005). Annually, large numbers of captive-reared mallards are introduced into the wild in Europe for hunting purposes (probably >1 million birds; Champagnon et al. 2009). Throughout Europe, mallards are partially migratory in which northern breeding birds (i.e. Finland, Sweden, the Baltic, north-west Russia) migrate southwards to winter, while birds breeding in temperate regions (i.e. mostly Western Europe) are sedentary or dispersive (Scott and Rose 1996, van Toor et al. 2013).

Furthermore, wild mallards are frequently infected with LPAIV and harbour all HA and NA subtypes discovered in birds to date, the exceptions being H13 and H16 (Kawaoka et al. 1990, Röhm et al. 1996, Olsen et al. 2006). Mallards are capable to sustain LPAIV by themselves and function as a reservoir species (Nishiura et al. 2009). When experimentally infected with LPAIV, mallards show little to no clinical signs (Daoust et al. 2011, Kuiken

Box 1.1 Avian influenza virus

Avian influenza virus (AIV) is a pathogen of the type A influenza virus that is adapted to avian hosts. These viruses most commonly infect wild bird species and poultry (e.g. domestic chickens, turkeys, ducks), but are also known to infect a variety of mammals (e.g. swine, horses, whales), including humans. Just recently it was discovered that bats, besides wild birds, likely constitute an important reservoir for influenza viruses (Tong et al. 2013). Influenza A viruses are members of the *Orthomyxoviridae* family, with the genome consisting of eight segments of negative-stranded RNA, which code for 11 proteins (Webster et al. 1992). Influenza viruses are classified on the basis of the antigenic properties of two glycoproteins expressed on the surface of the virus particle: hemagglutinin (HA) and neuraminidase (NA). These proteins are involved in the binding of the viral particles to host cells, the release of genetic information and, after multiplication in the host cells, release of new viral particles (Perdue 2008). To date, 16 HA subtypes (H1 to H16) and 9 NA subtypes (N1 to N9) are found in wild birds (Olsen et al. 2006), with each virus having one HA and one NA in any combination. H13 and H16 subtypes are specific for gull species (Hinshaw et al. 1982, Fouchier et al. 2005). Recently, novel subtypes H17, H18, N10, and N11 were identified in fruit bats (Tong et al. 2012, Tong et al. 2013).

In most cases, AIVs are of a low pathogenic phenotype (LPAIV), causing only mild diseases. However, the LPAIV subtypes H5 and H7 may become highly pathogenic (HPAIV) after introduction into poultry, which may cause disease outbreaks, previously known as 'fowl plague', with up to 100% mortality in unprotected poultry flocks (Alexander 2007). The classification between LPAIV and HPAIV is based on clinical disease in poultry: LPAIV cause infections of the intestinal and respiratory tract, and HPAIV cause systemic infections (Suarez 2008). LPAIV may switch to HPAIV due to changes in the amino acid composition of the HA (Perdue 2008). In wild birds, LPAIV preferentially infects cells lining the intestinal tract (Daoust et al. 2012, Höfle et al. 2012), even though replication of LPAIV can also be observed in the respiratory tract (Kida et al. 1980). LPAIV is transmitted primarily by the faecal-oral route, with virus particles in faeces shed into the surface water, which is likely a very efficient way to transmit viruses between waterfowl (Webster et al. 1992).

2013), and antibodies to the nucleoprotein of AIV persist up to eight months (Fereidouni et al. 2010). Mallards are partially protected against reinfection with a similar HA subtype (homologous immunity) and to a lesser extent to different HA subtypes (heterologous immunity; Fereidouni et al. 2009, Jourdain et al. 2010, Latorre-Margalef et al. 2013). Mallards are also an important study species for HPAIV, since they are closely related to domestic ducks (Pekin ducks, *Anas platyrhynchos domesticus*) and show hardly any clinical signs when experimentally infected with this virus (Keawcharoen et al. 2008).

Duck decoy

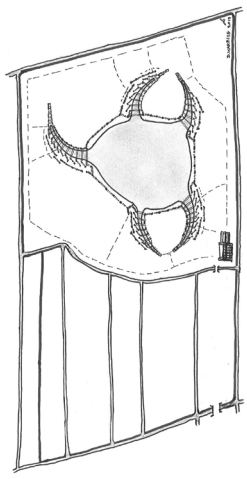

Figure 1.1 Duck decoy 'Roodnatkooi' (drawing D. Karelse).

Most of the work of this study is conducted at the duck decoy 'Roodnatkooi' (Fig. 1.1), located near Oud Alblas in the Alblasserwaard (51°52′38″N, 4°43′26″E), the Netherlands. A duck decoy is originally a Dutch invention (called 'eendenkooi' in Dutch) where in former times wild ducks were captured for consumption. Nowadays only few duck decoys maintain this function in the Netherlands, decoys being frequently used to support scientific research. A duck decoy often has four catching pipes (one for each wind direction), with the 'Roodnatkooi' having five catching pipes, which are surrounded by screens of reed and are connected to a large pond (Payne-Gallwey 1886). The catching pipes are used as swim-in traps, into which ducks are lured with grain, and the use of tame ducks and a dog. Ducks are naturally curious and when they see a predator, in this case the dog, they tend to follow it. The dog runs in between the screens, not always visible, luring the ducks towards the end of the catching pipe. At the start of the catching pipe, the decoy man (called 'kooiker' in Dutch) shows himself, after which the wild ducks fly further into the catching pipe to be trapped. The duck decoy 'Roodnatkooi' dates from 1830 and is located at 10 ha of peat land. In the surrounding of the duck decoy (approximately 1000 m distance from the decoy border) no disturbances are allowed. The duck decoy is privately owned and operated by Teunis de Vaal.

Thesis outline

In Europe, the population of mallards consists of both migratory and resident individuals. A frequently used technique to assess a bird's origin is by analysing hydrogen stable isotopes (δ^2H) in feathers. In part I, I describe how this technique can be used to assess the origin and migratory strategy of mallards. Besides validating the technique for mallards specifically, **Chapter 2** also evaluates whether a higher provenance accuracy of birds can be established when accounting not only for species, but also for variation in age and year.

In part II, I describe temporal dynamics of LPAIV infections in free-living mallards on a local, epidemiologically relevant spatial scale at the wintering grounds. The dynamics of LPAIV and other infectious diseases are likely to occur at small spatial scales, probably leading to disease clusters of limited geographic size (Barlow 1991). In contrast to other studies, we sampled a single species comprehensively throughout a complete annual cycle, measuring both current and past LPAIV infection. In autumn, the period that

resident and migratory hosts congregate, we distinguished three mallard populations, namely residents, local migrants (i.e. short distance) and distant migrants (i.e. long distance). These populations were assessed based on feather δ^2H and several criteria, such as time of capture, recapture rate and moult. In **Chapter 3**, we showed that LPAIV infection in mallards exhibits seasonal variation with a minor infection peak in summer and a dominant peak in autumn. Antibodies against AIV peaked in winter and in spring. We tested the validity of three suggested drivers for these seasonal dynamics in LPAIV infections, namely host density, immunologically naïve juveniles and increased susceptibility in migrants. Based on the findings of Chapter 3, we studied the role of resident and migratory hosts during the LPAIV epizootic in autumn, which is described in **Chapter 4**. The LPAIV epizootic was caused by an H3 LPAIV subtype. We investigated the role of migratory and resident hosts during this epizootic, by connecting mallard's migratory strategy with H3 virus kinship, H3 virus prevalence and shedding, and H3-specific antibodies.

In part III, I explore associations between LPAIV infection and body condition, immune status and movement behaviour of free-living mallards. It is often assumed that mallards are asymptomatic carriers of LPAIV, but supportive evidence from wild birds is largely lacking. In **Chapter 5** we investigated whether LPAIV infected mallards differed in body condition and immune status (i.e. using five immunological parameters) from non-infected individuals. We tested whether potential differences were associated with variation in body mass and immune status between age class, sex and migratory strategy. We discuss the potential of a co-evolution between mallards and LPAIV. In **Chapter 6** we focussed on consequences of previous LPAIV infection, in the form of maternal AIV antibodies, for offspring protection. In a field and experimental setting, we investigated which factors correlated with AIV antibody concentration in eggs, namely female body condition, female AIV antibody concentration, egg laying order, egg size and offspring sex. Associations between LPAIV infection and host movement behaviour were examined in **Chapter 7**. We sampled mallards for current LPAIV infection and fitted them with GPS loggers. We tested whether LPAIV infection was associated with lower local and regional movements within individuals and among birds.

In part IV, I synthesize the results of my thesis. In **Chapter 8** the implications of our findings for our understanding of wild bird-LPAIV interactions and particularly with respect to the role of resident and migratory hosts are discussed. Furthermore, we provisionally elaborate on (i) spatial variation of LPAIV infection dynamics in wild birds (**Box 8.1**), (ii) the potential for epidemiological models to evaluate the true role of the suggested drivers of seasonal dynamics of LPAIV infections in wild birds (**Box 8.2**) and (iii) associations between LPAIV infection and bird's local survival (**Box 8.3**).

I Assessing origin and migratory strategy

2 Improving provenance studies in migratory birds when using feather hydrogen stable isotopes

Jacintha G.B. van Dijk, Włodzimierz Meissner & Marcel Klaassen

Hydrogen stable isotopes (δ^2H) in feathers are used to determine the origin and migration strategy of birds. To identify the geographic location of the site of feather synthesis, calibration curves for the relation between feather δ^2H and amount-weighted growing-season δ^2H in precipitation are used to generate feather δ^2H isoscapes. Factors like species, age and year might generate isotopic variation in calibration curves, but the extent to which accounting for variation may improve calibration curves and hence provenance determination of birds, is unknown. We compared three European calibration curves: (i) an existing multi-species curve, uncorrected for age and year variation, and two species-specific calibration curves, based on mallard (*Anas platyrhynchos*) feathers, of (ii) varying age and year, and (iii) juvenile natal origin, corrected for year variation. Calibration curves using ordinary least square linear regression (OLS) as opposed to standard major axis regression showed least bias in estimation. As expected, we found that a single species (mallard) OLS calibration curve corrected for age and year yielded the highest coefficient of determination, but was still surprisingly similar to the other two calibration curves. Nevertheless, when using feathers of known-origin to assess provenance accuracy, the calibration curve that accounted for species, age and year variation yielded the best prediction in as many as 59% of the cases. Our study is the first to demonstrate implications of isotopic variation on assessing the origin of individual birds, but also highlights the relatively small gain in precision that is achieved by generating species, age and year specific calibration curves rather than resorting to more general alternatives.

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Introduction

Hydrogen stable isotope ratio measurements (δ^2H) of feathers are increasingly used to determine the origin and migration strategy of birds. For instance, studies have used feather δ^2H to link breeding and wintering populations (Franks et al. 2012, Garcia-Perez et al. 2013, Reichlin et al. 2013), assess dual breeding ranges and moult locations (Knoche et al. 2007, Norris et al. 2009, Rohwer et al. 2011), mixing of breeding populations on the wintering grounds (Pain et al. 2004, de la Hera et al. 2012) and cross-hemispheric migration patterns (Bairlein et al. 2012). The growing interest of feather δ^2H in bird studies increases the need for precision and a more accurate assessment of the origin of individual birds.

The major principle behind the use of feather δ^2H in provenance studies is the close correlation with amount-weighted growing-season δ^2H in precipitation (hereafter called 'precipitation δ^2H'; Hobson and Wassenaar 1997, Bowen et al. 2005). Precipitated water is passed through the food web and incorporated into feathers during the period of growth (Hobson 1999). Across the globe, precipitation δ^2H varies systematically with latitude and altitude, largely driven by temperature distillation of the heavy isotope from air masses (Dansgaard 1964), enabling assessment of the geographic location where an individual moulted its feathers. For an accurate assignment of the moulting location of individuals, a calibration curve is needed to convert feather δ^2H to precipitation δ^2H. Several avian calibration curves have been created, either based on multiple species (Hobson and Wassenaar 1997, Lott and Smith 2006) or single species (Meehan et al. 2001, Clark et al. 2009, Hobson et al. 2009a), and varying in species composition of the feathers analysed, temporal range of precipitation data (i.e. growing-season or mean annual) and geographic range (continent) (Hobson et al. 2004, Bowen et al. 2005, Hobson et al. 2009b).

Several factors might generate isotopic variation and thus inaccuracy in identifying the geographic location of the site of feather synthesis. Species may differ in their diet, causing the isotopic composition in their feathers to vary (Hobson et al. 2012). Feather δ^2H of juvenile and adult birds may differ (Smith and Dufty 2005, Marquiss et al. 2012), causing variation to increase when combining them in the same analysis. Feather δ^2H might also differ annually, as precipitation δ^2H values fluctuate as a result of regional climatic and hydrological processes (e.g. temperature, amount of precipitation, evaporation, drought; Clark and Fritz 1997, Marshall et al. 2007). But to what extent accounting for these factors yields calibration curves that significantly improve provenance predictions is unknown.

To assess the extent to which calibration curves and, hence, provenance determination in birds can be improved through accounting for species, age and year variation, we compared three European calibration curves: (i) an existing multi-species curve based on feathers of passerine species of varying age, collected across several years (Bowen et al.

2005), (ii) a species-specific curve based on feathers of varying age collected in two years and (iii) a species-specific curve based on juvenile feathers of natal origin, corrected for year differences. Bowen et al. (2005) executed their study to demonstrate the global application of hydrogen stable isotopes to wildlife forensics. We expected that accounting for species, age and year variation would contribute significantly to a more accurate assessment of the origin of birds.

Materials and methods

Feather sampling

For the species-specific calibration curves, feathers of mallards (*Anas platyrhynchos*) were collected across Europe. In 2010 and 2011, 215 feathers of juvenile mallards were obtained from 38 locations across 14 European countries (Fig. 2.1, Appendix 2.1 Table S2.1). At four locations feathers were collected in both years. Juvenile birds used for feather sampling were either captured, shot or found dead close to their natal site from June till mid-September. Additionally, in 2010, at Tukums, Latvia, (n = 3) and Oud Alblas, the Netherlands, (n = 4) feathers of full-grown juveniles were collected in October from birds banded as chicks in the same area. Feathers of moulting adult mallards were collected in Oud Alblas, from June until September 2010. Aging was based on plumage characteristics (Boyd et al. 1975). Primary feathers (mostly P1) of the right wing (n = 204) and few juvenile primary coverts (n = 11) were used in the analyses. In live birds a small piece (1-2 cm) of the tip of the feather was collected, whereas the whole feather was collected in dead individuals. Feathers were placed in a plastic zip-lock bag or paper envelope and kept at room temperature until stable hydrogen isotope analysis.

Stable isotope analysis

Feathers obtained in 2010 and 2011 were prepared and analysed in 2011 and 2012, respectively. Feathers were cleaned with 2:1 chloroform:methanol solvent mixture to remove surface contaminants and oils, and air-dried overnight under a fume hood. Feather samples (~0.33-0.37 mg) were placed into 5.0 x 3.5 mm silver capsules, folded into tiny balls and stored in 96-well trays. Each tray contained duplicates of eight samples of which the average value was used in the analysis. Trays were shipped to the Colorado Plateau Stable Isotope Laboratory (Northern Arizona University, Flagstaff, Arizona, USA) for hydrogen stable isotope analysis, which was performed on a Thermo-Electron Delta Plus XL IRMS (via CONFLO II) equipped with a 1400 C TC/EA pyrolysis furnace. 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 and keratin standards (Appendix 2.2 Table S2.2) are reported in units per mil (‰) relative to the Vienna Standard Mean Ocean Water-Standard Light Antarctic Precipitation (VSMOW-SLAP) standard scale. Feathers and keratin standards resided in the Stable Isotope Laboratory for over three weeks for all exchangeable H to equilibrate with local water vapour (Wassenaar and Hobson 2003).

Statistical analysis

We used general linear model (GLM) analysis to determine differences in feather δ^2H between juveniles (n = 19) and adults (n = 94), collected at Oud Alblas, the Netherlands, in 2010. The age-related fractionation factor was the difference between mean feather δ^2H of juveniles and adults. Annual differences in feather δ^2H were determined for those locations where feathers had been collected in 2010 and 2011, using a GLM with year, location and their interaction as fixed factors.

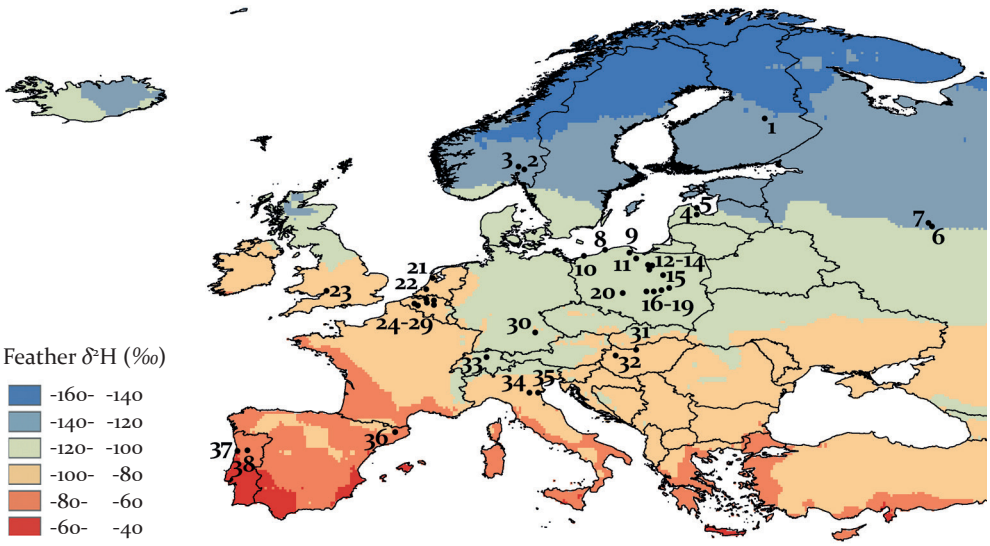

Figure 2.1 European feather δ^2H (‰) isoscape of mallards with the 38 locations where feathers were collected in 2010 and 2011. Numbers correspond to the location numbers in Appendix 2.1 Table S2.1. The feather δ^2H isoscape is based on the ordinary least-square linear regression (OLS) calibration curve of juvenile mallard feathers corrected for year, and the amount-weighted growing-season δ^2H in precipitation surface of Bowen et al. (2005).

The multiple-species calibration curve published in Bowen et al. (2005) was based on European feather δ^2H data published by Hobson et al. (2004) and precipitation δ^2H. We created two calibration curves for mallards: (i) including feathers of both age classes and years (n = 309) (hereafter called 'uncorrected calibration curve') and (ii) including juvenile feathers of natal origin with an average for those locations where feathers had been collected in both years (n = 148) (hereafter 'corrected calibration curve'). Precipitation δ^2H values from feather-collection locations were obtained from the European precipitation δ^2H map (Bowen et al. 2005). The relation between feather δ^2H and precipitation δ^2H was calculated using (i) standard major axis regression (SMA) and (ii) ordinary least-square linear regression (OLS). The preferred method when determining the best fit of a bivariate relationship of which the variables have some level of uncertainty is debated (Smith 2009). As the method used has inferences on the origin birds are assigned to, we applied both SMA and OLS to calculate calibration curves. The method with the highest predictive ability, i.e. lowest bias in estimation, was assessed by constructing a predictive relationship using ~25% of the data which was then validated using the remaining ~75% of data. The method with the highest predictive ability was used in further analysis. We used ANCOVA to compare the slopes and intercepts of the OLS calibration equations.

To assess the extent in which the three OLS calibration curves differed in their capacity to determine the correct origin of birds based on feather δ^2H, we used the δ^2H values of juvenile feathers to calculate precipitation δ^2H for each calibration curve. Knowing the natal origin of these juvenile feathers, we determined the distance between the natal site and the nearest possible location (i.e. based on the calculated precipitation δ^2H) allocated by each calibration curve. To find the location (latitude, longitude) belonging to the precipitation δ^2H values calculated by the calibration curves, the European precipitation δ^2H map of Bowen et al. (2005) was used, in which the location nearest to the natal site was chosen. The distance between the natal site and nearest location (ΔD, km) was calculated using Haversine's formula (Robusto 1957). To check for any differences in ΔD across the three OLS calibration curves we used generalized linear mixed model (GLMM) analysis, with natal site as random factor. For each calibration curve we also calculated the proportion of cases where ΔD was lowest (i.e. proportion of cases that one of the three calibration curves yielded the best prediction). A European mallard feather δ^2H isoscape was created in which the precipitation δ^2H values of the map of Bowen et al. (2005) were transformed to feather δ^2H values using the corrected mallard OLS calibration curve. The feather δ^2H isoscape was created using Spatial Analyst inverse distance weighing (ArcView 9.3, ESRI). All statistical analyses were conducted using R 2.15.0 (R Development Core Team 2012). Package lme4 was used to fit OLS models and regression lines, and package smatr to apply SMA.

Results

Feather δ^2H was 7.0 ± SE 2.7‰ lower in 2011 compared to 2010 ($F_{1,71}$ = 16.70, P < 0.001; Fig. 2.2a), with no interaction effect between year and location, for those locations where feathers had been collected in both years. Feather δ^2H differed between juveniles and adults ($F_{1,113}$ = 11.10, P = 0.001), with juveniles having a lower feather δ^2H than adults (Fig. 2.2b). The age-related fractionation factor was -6.8‰ ± SE 2.1‰.

The coefficient of determination (r^2) for the feather δ^2H/precipitation δ^2H regression was largest for the corrected mallard calibration curve, when applying both SMA and OLS (Fig. 2.3c), while the multi-species curve (Fig. 2.3a) had a slightly larger r^2 than the uncorrected mallard curve (Table 2.1, Fig. 2.3b). For all three calibration curves, the one calculated by OLS generated a higher predictive ability than when SMA was used. However, the predictive ability of the three OLS calibration curves was relatively similar (Table 2.1). The two mallard OLS calibration curves did not differ in slope (t = 0.91, P = 0.361) and intercept (t = 0.43, P = 0.668). Slopes differed between the multi-species OLS calibration curve and the uncorrected and corrected mallard OLS calibration curve (t = 3.72, P < 0.001 and t = 2.56, P = 0.011, respectively), which was also apparent for the intercepts (t = -15.44, P < 0.001 and t = -13.98, P < 0.001, respectively).

ΔD differed between the three OLS calibration curves (X^2 = 222.59, P < 0.001). The mean ΔD (± SE) for the corrected mallard OLS calibration curve was 332 ± 30 km,

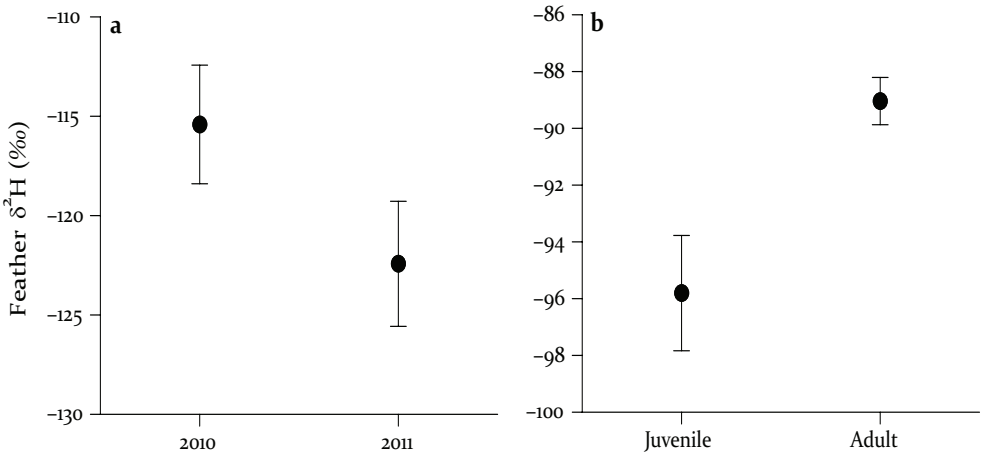

Figure 2.2 Feather δ^2H (mean ± SE, ‰) of (a) juvenile mallard feathers collected in 2010 (n = 36) and 2011 (n = 35) at four locations in Europe and (b) juvenile (n = 19) and adult (n = 94) mallard feathers collected at Oud Alblas, the Netherlands, in 2010.

for the OLS uncorrected mallard curve 373 ± 26 km and for the OLS multi-species curve 766 ± 28 km. In 59% of the cases the corrected mallard OLS calibration curve yielded the best prediction, versus 31% for the uncorrected OLS mallard curve and only 10% for the OLS multi-species curve. Using the corrected mallard OLS calibration curve, a feather δ^2H isoscape for mallards in Europe was created (Fig. 2.1).

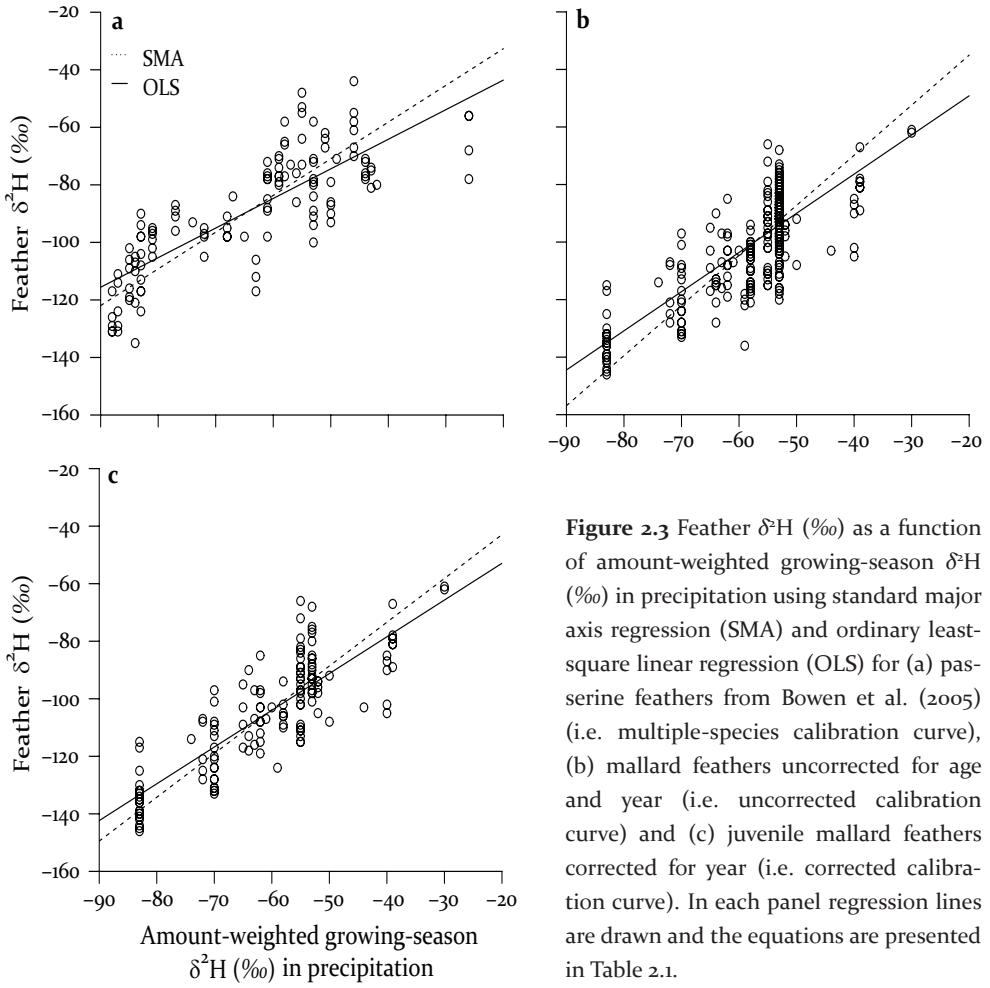

Figure 2.3 Feather δ^2H (‰) as a function of amount-weighted growing-season δ^2H (‰) in precipitation using standard major axis regression (SMA) and ordinary least-square linear regression (OLS) for (a) passerine feathers from Bowen et al. (2005) (i.e. multiple-species calibration curve), (b) mallard feathers uncorrected for age and year (i.e. uncorrected calibration curve) and (c) juvenile mallard feathers corrected for year (i.e. corrected calibration curve). In each panel regression lines are drawn and the equations are presented in Table 2.1.

Discussion

As a result of a reduction in the sources of potential isotopic variance by limiting the data used to a single species (mallard), a single age group (juveniles) and a limited time span (2010 and 2011), the species-specific OLS calibration curve corrected for age and year

variation yielded a higher coefficient of determination for the feather δ^2H/precipitation δ^2H regression compared to the uncorrected species-specific and multi-species OLS calibration curve. The predictive ability of the three calibration curves was relatively similar, suggesting that variation in species, age and year causes little bias in estimating the correct origin of birds. Still, when using feathers of known-origin to assess provenance accuracy, the calibration curve that accounted for species, age and year variation yielded the most accurate assessment of the origin of individuals in as many as 59% of all cases. Variation in species likely had a larger effect on provenance determination than age and year variation. For each calibration curve, least bias in estimation was shown when applying OLS instead of SMA.

The multi-species calibration curve of Bowen et al. (2005) was created to test the feather δ^2H/precipitation δ^2H relationship of passerine birds in Europe. Even though the coefficient of determination of this multi-species curve is slightly higher than for the uncorrected mallard curve, suggesting that correcting for age and year is more important than for species, a less accurate assessment of the origin of individuals is obtained (i.e. 10%) than when using a calibration curve specific for mallards (i.e. 31-59%). The (limited) extent to which a species-specific calibration curve can be judged 'better' than a multi-species curve (i.e. six times more frequently determining accurate provenance, but marginally better in terms of distance) is a valuable finding, notably for those considering creating a species-specific calibration curve themselves.

Table 2.1 Parameters of the existing multi-species curve of Bowen et al. (2005) (n = 123) and the two mallard calibration curves (i.e. uncorrected (n = 309) and corrected (n = 148) for age and year) calculated by standard major axis regression (SMA) and ordinary least-square linear regression (OLS), including the 95% CI (upper/lower) or SE of the intercept (β_0) and slope (β_1) of respectively the SMA and OLS calibration equation. The coefficient of determination (r^2) for the feather δ^2H/precipitation δ^2H regression and the predictive ability (mean ± SE) is given for each calibration curve and method used.

Calibration curve	Method	β_0	95% CI/ SE	β_1	95% CI/ SE	r^2	P	Predictive ability mean slope ± SE
Multiple species	SMA	-7.2	1.8/-16.2	1.28	1.42/1.15	0.65	<0.001	0.80 ± 0.06
Multiple species	OLS	-23.0	4.5	1.03	0.07	0.65	<0.001	0.99 ± 0.07
Uncorrected	SMA	-0.2	6.9/-7.3	1.74	1.87/1.62	0.61	<0.001	0.86 ± 0.05
Uncorrected	OLS	-21.9	3.6	1.36	0.06	0.61	<0.001	0.96 ± 0.05
Corrected	SMA	-12.5	-4.1/-20.9	1.52	1.66/1.39	0.71	<0.001	0.81 ± 0.05
Corrected	OLS	-27.4	4.2	1.28	0.07	0.71	<0.001	0.97 ± 0.06

Annual variation in feather δ^2H may be caused by the amount of precipitation (Coulton et al. 2009, Haché et al. 2012). In our study, the annual amount of precipitation (GNIP database, IAEW-WMO 2001) varied significantly between years 1996 to 2009 for those countries where feathers had been collected in both years (linear model: $F_{13,488} = 2.63$, $P = 0.001$), potentially explaining the detected annual variation in δ^2H of mallard feathers. Besides annual variation, feather δ^2H varied between juveniles and adults that had grown their feathers at the same location in the same year. Feathers of juvenile mallards were more depleted (6.8‰) in hydrogen than adult feathers, which could potentially be caused by differences in physiology or diet (Langin et al. 2007, Studds et al. 2012). Similar results were found in other bird species (e.g. wood thrushes (*Hylocichla mustelina*): 4.7‰, American redstarts (*Setophaga ruticilla*): 12‰, Bicknell's thrush (*Catharus bicknelli*): 12‰, common crossbills (*Loxia curvirostra*): 17.6‰; Powell and Hobson 2006, Langin et al. 2007, Marquiss et al. 2012, Studds et al. 2012), showing the generality of the differences in feather δ^2H between age classes within species. In case of predicting the origin of adult mallards in Europe, 6.8‰ should be subtracted from the feather δ^2H values before using the corrected mallard calibration curve or the feather δ^2H isoscape.

In conclusion, we demonstrate that the predictive ability of calibration curves is only marginally affected when variation in species, age and year is being accounted for. Our study highlights that only relatively small gain in precision can be achieved by generating species, age and year specific calibration curves, rather than resorting to more general, already available alternatives.

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II Pathogen dynamics

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

Jacintha G.B. van Dijk, Bethany J. Hoye, Josanne H. Verhagen, Bart A. Nolet, Ron A.M. Fouchier & Marcel Klaassen

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. 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). 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. 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. 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.

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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 and 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 dispersal, and the addition of juveniles to the population (Rosatte 1984, Loveridge and 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 et al. 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. 1990b, 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 et al. 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 et al. 2011, Lachish et al. 2012, Leighton et al. 2012), despite the importance of movement behaviour to pathogen transmission (Altizer et al. 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 programs 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. 1990b, 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 et al. 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 population consists of both migratory and resident birds. Birds breeding in western Europe (e.g. 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 and 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 program executed by the Department of Viroscience 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 approximately 15 individuals per visit (n = 1109; Appendix 3.1 Table S3.1). 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 et al. (1975). In April, the month that the first chicks hatched, juveniles from the previous year were assigned as adults, being approximately one 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 Department of Viroscience of Erasmus MC, and analysed within seven 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 approximately 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 (i.e. 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, Woerden, 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 hemagglutinin (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 hemagglutination 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 and Fry 1987). Local precipitation is incorporated into feathers during the period of growth (Hobson and Clark 1992), 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 et al. 2005, van Dijk et al. 2014a). 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 3.2 Fig. S3.1).

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 analyser (TC/EA) pyrolysis furnace at the Colorado Plateau Stable Isotope Laboratory (Northern Arizona University, Flagstaff, Arizona, 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 (~0.33-0.37 mg) were placed into 5.0 x 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 Water-Standard Light Antarctic Precipitation (VSMOW-SLAP) standard scale and calibrated using the comparative equilibration technique with pre-calibrated keratin standards (Wassenaar and 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; approximately 90% of viral particles are shed through cloacal and oral routes within 3 to 4 days, with a maximum shedding duration of 18 days (Hénaux and 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 the 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 3.1 Table S3.1). 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 (X^2 = 182.86, P < 0.001) 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. 3.1a). 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 3.3 Fig. S3.2).

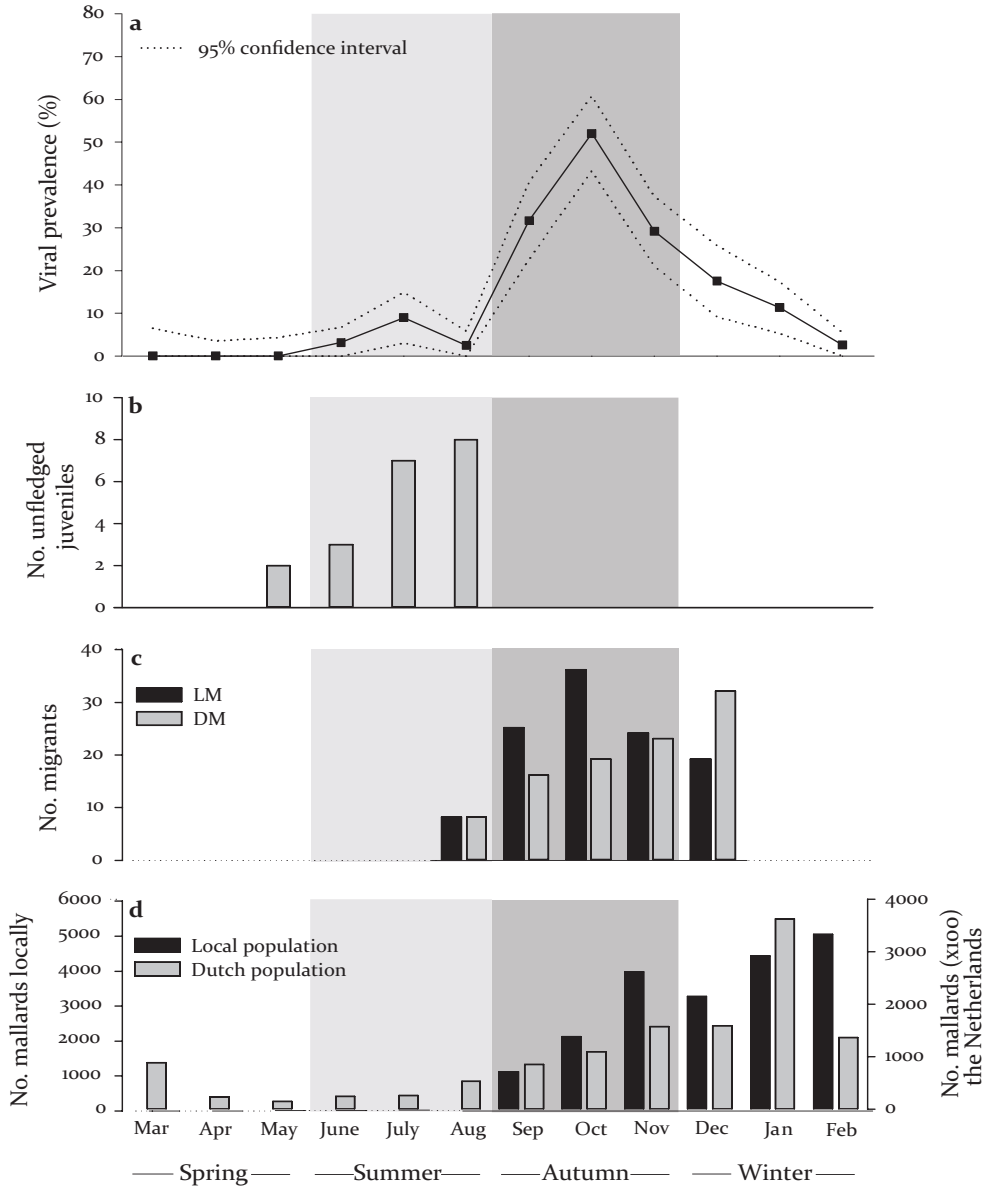

Figure 3.1 Monthly prevalence (± 95% CI) of LPAIV infection and population numbers of mallards, from March 2010 until February 2011. (a) Viral prevalence (i.e. current infection), (b) number of unfledged juveniles of the resident population, (c) number of local migrants (i.e. coming from a short distance, *LM*) and distant migrants (i.e. coming from a long distance, *DM*) 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.

Prevalence of AIV antibodies also differed among seasons (X^2 = 40.51, P < 0.001), with seroprevalence being notably higher in winter and spring (November until May), and lower in summer and autumn (June until October) (Fig. 3.2).

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. 3.1d). The LPAIV infection peak in summer and autumn did not correspond with the highest density of mallards (Fig. 3.1).

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. 3.1b). In June juveniles were between 4.5 and 8 weeks old, whereas in July juveniles were either 3 to 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 to just 4.1% of adults (n = 244). Overall, juveniles were more likely to be infected with LPAIV than adults (juveniles: 32%, adults: 13%; X^2 = 7.03, P = 0.008; Fig. 3.3a), with no effect of sex (♂: 17%, ♀: 15%; X^2 = 0.10, P = 0.758), or an interaction effect between age and sex (X^2 = 0.02, P = 0.891), age and season (X^2 = 1.20, P = 0.573) and season and sex (X^2 = 0.10, P = 0.992).

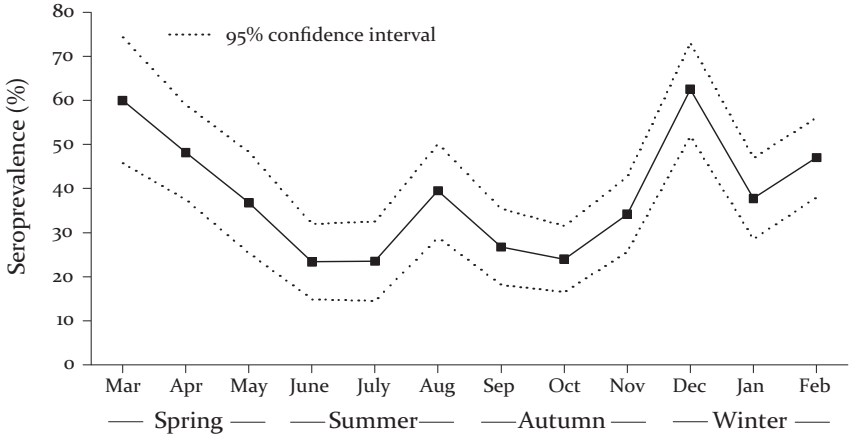

Figure 3.2 Monthly prevalence (± 95% CI) of AIV antibodies (i.e. past infection) in mallards from March 2010 until February 2011.

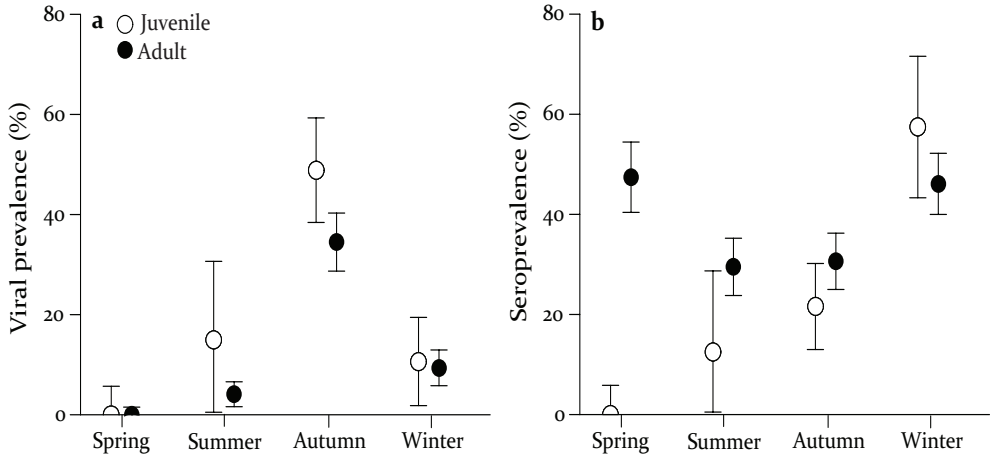

Figure 3.3 Seasonal prevalence (± 95% CI) of LPAIV infection in juvenile (<1 year) and adult (>1 year) mallards. (a) Viral prevalence and (b) seroprevalence.

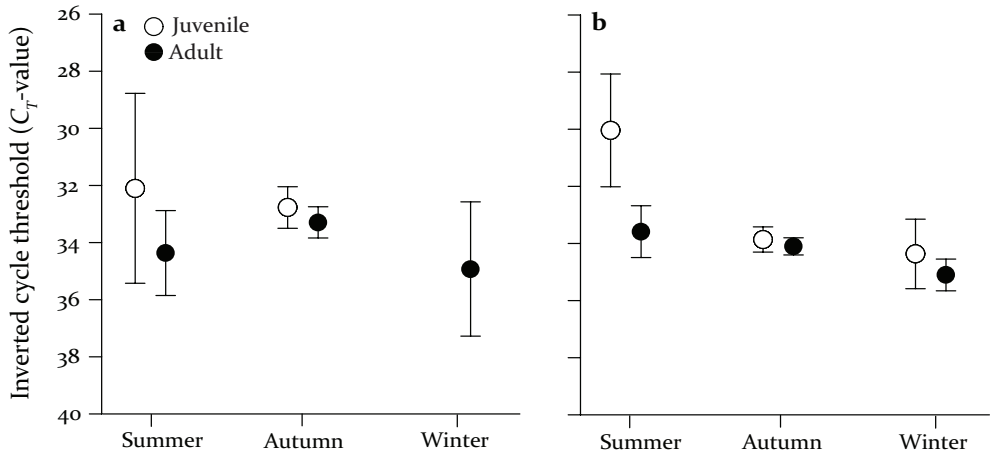

Figure 3.4 Degree of viral shedding (i.e. C_T-value) (mean ± SE) of LPAIV-positive juvenile (<1 year) and adult (>1 year) mallards detected in (a) cloacal and (b) oropharyngeal samples. The C_T-value is inversely proportional to the number of virus particles in a sample, with lower C_T-values indicating large quantities of virus. Note: inverted y-axis.

The quantity of viral particles shed from the cloaca did not vary between juveniles and adults (X^2 = 2.17, P = 0.141; Fig. 3.4a), with no effect of sex (X^2 = 1.47, P = 0.225), season (X^2 = 0.71, P = 0.399), nor an interaction effect between age and sex (X^2 = 3.37, P = 0.067). There was an interaction effect between season and sex (X^2 = 11.30, P = 0.004), 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 (X^2 = 0.99, P = 0.320; Fig. 3.4b), with no effect of sex (X^2 = 0.92, P = 0.337). There was a significant effect of season (X^2 = 6.71, P = 0.035), 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 = 9.88, P = 0.007).

There was no difference in seroprevalence between juveniles and adults (juveniles: 31%, adults: 38%; X^2 = 0.21, P = 0.644; Fig. 3.3b). 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 = 10.74, P = 0.013), 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 = 7.53, P = 0.006), with females more likely to be AIV seropositive than males (♂: 32%, ♀: 41%), and an interaction effect with season (X^2 = 11.84, P = 0.008), with the highest proportion of females having AIV antibodies in spring and winter. There was no interaction effect between age and sex (X^2 = 0.00, P = 0.962).

Migrants

Peak prevalence of LPAIV infection in autumn corresponded with the arrival of migrants (Fig. 3.1c). Both local and distant migrants were more often infected with LPAIV than residents (respectively, 44%, 41% and 12%; X^2 = 37.18, P < 0.001; Fig. 3.5a), with no effect of age (X^2 = 0.59, P = 0.444), sex (X^2 = 0.01, P = 0.916), but with a significant effect of month (X^2 = 49.10, P < 0.001). There was a significant interaction effect between a bird's origin and month (X^2 = 16.03, P = 0.042), with distant migrants most often infected with LPAIV in October. Other interactions were not-significant (P > 0.05). Viral prevalence did not differ between local and distant migrants (P = 0.711).

Residents, local and distant migrants were shedding similar quantities of viral particles from the cloaca ($F_{2,36}$ = 1.63, P = 0.210), with no age ($F_{1,36}$ = 1.30, P = 0.262), sex ($F_{1,36}$ = 2.14, P = 0.153), month ($F_{2,36}$ = 0.13, P = 0.877) or interaction effects (all P > 0.05). Similarly, a bird's origin had no effect on the degree of viral shedding in the oropharynx ($F_{2,85}$ = 1.22,

$P = 0.302$) , with no age ($F_{1,85} = 0.57$, $P = 0.452$), sex ($F_{1,85} = 0.09$, $P = 0.770$), month ($F_{4,85} = 0.77$, $P = 0.547$) or interaction effects (all $P > 0.05$).

Seroprevalence was similar among residents, local and distant migrants (respectively, 33%, 31% and 38%; $X^2 = 0.76$, $P = 0.685$; Fig. 3.5b), with no effects of age ($X^2 = 1.65$, $P = 0.199$) and sex ($X^2 = 0.00$, $P = 0.960$), but with a significant month effect ($X^2 = 27.18$, $P < 0.001$). There was a significant interaction between sex and month ($X^2 = 11.11$, $P = 0.025$), 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 ($P > 0.05$).

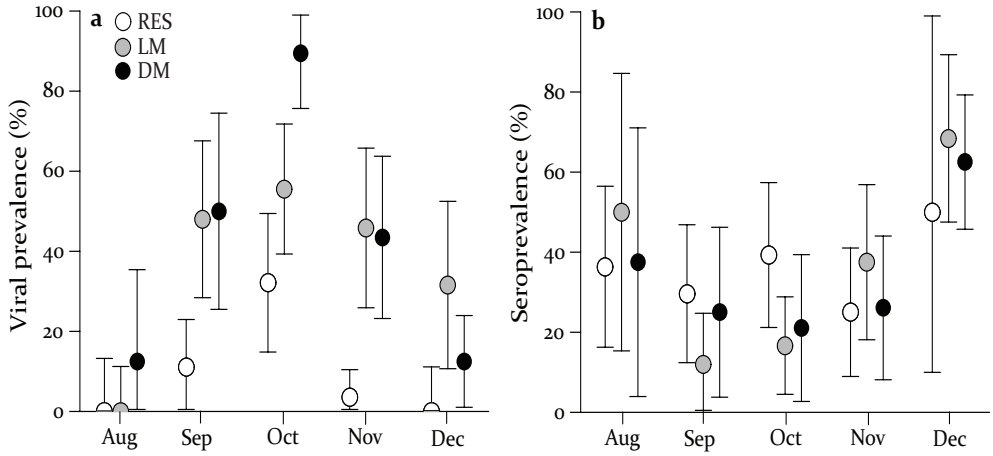

Figure 3.5 Prevalence (± 95% CI) of LPAIV infection in mallards for residents (*RES*), local migrants (*LM*) and distant migrants (*DM*) from August until December. (a) Viral prevalence and (b) seroprevalence.

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 2005-2009; 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 et al. 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 et al. 2012). The importance of juveniles driving disease dynamics has also been shown in a wide range of other host-parasite systems (e.g. Young and 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 and Staszewski 2008), reach minimum levels 2 weeks after hatching in mallards (Liu and 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. California, 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 and 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. 3.5). 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 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 et al. 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 lifespan (Kida et al. 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 leukocyte concentrations, haptoglobin, hemolysis-hemagglutination), while AIV subtypes of antibodies may be assessed using a hemagglutination 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.

Acknowledgements

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4 Migratory birds reinforce local circulation of avian influenza viruses

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**contributed equally to this study*

Migratory and resident hosts have been hypothesized to fulfil distinct roles in infectious disease dynamics. However, the contribution of resident and migratory hosts to wildlife infectious disease epidemiology, including that of low pathogenic avian influenza virus (LPAIV) in wild birds, has largely remained unstudied. During an autumn H3 LPAIV epizootic in free-living mallards (*Anas platyrhynchos*), a partially migratory, LPAIV key host species, we identified resident and migratory host populations using stable hydrogen isotope analysis of flight feathers. We investigated the role of migratory and resident hosts separately in the introduction and maintenance of H3 LPAIV during the epizootic. To test this we analysed (i) H3 virus kinship, (ii) temporal patterns in H3 virus prevalence and shedding, and (iii) H3-specific antibody prevalence in relation to host migratory strategy. We demonstrate that the H3 LPAIV strain causing the epizootic most likely originated from a single introduction, followed by local clonal expansion. The H3 LPAIV strain was genetically unrelated to H3 LPAIV detected both before and after the epizootic at the study site. During the LPAIV epizootic, migratory mallards were more often infected with H3 LPAIV than residents, whereas residents shed more H3 virus at the start of the epizootic. Low titres of H3-specific antibodies were detected in only a few residents and migrants. Our results suggest that in this LPAIV epizootic, a single H3 virus was introduced into susceptible resident birds, followed by a period of virus amplification, importantly associated with the influx of migratory mallards. Thus migrants are suggested to act as local amplifiers rather than the often suggested role as vectors importing novel strains from afar. Our study exemplifies that a multifaceted interdisciplinary approach offers promising opportunities to elucidate the role of migratory and resident hosts in infectious disease dynamics in wildlife.

Plos One (in revision)

Introduction

Migratory and resident (i.e. sedentary) hosts are thought to fulfil different, non-mutually exclusive, roles in infectious disease dynamics in wild animal populations, although empirical evidence is largely lacking. For one, migratory hosts may transport pathogens to new areas, resulting in the exposure and potential infection of new host species, thereby contributing to the global spread of infectious diseases (Altizer et al. 2011). Resident hosts, immunologically naïve to these novel pathogens, may subsequently act as local amplifiers. For instance, the global spread of West Nile virus (WNV) is considered to be greatly facilitated by migratory birds introducing the virus to other wildlife and humans in many parts of the world (Rappole and Hubálek 2003). Similarly, the introduction of Ebola virus into humans in the Democratic Republic of Congo, Africa, in 2007 coincided with massive annual fruit bat migration (Leroy et al. 2009).

Additionally, migratory hosts may amplify pathogens upon arrival at a staging site, either because they are immunologically naïve to locally circulating pathogens (Leighton 2002) and/or as a consequence of reduced immunocompetence due to the trade-off between investment in immune defences and long-distance flight (Altizer et al. 2011). Correspondingly, pathogen prevalence or the risk of disease outbreaks may locally be reduced when migratory hosts depart (Altizer et al. 2011). Consistent with the role for migrants, residents in this scenario are suggested to act as reservoirs, permanently maintaining pathogens within their population and transmitting them to other hosts, including migrants (Haydon et al. 2002, Waldenström et al. 2002). Given these potentially distinct roles for migratory and resident hosts in the spatial and temporal spread of infectious diseases, it is important to differentiate between these two hosts when aiming to improve our understanding of the ecology, epidemiology and persistence of diseases in wild animal populations.

Wild bird populations are considered the reservoir hosts of low pathogenic avian influenza A viruses (LPAIV). Predominantly birds from wetlands and aquatic environments (orders *Anseriformes* and *Charadriiformes*) are infected with LPAIV (Webster et al. 1992), causing transient and mainly intestinal infections (Daoust et al. 2012, Höfle et al. 2012), with no or limited signs of disease (Kuiken 2013). LPAIV can be classified in subtypes based on antigenic and genetic variation of the viral surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). All subtypes that have been recognized to date, notably HA subtypes 1 through 16 (H1-H16) and NA subtypes 1 through 9 (N1-N9), have been found in wild birds (Olsen et al. 2006). Recently, novel influenza viruses were identified in fruit bats that are distantly related to LPAIV (H17N10, H18N11), indicating that bats, alongside wild birds, harbour influenza viruses and might play a distinct role in the dynamics of this infectious disease (Tong et al. 2012, Tong et al. 2013).

Despite a large number of studies on the ecology and epidemiology of LPAIV in wild birds, only few studies have focussed on the role of resident and migratory hosts in the dynamics of this infectious disease. Resident bird species likely facilitate LPAIV transmission, while migratory bird species harbour high LPAIV subtype diversity after arrival at the wintering grounds (Stallknecht et al. 1990b, Ferro et al. 2010). In most of these studies resident and migratory hosts belonged to different bird species, with presumably different LPAIV susceptibility. However, many bird species are composed of a mixture of resident and migratory individuals, so called partial migrants (Lack 1943). Individuals that belong to the same species but use distinct migratory strategies, may differ in morphology and behaviour (e.g. body size, dominance; Chapman et al. 2011), immune status and pathogen exposure. As a consequence, resident and migratory individuals of a single species may respond differentially to LPAIV infection and hence their contribution to local, and consequently global, LPAIV infection dynamics may differ. Hill et al. (2012) investigated the role of migratory and resident hosts of a single bird species in LPAIV infection dynamics. No differences were detected in LPAIV prevalence between migratory and resident host populations (Hill et al. 2012). However, migrants likely introduced LPAIV subtypes from their breeding areas to the wintering grounds, and residents likely acted as LPAIV reservoirs facilitating year-round circulation of limited subtypes (Hill et al. 2012). A similar study in the same species conducted at a local scale instead of a macro-ecological scale, showed that susceptible migratory hosts were more frequently infected with LPAIV than residents, which had probably driven the epizootic in autumn (van Dijk et al. 2014b). LPAIV epizootics in wild birds are likely to take place at local spatial and temporal scales, since LPAIV infections are generally short (i.e. up to a week; Latorre-Margalef et al. 2009), and most virus particles are shed within the first few days after infection (Hénaux and Samuel 2011). Yet, the precise role of migratory and resident hosts during local LPAIV epizootics in terms of virus introduction and reinforcement, including host immunity, has remained largely unstudied.

We build on the study of van Dijk et al. (2014b) to investigate the role of migratory and resident hosts of a single bird species during a local LPAIV epizootic. Throughout an H3 epizootic at the wintering grounds in autumn 2010, we sampled a partly migratory bird species, the mallard (*Anas platyrhynchos*), and connected host migratory strategy with (i) H3 virus kinship, (ii) H3 virus prevalence and shedding, and (iii) H3-specific antibody prevalence. H3 LPAIV is a dominant subtype in wild ducks in the northern hemisphere (Krauss et al. 2004, Munster et al. 2007). This study provides a detailed description of a monophyletic H3 LPAIV epizootic importantly associated with the influx of migratory mallards.

Materials and methods

Study species and site

Mallards are considered a key LPAIV host species, together with other dabbling duck species of the *Anas* genus, harbouring almost all LPAIV subtype combinations found in birds to date (Olsen et al. 2006). Mallards are partially migratory: meaning that the population exists of both migratory and resident birds. Along the East Atlantic Flyway, mallards breeding in Scandinavia, the Baltic, and north-west Russia migrate to winter at more southern latitudes in autumn, congregating with the resident populations that breed in Western Europe, including the Netherlands (Scott and Rose 1996).

During the 2010 LPAIV epizootic described here, free-living mallards were caught in swim-in traps of a duck decoy (Payne-Gallwey 1886). The duck decoy was located near Oud Alblas (51°52′38″N, 4°43′26″E), situated in the province of Zuid-Holland in the Netherlands. This sampling site is part of the national wild bird avian influenza virus (AIV) surveillance program, executed by the Department of Viroscience of Erasmus MC in Rotterdam, the Netherlands, where mallards, free-living and hunted in the near surrounding, were sampled for LPAIV from 2005 onwards.

Sampling

During the LPAIV epizootic, the duck decoy was visited, on average, 7 times per month capturing approximately 11 birds per visit. Each captured mallard was marked with a metal ring, aged (juvenile: <1 year, adult: >1 year) and sexed based on plumage characteristics (Boyd et al. 1975). For virus detection, cloacal and oropharyngeal samples were collected using sterile cotton swabs as LPAIV may replicate in both the intestinal and respiratory tract of wild birds (Fouchier and Munster 2009). Swabs were stored individually in transport medium (Hank's balanced salt solution with supplements; Munster et al. 2009) at 4 °C, and transported to the laboratory for analysis within seven days of collection. For detection of antibodies to AIV, blood samples (<1 ml, 2% of the circulating blood volume) were collected from the brachial vein, which were allowed to clot for approximately 6 h before centrifugation to separate serum from red blood cells (Hoye 2012). Serum samples were stored at -20 °C until analysis. To determine a bird's migratory strategy using stable hydrogen isotope analysis, the tip (1-2 cm) of the first primary feather of the right wing was collected and stored in a sealed bag at room temperature. Of recaptured birds, both swabs and a blood sample were collected.

Stable isotope analysis

In the study of van Dijk et al. (2014b), the origin (and hence, migratory strategy) of mallards sampled during the 2010 LPAIV epizootic was determined using stable hydrogen isotope analysis in feathers. Stable isotope signatures in feathers reflect those of local food webs (Peterson and Fry 1987). During the period of growth (i.e. moult), local precipitation is incorporated into these feathers (Hobson 1999), causing the stable hydrogen isotope (δ^2H) ratio in feathers to be correlated with δ^2H of local precipitation (Hobson and Wassenaar 1997). Across Europe, a gradient of δ^2H in feathers is found in mallards (van Dijk et al. 2014a). Based on feather δ^2H and additional criteria, van Dijk et al. (2014b) classified mallards as resident, local migrant (i.e. short distance) and distant migrant (i.e. long distance). A resident bird had grown its feathers near the duck decoy (was captured during moult) and were recaptured multiple times either before or during the LPAIV epizootic. A local and distant migratory bird was seen and sampled once, i.e. only during the LPAIV epizootic and was not captured before this epizootic. Based on feather δ^2H values of local and distant migrants, and using a European feather δ^2H isoscape of mallards, local migrants originated roughly from central Europe and distant migrants roughly from north-eastern Europe (van Dijk et al. 2014a). We used similar criteria to assess the migratory strategy of mallards caught during the H3 LPAIV epizootic. For 149 individual birds in this study we were unable to assign them to either the resident or migratory population and these were excluded from analyses, except the genetic analysis.

For full details on the stable hydrogen isotope analysis, see van Dijk et al. (2014a). In short, feathers were cleaned and air-dried overnight. Feather samples were placed into silver capsules, stored in 96-well trays and shipped to the Colorado Plateau Stable Isotope Laboratory (Northern Arizona University, Flagstaff, Arizona, USA). Stable hydrogen isotope analyses were performed on a Delta Plus XL isotope ratio mass spectrometer (IRMS) equipped with a 1400 C TC/EA pyrolysis furnace. Feather δ^2H values are reported in units per mil (‰) relative to the Vienna Standard Mean Ocean Water-Standard Light Antarctic Precipitation (VSMOW-SLAP) standard scale.

Virus detection, isolation and characterization

As part of the national wild bird AIV surveillance program, including the 2010 LPAIV epizootic, LPAIV infection of free-living and hunted mallards was assessed using cloacal and oropharyngeal swab samples. RNA from these samples was isolated using the MagnaPure LC system with a MagnaPure LC Total nucleic acid isolation kit (Roche Diagnostics, Almere, the Netherlands) and analysed using a real-time reverse transcriptase-PCR (RT-PCR) assay targeting the matrix gene. Matrix RT-PCR positive samples were used for the

detection of H5 and H7 influenza A viruses using HA specific RT-PCR tests (Fouchier et al. 2004, Munster et al. 2009). All matrix positive samples were used for virus isolation and characterization as described previously (Munster et al. 2009).

Matrix RT-PCR positive samples collected during the 2010 LPAIV epizootic for which virus culture was not successful, were screened for the presence of H3 influenza A viruses using a H3 specific RT-PCR test (n = 126). Additionally, matrix RT-PCR positive samples collected half year prior to the LPAIV epizootic (November 2009-July 2010) were screened for the presence of H3 influenza A viruses to determine whether H3 LPAIV was detected in mallards prior to the epizootic (n = 20). Amplification and detection were performed on an ABI 7500 machine with the taqman Fast Virus 1 Step Master mix reagents (Applied Biosystems, Nieuwerkerk aan den IJssel, the Netherlands) and 5 µl of eluate in an end volume of 30 µl using 10 pmol Oligonucleotides RF3226 (5'-GAACAACCGGTTC-CAGATCAA -3') and 40 pmol RF3227 (5'-TGGCAGGCCCACATAATGA-3') and 10 pmol of the double-dye labelled probe RF3228 (5'-FAM-TCCTRTGGATTTCCTTTGCCATATCAT-GC-BHQ-3'). Primers and probe were designed with the software package Primer Express version 3.01 (Applied Biosystems, Nieuwerkerk aan den IJssel, the Netherlands), based on avian H3 nucleotide sequences obtained from Genbank (www.ncbi.nlm.nih.gov).

The degree of virus shedding from the cloaca and the oropharynx during the LPAIV epizootic was based on the cycle threshold (C_T) value, i.e. first real-time matrix RT-PCR amplification cycle in which matrix gene amplification was detected. The C_T-value is inversely proportional to the amount of viral RNA in a sample.

Sequence analysis and phylogeny

To investigate H3 LPAIV diversity in time and space among resident and migratory mallards during the LPAIV epizootic, we performed a genetic analysis focussed on the HA segment, one of the two most variable gene segments of LPAIV. Nucleotide sequences of the HA gene segment were obtained from virus isolates that were previously characterized by hemagglutination inhibition (HI) assay as H3 LPAIV. RT-PCR and sequencing of the HA segment was performed using HA specific primers (5'-GGATCTGCTGCTTGTCCTGT-3' and 5'- GRATA-AGCATCTATTGGAC-3'), as described previously (Hoffmann et al. 2001).

A total of 86 HA gene segments of 1576 nt in length were included in the genetic analysis. The genetic analysis comprised H3 nucleotide sequences obtained from (i) residents and migratory mallards during the 2010 LPAIV epizootic (n = 23), (ii) additional H3 LPAIV isolates from the national wild bird surveillance program of Erasmus MC (n = 43) and (iii) a BLAST analysis using public databases available as of 29 November 2013 (www.ncbi.nlm.nih.gov, http://www.gisaid.com), from which only European virus sequences with

a known isolation date were retrieved (n = 20). Duplicate and incomplete sequences were removed. Nucleotide sequences were aligned using the software MAFFT version 7 (http://mafft.cbrc.jp/alignment/software/).

H3 nucleotide sequences were labelled based on sampling site, year of virus isolation, and host migratory strategy (i.e. resident, local migrant, distant migrant). During the 2010 LPAIV epizootic, H3 nucleotide sequences were obtained from 23 viruses, isolated from residents (n = 3), from local migrants (n = 13), from distant migrants (n = 2) and from birds of which the migratory strategy could not be assessed (n = 5). This was supplemented with 12 H3 nucleotide sequences obtained from viruses isolated from mallards sampled in the duck decoy in different years, notably in 2008 (n = 11) and 2011 (n = 1). There were 31 H3 nucleotide sequences from virus samples collected at other sampling locations in the Netherlands and elsewhere in Europe between 1999 and 2011. Of these virus samples, 18 originated from the province of Zuid-Holland (5 to 30 km from the duck decoy), i.e. from Berkenwoude (n = 13) (51°57'00"N, 4°41'36"E), Lekkerkerk (n = 2) (51°53'41"N, 4°39'24"E), Oudeland van Strijen (n = 2) (51°46'56"N, 4°30'56"E) and Vlist (n = 1) (51°59'13"N, 4°45'56"E). Eleven viruses were isolated from birds in coastal regions in the Netherlands (i.e. 115 to 200 km from the duck decoy), i.e. Schiermonnikoog (n = 1) (53°28'41"N, 6°9'24"E), Vlieland (n = 1) (53°16'42N, 5°01'22"E), Westerland (n = 8) (52°53'39"N, 4°56'32"E) and Wieringen (n = 1) (52°54'00"N, 4°58'11"E). Outside the Netherlands, 2 H3 sequences were from viruses isolated in Hungary in 2009. The 20 H3 nucleotide sequences retrieved from the public databases originated from multiple locations throughout Europe (i.e. Belgium, Czech Republic, Germany, Iceland, Italy and Switzerland) and Russia.

A Maximum Likelihood (ML) phylogenetic tree was generated using the PhyML package version 3.1 using the GTR+I+G model of nucleotide substitution, performing a full heuristic search and subtree pruning and regrafting (SPR) searches. The best-fit model of nucleotide substitution was determined with jModelTest (Posada 2008). Trees were visualized using the Figtree program, version 1.4.0 (http:// tree.bio.ed.ac.uk/software/figtree). Overall rates of evolutionary change (i.e. number of nucleotide substitutions per site per year) and time of circulation to the most recent common ancestor (TMRCA) in years was estimated using the BEAST program version 1.8.0 (http://beast.bio.ed.ac.uk/). To accommodate variation in the molecular evolutionary rate among lineages, the uncorrelated log-normal relaxed molecular clock was used. Isolation dates were used to calibrate the molecular clock. Three independent Bayesian Markov Chain Monte Carlo (MCMC) analyses were performed for 50 million states, with sampling every 2,000 states. Convergence and effective sample sizes of the estimate were checked with Tracer v1.6 (http://tree.bio.ed.ac.uk/software/tracer/). Uncertainty in parameter estimates was

reported as the 95% highest posterior density (HPD) (Westgeest et al. 2014). Nucleotide sequences presented in Fig. 5 are online available under the accession numbers as listed in the Appendix 4.1 Table S4.1.

Serology

To assess whether mallards had H3-specific antibodies during the 2010 LPAIV epizootic, all sera were first tested for the presence of AIV antibodies specific for the nucleoprotein (NP) using a multispecies blocking enzyme-linked immunosorbent assay (bELISA MultiS-Screen Avian Influenza Virus Antibody Test Kit; IDEXX Laboratories, Hoofddorp, the Netherlands), following manufacturer's instructions. Each plate contained two positive and two negative controls. Samples were tested in duplicate. An infinite M200 plate reader (Tecan Group Ltd, Männedorf, Switzerland) was used to measure the absorbance (i.e. OD-value) at 620 nm. Samples were considered positive for the presence of NP antibodies when signal-to-noise ratios (i.e. mean OD-value of the sample divided by the mean OD-value of the negative control) were <0.5. NP antibody positive serum samples were subsequently tested for the presence of H3-specific antibodies using the HI assay according to standard procedures (Hirst 1943). Briefly, sera were pre-treated overnight at 37 °C with receptor destroying enzyme (Vibrio cholerae neuraminidase) and incubated at 56 °C for 1 h. Two-fold serial dilutions of the antisera, starting at a 1:10 dilution, were mixed with 4 hemagglutinating units of A/Mallard/Netherlands/10/2010 (H3N8) in 25 µl and were incubated at 37 °C for 30 min. Subsequently, 25 µl 1% turkey erythrocytes was added and the mixture was incubated at 4 °C for 1 h. Hemagglutination inhibition patterns were read and the HI titre was expressed as the reciprocal value of the highest dilution of the serum that completely inhibited agglutination of turkey erythrocytes.

Statistical analysis

Birds were considered LPAIV positive when either cloacal or oropharyngeal swabs were positive. To exclude samples of birds that had been sampled twice within the same infectious period during the 2010 LPAIV epizootic, we used an interval of at least 30 days between the day that a bird tested LPAIV positive and the next sampling day. Mallards may shed virus up to 18 days (Hénaux and Samuel 2011).

During the LPAIV epizootic, 709 cloacal and oropharyngeal swabs were collected from 472 mallards of which 129 individuals were recaptured. Of these swabs, 84 tested positive for H3 LPAIV, 35 tested LPAIV positive but H3 negative (i.e. matrix-positive H3-negative), and 583 swabs tested LPAIV negative. Of 7 matrix-positive swabs we were unable to determine H3-positivity. To test H3 virus prevalence and shedding, we included H3-

positive and H3-negative swabs (i.e. matrix-negative and matrix-positive). Swabs from birds of which the migratory strategy could not be assessed (n = 269) or with undefined age and sex (n = 13) were excluded. Thus in total we included 420 cloacal and oropharyngeal swabs from 305 individual birds, of which 55 birds were sampled more than once (Appendix 4.2 Table S4.2).

During the LPAIV epizootic, 428 serum samples were collected from 364 mallards of which 52 individuals were recaptured. Of these serum samples, 9 tested positive for H3-specific antibodies, 98 tested positive for LPAIV antibodies but negative for H3-specific antibodies (i.e. NP-positive H3-negative), and 321 sera tested negative for LPAIV antibodies. To investigate H3-specific antibody prevalence, we included H3-specific antibody positive and H3-specific antibody negative sera (i.e. NP-negative and NP-positive). Sera from birds of which the migratory strategy could not be assessed (n = 96) or with undefined age and sex (n = 5) was excluded. Thus in total we included 320 sera samples from 281 individual birds, of which 30 birds were sampled more than once (Appendix 4.2 Table S4.2).

A generalized linear mixed model (GLMM) was used in the analysis of H3 virus prevalence, with bird group (i.e. resident, local migrant, distant migrant), age, sex and month as fixed factors, all two-way interactions with bird group, and individual bird as random factor. A general linear model (GLM) was used to test for differences in prevalence of H3-specific antibodies, with bird group and month as fixed factors. Linear models (LMs) were used to determine differences in the degree of virus shedding of H3 LPAIV-particles based on viral RNA from the cloaca and the oropharynx (i.e. C_T-value) with bird group, age, sex and month as fixed factors, and all two-way interactions with bird group. The interactions between bird group and age, bird group and sex, and bird group and month were tested to assess whether H3 virus prevalence and the degree of H3 virus shedding differed per age class, sex and month for the three bird groups. The fixed factors age and sex were merely included in the models to conduct the interactions. A Tukey's post hoc test was performed to detect differences in H3 LPAIV prevalence between the three bird groups and months. All analyses were conducted using R 2.14.1 (R Development Core Team 2012). Package lme4 was used to fit the GLMM (Bates et al. 2012) and multcomp to perform a Tukey's post hoc test (Hothorn et al. 2008).

Results

Virus prevalence

Each year, from 2005 until 2011, LPAIV prevalence in mallards peaked between the end of summer (August) and the beginning of winter (December), with some exceptions in March 2009 and June 2011 (Fig. 4.1a). Detection of the various HA subtypes varied per year, with most virus isolates found in autumn, notably H2 to H8, H10 and H12. H3 LPAIV was isolated from mallards every year, except in 2007 and 2009, and was the dominant HA subtype in 2006, 2008 and 2010 (Fig. 4.1b).

During the 2010 LPAIV epizootic, mallards were infected with H3 LPAIV (84 of 709, 12%) and with other LPAIV subtypes, namely H4, H6 and H10 (35 of 709, 5%; Fig. 4.1b). The H3 LPAIV epizootic started on the 12th of August 2010 (Fig. 4.2a) and H3 virus prevalence differed between months (Table 4.1). H3 virus prevalence increased in September, peaked in October, and decreased in November and December (Fig. 4.2a and 4.2c). Shortly before the 2010 LPAIV epizootic, a single mallard of unknown origin was infected with H3 LPAIV on 10 February 2010, followed by a period of five months where no H3 infections were detected among 536 mallards sampled.

Local and distant migrants were more often infected with H3 LPAIV (37 of 113, 33% and 22 of 98, 22%, respectively) than residents (20 of 209, 10%; Fig.4.2c, Table 4.1). The peak month of the H3 epizootic differed between the three mallard populations (Table 4.1): in local migrants H3 LPAIV infection peaked in September, whereas in residents and distant migrants infection peaked in October (Fig. 4.2c). At the start of the H3 epizootic (12th of August), three residents and one local migrant were infected with H3 LPAIV, with their populations constituting respectively 88% and 12% of the sampled mallard population. Two weeks later (26th of August), the first distant migrant infected with H3 LPAIV was detected (44% of the sampled mallard population). In September and October, most mallards infected with H3 LPAIV were local migrants (respectively 12 of 22 and 15 of 35 total H3 LPAIV positives), while local migrants comprised respectively 24% and 40% of the sampled mallard population. In October, 11 residents and nine distant migrants were infected with H3 LPAIV, the latter constituting only 17% of the sampled mallard population. In November, only nine local and five distant migrants were infected with H3 LPAIV (comprising respectively 29% and 25% of the sampled mallard population). The last month of the H3 epizootic, only one distant migrant and two residents were infected with H3 LPAIV, although distant migrants and residents constituted respectively 43% and 32% of the sampled mallard population.

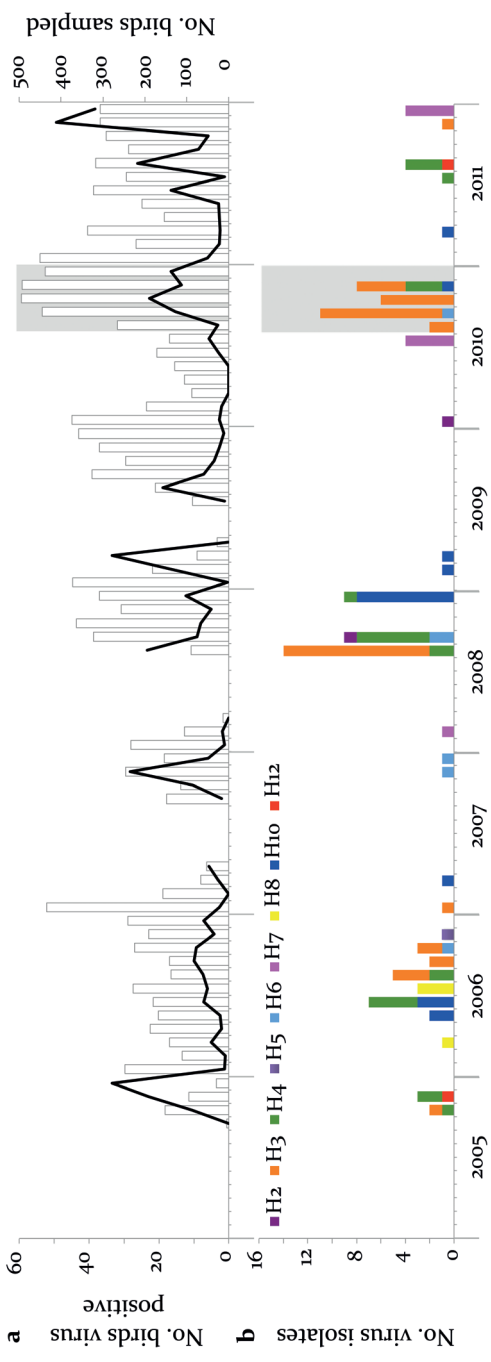

Figure 4.1 LPAIV prevalence and subtype diversity in mallards sampled at Oud Alblas, the Netherlands, 2005-2011. The grey-shaded area indicates the H3 LPAIV epizootic from August until December 2010. (a) Number of free-living and hunted birds sampled (bars, right y-axis) and percentage of birds tested virus positive based on M RRT-PCR (line, left y-axis). (b) Number of virus isolates per HA subtype: H2 (purple), H3 (orange), H4 (green), H5 (light purple), H6 (light blue), H7 (pink), H8 (yellow), H10 (dark blue) and H12 (red).

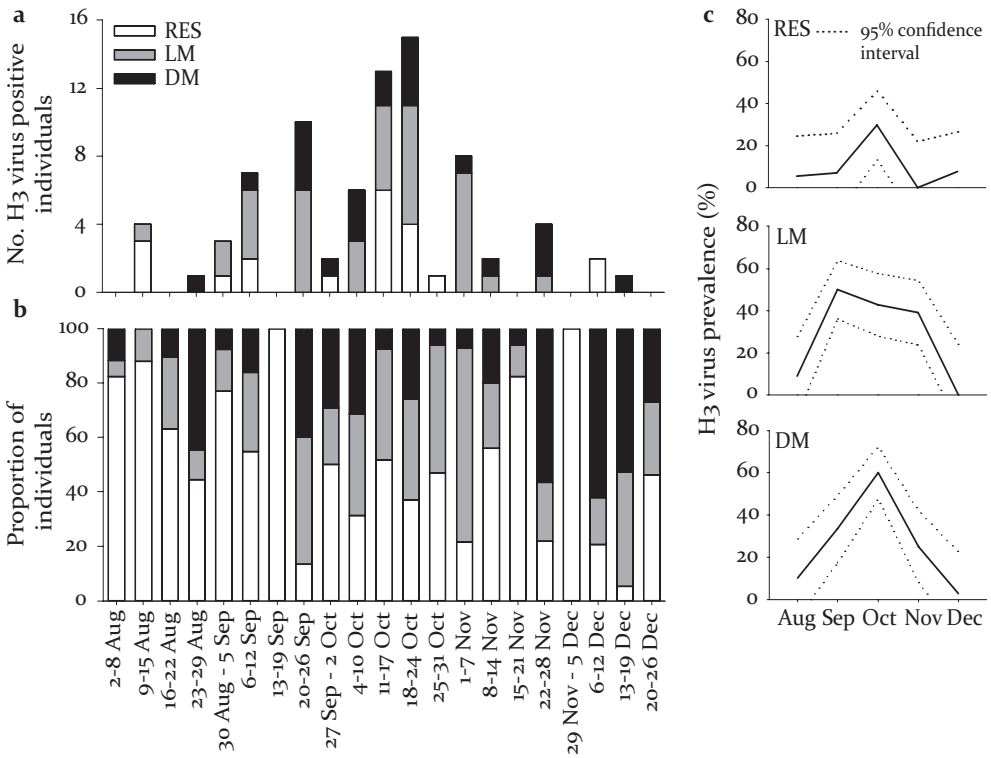

Figure 4.2 H3 LPAIV prevalence in residents, local and distant migratory mallards during the H3 epizootic in 2010. For residents (*RES*), local migrants (*LM*) and distant migrants (*DM*) the (a) number of H3 virus positive individuals per week, (b) proportion of individuals sampled per week and (c) H3 virus prevalence per month are depicted.

Virus shedding

H3 virus shedding from the cloaca and oropharynx did not differ between the three mallard populations (Table 4.1). However, the amount of H3 virus shed from the cloaca and the oropharynx differed per month for residents, local and distant migrants (Table 4.1). In August, only residents shed H3 virus particles from the cloaca (Fig. 4.3a). In the same month, residents shed more H3 virus particles from the oropharynx than migrants (Fig. 4.3b).

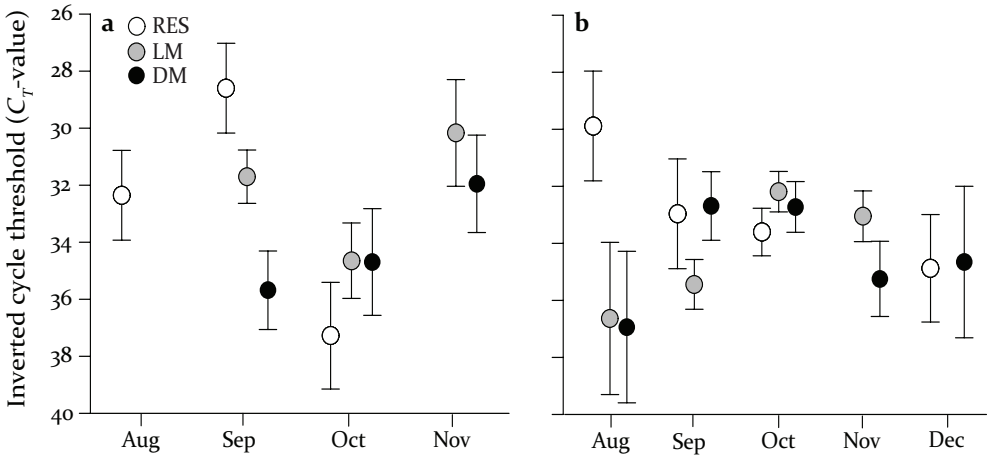

Figure 4.3 H3 LPAIV shedding by residents, local and distant migratory mallards during the H3 epizootic in 2010. Virus shedding was based on C_T-values of the M RT-PCR (mean ± SE) of (a) cloacal and (b) oropharyngeal swabs of residents (*RES*), local migrants (*LM*) and distant migrants (*DM*). The C_T-value is inversely proportional to the amount of viral RNA in a sample, with lower C_T-values indicating a high amount of virus. Note: inverted y-axis.

Antibody prevalence

During the H3 epizootic, the proportion of local and distant migrants with H3-specific antibodies (3 of 106, 3% and 4 of 96, 4% respectively) was similar to that in residents (2 of 118, 2%; $X^2 = 0.54$, $P = 0.762$; Fig. 4.4). There were no differences in H3-specific antibodies between months ($X^2 = 7.00$, $P = 0.136$). During the H3 epizootic, H3-specific antibodies were detected on four sampling dates. On the 5th of August, before the start of the H3 epizootic, one distant migrant had H3-specific antibodies (while distant migrants constituted 14% of the sampled mallard population). During the H3 epizootic, the first resident with H3-specific antibodies was sampled on the 21st of September, with 9% of the sampled mallard population comprised of residents. After the peak of the H3 epizootic (1st of November), two local migrants, one distant migrant and one resident had antibodies specific for H3 LPAIV. That day, local migrants constituted the largest proportion of the sampled mallard population (71%). At the end of the epizootic (21st of December), only migrants (local migrant: 1, distant migrant: 2) had specific antibodies against H3 LPAIV (constituting 38% and 44% of the sampled mallard population, respectively).

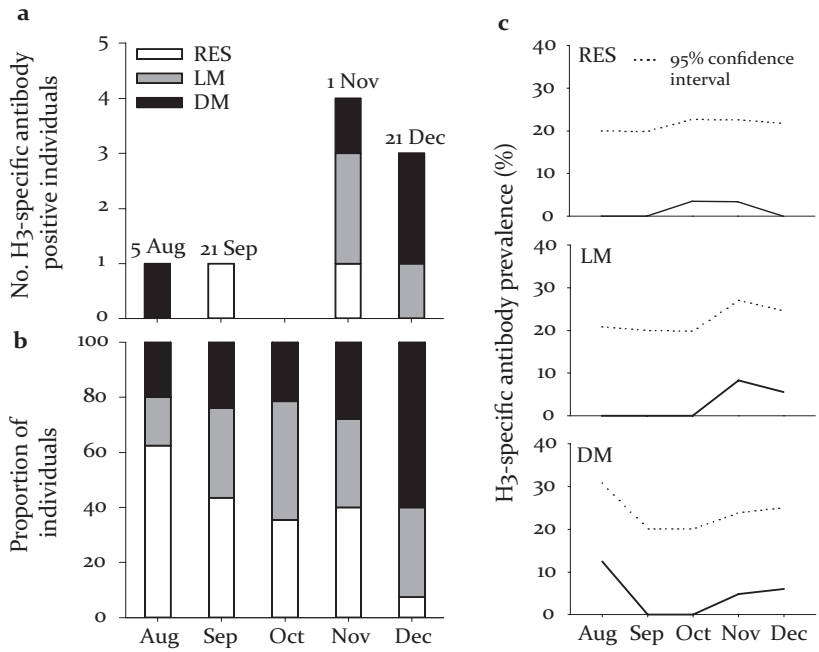

Figure 4.4 H3-specific LPAIV antibody prevalence in residents, local and distant migratory mallards during the H3 epizootic in 2010. For residents (*RES*), local migrants (*LM*) and distant migrants (*DM*) the (a) number of H3-specific antibody positive individuals, (b) proportion of individuals sampled and (c) H3-specific antibody prevalence per month are depicted.

Table 4.1 Linear model test results on migratory strategy and H3 LPAIV prevalence and shedding during the H3 epizootic in 2010. Next to migratory strategy are age, sex, month and two-way interactions included. Virus shedding was based on C_T-value of the M RT-PCR of cloacal and oropharyngeal swabs. Significant values ($P < 0.05$) are shown in bold.

	H3 virus prevalence		Degree of H3 virus shedding			
			Cloaca		Oropharynx	
Variable	X^2	*P*-value	*F*-value	*P*-value	*F*-value	*P*-value
Age	0.14	0.705	0.29	0.612	0.13	0.715
Sex	0.66	0.417	0.67	0.450	2.00	0.163
Month	44.93	**<0.001**	4.80	0.062	1.18	0.331
Migratory strategy	23.68	**<0.001**	4.69	0.071	0.01	0.992
Migratory strategy*age	0.78	0.678	0.17	0.698	1.27	0.289
Migratory strategy*sex	0.56	0.757	6.46	0.052	0.31	0.733
Migratory strategy*month	21.51	**0.006**	6.84	**0.047**	2.66	**0.025**

Virus kinship

The HA gene sequences of the H3 LPAIV strains isolated from free-living mallards during the H3 epizootic were monophyletic, suggesting the outbreak resulted from a single virus introduction. Although migratory mallards kept arriving at the study site during the H3 epizootic, the genetic analysis indicates that no other H3 LPAIVs were introduced. The estimated time to the most recent common ancestor of the H3 LPAIV strains of the epizootic was spring 2008 (TMRCA 31 May 2008, LHPD95% 13 June 2009, UHPD 4 January 2007). The H3 LPAIV strain detected in a single mallard at our study site prior to the H3 epizootic (10th of February 2010) differed from the H3 LPAIV strains of the epizootic (HA could only be sequenced partially and is not shown in the tree), and was therefore unlikely to have seeded the outbreak. Furthermore, the H3 LPAIV strains isolated during the H3 epizootic were not closely related to isolates obtained from mallards at our study site in autumn 2008 (sequence identity 0.958 - 0.967), or November 2011 (sequence identity 0.954 - 0.957; Fig. 4.5). However, the H3 LPAIV strains isolated from the H3 epizootic were genetically closely related to H3 isolates from mallards at two sampling sites 8 to 12 km away from the study site one year later, in autumn 2011 (i.e. locations Berkenwoude and Vlist; Fig. 4.5).

H3 LPAIV strains isolated from the resident, local and distant migratory population belonged to the same cluster with little variation in nucleotide sequences (sequence identity 0.995 - 1; detail of Fig. 4.5). No consistent substitutions were detected in the nucleotide sequences that correlated with the migratory strategy of birds. Evolutionary divergence of the HA of H3 LPAIV was 2.5^{e-3} nucleotide substitutions per site per year, which is lower than reported by Hill et al. (2012): 1.38 ($\pm$ 0.40)$^{e-2}$.

Discussion

Studying the role of resident and migratory hosts in the spread and circulation of pathogens in animal populations is crucial for increasing our understanding of the ecology and epidemiology of infectious diseases in wildlife. We studied virus and antibody prevalence in free-living mallards during an autumn LPAIV epizootic of subtype H3 at a local scale, focussing on the distinct role that resident and migratory hosts might have played in the introduction and circulation of this virus subtype. Although alternative interpretations cannot be entirely excluded, our findings suggest that the H3 LPAIV causing the epizootic was introduced in resident mallards, in which virus amplification was importantly associated with the arrival of migratory mallards.

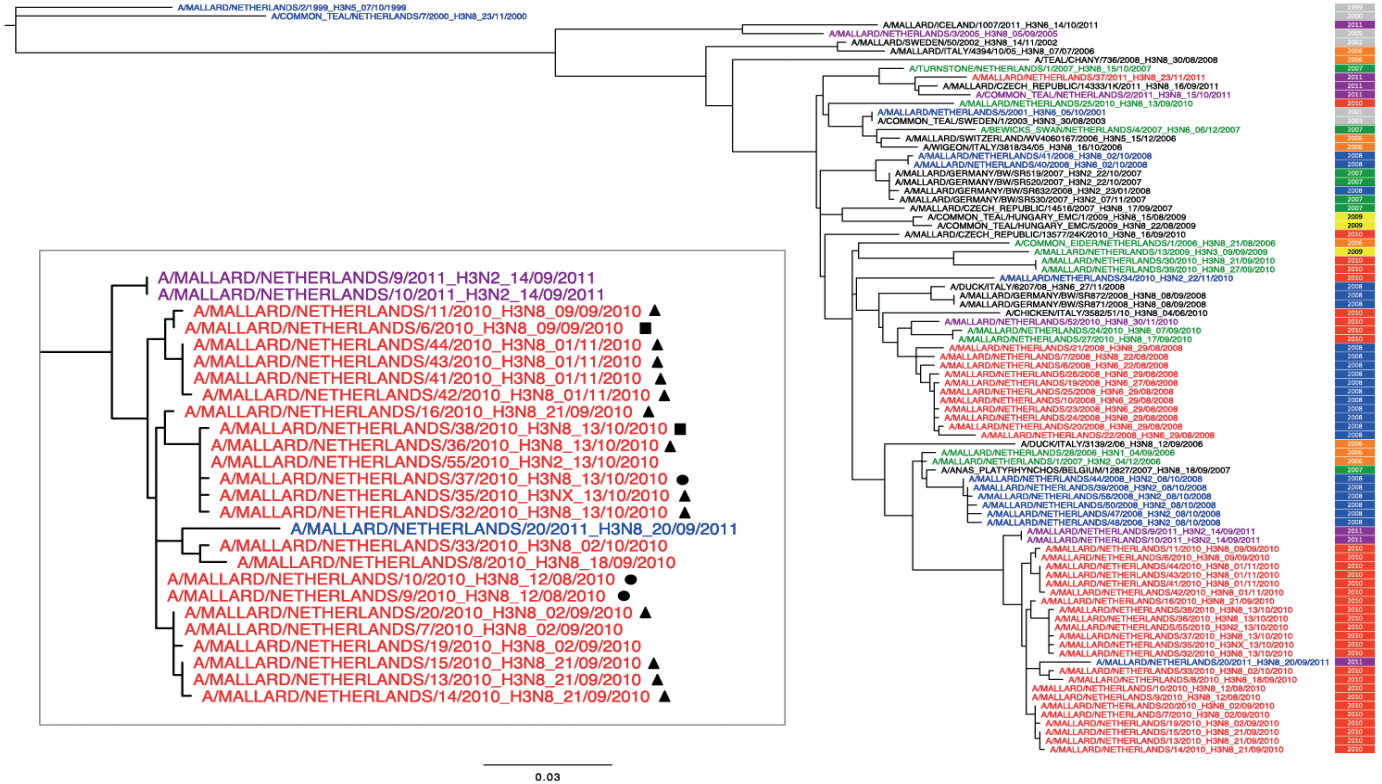

Figure 4.5 Phylogenetic analysis of HA gene of LPAIV H3 isolated during the H3 epizootic in 2010. The Maximum Likelihood (ML) tree contains samples of wild birds collected at various locations in and outside the Netherlands from 1999 until 2011. Each sampling location within the Netherlands is grouped by colour: Oud Alblas (red); Berkenwoude (blue); Lekkerkerk aan de IJssel, Oudeland van Strijen and Vlist (purple); Schiermonnikoog, Vlieland, Westerland and Wieringen (green). Locations are closely situated to the study site , except the locations shown in green, which are located at the coast. Year of virus isolation is listed next to isolate and grouped by colour. Detail of ML tree contains samples of the H3 LPAIV epizootic and migratory strategy of mallards: residents (*RES*; circle), local migrants (*LM*; triangle) and distant migrants (*DM*; square).

H3 LPAIV isolations from residents, local and distant migrants belonged to the same genetic cluster (Fig. 4.5). However, we cannot fully exclude the possibility that novel introductions of H3 LPAIV by migratory birds occurred that were subsequently outcompeted by the dominant epizootic H3 LPAIV strain and thus remained undetected during our monitoring (i.e. competitive exclusion principle; Hardin 1960). For instance, another H3 LPAIV epizootic in the area (i.e. Berkenwoude in 2008) resulted from multiple virus introductions. The H3 LPAIV that induced the 2010 epizootic was closely related to H3 LPAIV strains isolated in the near surrounding one year after the epizootic (i.e. Berkenwoude and Vlist in 2011). This suggests that H3 LPAIV of the epizootic was maintained locally during winter to be isolated the next year. H3 virus prevalence in migratory mallards was higher (especially in distant migrants) and more prolonged (especially in local migrants) than in resident individuals. This finding corresponds with that of van Dijk et al. (2014b) who found a three-fold increase in overall (i.e. non LPAIV-subtype specific) virus prevalence in migratory mallards. However, during the peak of the H3 epizootic many residents were also infected with H3 LPAIV, which may be a consequence of the local amplification and increased viral deposition in the environment (i.e. water and sediment) at the study site. The local amplification may thus be a self-reinforcing process.

At the start of the H3 epizootic, almost exclusively resident birds were infected with H3 LPAIV and shed high amounts of H3 virus. However, it is not surprising that the majority of H3 infections were found in residents, since the sampled mallard population consisted mainly out of resident birds (88%). What is remarkable though is that one week after detection of the first H3 LPAIV infections, no migrants were infected while a large proportion of the sampled mallard population consisted of migrants (~40%). Either migratory birds were not, or to a lesser extent, susceptible to H3 LPAIV infection, or contact rates and the amount of H3 virus particles in the surface water were still too low to infect arriving migrants. Based on quantity of viral RNA derived from corresponding C_T-value (a C_T-value of ~3 higher is a 10-fold increase), residents shed approximately 100-fold higher quantity of H3 virus from the oropharynx than local and distant migrants (sampled respectively on the 12th and 26th of August). Nonetheless, the amount of virus shed depends highly on the stage of infection, which was unknown for the mallards sampled in this study. Besides, co-infections with additional LPAIV strains cannot be excluded, potentially causing a false measure of H3 virus shedding.

During the H3 epizootic, H3-specific antibodies were detected in both resident and migratory mallards, albeit in very few individuals and at low titres. A week before the start of the H3 epizootic, H3-specific antibodies were found in a distant migrant (5th of August). We cannot exclude that this individual was infected with H3 LPAIV either during

migration, at a stop-over site or at the breeding grounds. Hypothetically, this individual could have been infected with H3 LPAIV when transiting through southern Sweden (i.e. feather hydrogen stable isotope -129.2‰ suggest it originated from southern Scandinavia, Baltic States or Russia; van Dijk et al. 2014a), introducing this virus to the wintering grounds. H3 LPAIV is detected frequently in mallards sampled in southern Sweden in early autumn (Latorre-Margalef et al. 2014). Although our genetic analysis does not support this theory, it should be noted that only few H3 LPAIV originating from Sweden or other northern European countries were available and were included in the genetic analysis.

Several local and distant migrants had H3-specific antibodies after the peak of the H3 epizootic. Since these birds were captured once during the H3 epizootic, we cannot exclude that an H3 LPAIV infection outside the study site triggered this antibody response (i.e. genetically different H3 LPAIV were isolated at other locations in the Netherlands). Resident mallards with H3-specific antibodies most likely have been infected by the H3 LPAIV of the epizootic. Only 20% (1 of 5) of residents that had been infected with H3 LPAIV during the epizootic had H3-specific antibodies when recaptured (i.e. recaptured within 31 days since longevity of detectable HA specific antibodies is short; Curran et al. 2013). The absence of H3-specific antibodies following H3 infection could potentially be caused by the sensitivity of the HI assay, thus that H3-specific antibody titres were below the detection level, or that no H3-specific antibodies were generated.

In conclusion, by combining virology, serology and phylogeny analyses with stable isotopes we demonstrate that a local H3 LPAIV epizootic in mallards was likely induced by a single virus introduction into susceptible residents, followed by a period of local virus amplification that was associated with the influx of migratory mallards. In addition to the study of Hill et al. (2012), who showed long-distance movement of LPAIV genes by migrating mallards on a macro-ecological scale, we showed an association between local amplification of H3 LPAIV and the arrival of migratory mallards at the wintering grounds at a much smaller ecological scale. We suggest an alternative role for migrating mallards as local amplifiers, based on the difference in H3 LPAIV prevalence between resident and migratory mallards upon arrival at the wintering grounds. This study exemplifies the difficulty of elucidating the role of migratory and resident hosts in infectious disease dynamics in wildlife, but provides encouraging indications that the here presented multifaceted approach may open a window on these processes.

Acknowledgements

We thank Teunis de Vaal for catching mallards in the duck decoy at Oud Alblas and assisting with sampling the birds. Peter de Vries, Audrey van Mastrigt and Lennart Zwart are also thanked for their help in the field. We thank Ger van der Water, Judith Guldemeester and Kim Westgeest for logistical and technical assistance, and Richard Doucett and Melanie Caron of the Colorado Plateau Stable Isotope Laboratory for performing the stable hydrogen isotope analysis. This work was sponsored by grants from the Dutch Ministry of Economic Affairs, the Netherlands Organization for Scientific Research (NWO; grant 820.01.018) and contract NIAID NIH (HHSN266200700010C). Capturing free-living mallards was approved by the Dutch Ministry of Economic Affairs based on the Flora and Fauna Act (permit number FF/75A/2009/067 and FF/75A/2010/011). Handling and sampling of free-living mallards was approved by the Animal Experiment Committee of the Erasmus MC (permit number 122-09-20 and 122-10-20) and the Royal Netherlands Academy of Arts and Sciences (KNAW) (permit number CL10.02). Free-living mallards were released into the wild after sampling. All efforts were made to minimize animal suffering throughout the studies.

III Correlates of infection

5 Minor differences in body condition and immune status between avian influenza virus infected and non-infected mallards: a sign of co-evolution?

Jacintha G.B. van Dijk, Ron. A.M. Fouchier, Marcel Klaassen & Kevin D. Matson

Wildlife pathogens can alter host fitness. Low pathogenic avian influenza virus (LPAIV) infection is thought to have negligible impacts on wild birds; however, effects of infection in free-living subjects are in fact largely unstudied. We investigated the extent to which LPAIV infection was associated with body condition and immune status in free-living mallards (*Anas platyrhynchos*), a partially migratory key LPAIV host species, to assess their potential role as asymptomatic carriers of LPAIV. We sampled mallards throughout the species' annual autumn LPAIV infection peak, and we classified individuals according to age, sex and migratory strategy when analysing data on body mass, and five indices of immune status. Body mass was similar for LPAIV infected and non-infected birds. Migratory birds had a lower body mass than residents, when classified based on stable hydrogen isotope analysis. In females, LPAIV infected individuals exhibited reduced first lines of defence against infections (natural antibody and complement titres) compared to non-infected females. LPAIV infected local migrants (i.e. coming from a short distance) had higher titres of natural antibodies and complement than non-infected counterparts. Concentrations of the acute phase protein haptoglobin were higher in LPAIV infected males than in non-infected males. Avian influenza virus- (AIV-) specific antibody concentrations and ratios of heterophils to lymphocytes (H:L ratio) were similar in LPAIV infected and non-infected birds. Our study demonstrates weak associations between LPAIV infection and body condition and immune status of free-living mallards, which may support their role as asymptomatic carriers of LPAIV. The weak association between LPAIV infection and mallard's fitness raises the question whether this is a consequence of co-evolution between the two, which would likely explain the role of mallards as one of the key reservoirs of these viruses.

Ecology and Evolution (in revision)

Introduction

Wildlife pathogens can alter host fitness, for instance, by affecting an animal's ability to grow (Burthe et al. 2008), reproduce (de Crespigny and Wedell 2006) or survive (Burthe et al. 2008, Mayack and Naug 2009). Disease outbreaks can have detrimental effects on population numbers, for example the outbreak of rinderpest in African ungulates (Plowright 1982) or canine distemper in lions and seals (Roelke-Parker et al. 1996, Kuiken et al. 2006). Yet pathogens can also have more subtle effects which may impair host fitness, such as reduced foraging or decreased activity (Bradley and Altizer 2005, Venesky et al. 2009). Susceptibility to pathogen infection may differ between hosts. For example, West Nile virus (WNV) negatively impacted populations of North American corvids, while other passerines and members of other orders apparently tolerated infection without significant morbidity (LaDeau et al. 2007). Whether interspecific differences in the effects of pathogens result from different degrees of co-evolution (i.e. go through a process of reciprocal, adaptive genetic changes; Woolhouse et al. 2002) is poorly understood. A well-known example of co-evolution is the European rabbit–myxoma virus system, where phenotypic changes were observed in both pathogen and host after introduction of the virus into a naïve rabbit population (Fenner and Fantini 1999). Studying effects of pathogens on wildlife is useful for understanding the impacts on host fitness, and potential consequences for populations, and, more generally, the role of co-evolution.

A common pathogen that circulates naturally in wild birds is low pathogenic avian influenza virus (LPAIV). This virus predominantly infects birds inhabiting wetlands and aquatic environments (orders *Anseriformes* and *Charadriiformes*; Webster et al. 1992). Experimental infection studies under laboratory conditions show that LPAIV causes only mild disease in these species (Daoust et al. 2011, Höfle et al. 2012, Kuiken 2013). However, effects of LPAIV infection in free-living waterfowl are largely unstudied. Few studies showed that waterfowl that were naturally infected with LPAIV had a lower body mass than non-infected individuals (van Gils et al. 2007, Latorre-Margalef et al. 2009), but this result was observed in specific years only in some species (Kleijn et al. 2010). LPAIV infection may impair feeding, movement and even delay migration of waterfowl species (J.G.B. van Dijk unpublished data, van Gils et al. 2007), with other studies in the same species finding inconclusive results or no relationships (Latorre-Margalef et al. 2009, Hoye 2011). These limited studies, with often contradictory results, show that our understanding of effects of LPAIV infection on host fitness is still largely unclear.

Dabbling ducks of the *Anas* genus, and particularly mallards *(Anas platyrhynchos)*, are frequently infected with LPAIV (Olsen et al. 2006). Mallards are known to be infected with almost all LPAIV subtypes that are found in birds to date (H1-H16, N1-N9), the

exceptions being H13 and H16 (Kawaoka et al. 1990, Röhm et al. 1996, Olsen et al. 2006). Besides getting infected and causing secondary transmission, mallards are also likely to sustain the pathogen by themselves, i.e. they function as a reservoir species, which is probably not the case in other dabbling duck species (Nishiura et al. 2009). Experimentally infected mallards shed high LPAIV titres, but lack any signs of disease, while results on their immune response are contradictory: from a weak antibody response (Kida et al. 1980) to long-lasting antibody production (Fereidouni et al. 2010). However, it remains unclear how free-living mallards cope with natural LPAIV infections and whether their energetic and immunological statuses are impacted.

The question whether free-living waterfowl may carry LPAIV asymptomatically recently became more important with the outbreak of LPAIV H7N9 in China. This disease can be seriously pathogenic for humans, while wild birds, and poultry, show no clear symptoms of disease (Kreijtz et al. 2013). Since its emergence in February 2013, the H7N9 virus has led to more than hundred human deaths (World Health Organization 2014b). Therefore, studying effects of LPAIV infection in free-living waterfowl is critical to understand the role of these birds as potential carriers of this infectious disease.

The aim of our study was to investigate potential effects of LPAIV infection in free-living mallards in order to assess their ability to function as asymptomatic carriers of this virus. Autumn is generally the period that LPAIV infection in mallard populations is highest in the northern hemisphere (Munster et al. 2007, Latorre-Margalef et al. 2014, van Dijk et al. 2014b). During this peak, we comprehensively sampled mallards on their wintering grounds to assess the extent to which LPAIV infection was associated with body condition and immune status. We classified birds based on their age, sex and migratory strategy to investigate the interactions between these parameters and LPAIV infection status. Effects of LPAIV infection in juveniles may be more profound than in adults, since they are immunologically naïve and have less developed immune systems (Sol et al. 2003). Effects of LPAIV infection may also differ between males and females due to sexual differences in body condition, immune status and physiology in general (Zuk and McKean 1996). At the wintering grounds, migratory and resident mallard populations mix. Effects of LPAIV infection may differ between the two populations, since energetic demands of migration may compromise immune function and nutritional status in migratory birds (Owen and Moore 2006).

Materials and methods

Study species

Throughout Europe mallards are partially migratory: meaning that the population consist

of both migratory and resident birds. Birds that breed in Finland, Sweden, the Baltic and north-west Russia migrate in autumn to winter from Denmark to northern France and Britain. As a result, these migratory birds mix with resident individuals in north-western Europe, including the Netherlands (Scott and Rose 1996).

Sampling

From August until December 2010, coinciding with the major annual LPAIV infection peak, mallards were caught using swim-in traps (i.e. a duck decoy; Payne-Gallwey 1886) located near Oud Alblas (51°52′38″N, 4°43′26″E) in the Alblasserwaard, the Netherlands. On average, we visited the decoy six times per month and captured approximately nine individuals per visit. Each individual was marked with a metal ring and categorized based on plumage characteristics as male or female, and as juvenile (<1 year) or adult (>1 year) (Boyd et al. 1975). We measured tarsus length (nearest 0.01 mm; Byers and Cary 1991), head+bill length (nearest 0.1 mm) and wing length (maximum wing chord, nearest 1 mm; Baker 1993). A digital balance was used to measure body mass (nearest 1 g). We used sterile cotton applicators to swab the cloaca and the oropharynx, as LPAIV may replicate in both the intestinal and respiratory tract of wild birds (Fouchier and Munster 2009). Swabs were stored individually in transport medium (Hank's balanced salt solution with supplements; Munster et al. 2009) at 4 °C and transported to Erasmus MC for analysis within seven days of collection. Blood samples (<1 ml and <2% of the circulating blood volume) were collected from the brachial vein. Drops of fresh blood were used to make smears for leukocyte enumeration, and the remainder was allowed to clot for approximately 6 h before centrifugation to separate serum and cell fractions (Hoye 2012). Serum samples were stored at -20 °C for several months until analysis. We collected the tip (1–2 cm) of the first primary feather (P1) of the right wing for stable hydrogen isotope analysis. Feather samples were stored at room temperature in sealed plastic bags.

Virus detection

Cloacal and oropharyngeal swabs were used for detection of influenza A virus. For full details on RNA isolation and virus detection, see Munster et al. (2009). In short, RNA was isolated using a MagnaPure LC system with the MagnaPure LC Total nucleic acid isolation kit (Roche Diagnostics, Almere, the Netherlands). Influenza A virus was detected using a generic real-time reverse transcriptase PCR assay targeting the matrix gene.

Immune assays

We examined five immunological indices: (i) natural antibodies (NAbs), (ii) complement, (iii) avian influenza virus- (AIV-) specific antibodies, (iv) haptoglobin (Hp) and (v) ratio of heterophils to lymphocytes (H:L ratio).

A hemolysis-hemagglutination assay with rabbit red blood cells (HemoStat Laboratories, Dixon, California, USA) was used to quantify non-specific NAbs (i.e. agglutination score) and NAb-mediated complement activation (i.e. lysis score) in serum, as described by Matson et al. (2005). All scans of individual samples were randomized and scored blindly. NAbs and complement are part of the innate immune system, providing non-specific initial protection against foreign agents (Davison et al. 2008) and act as a first line of defence against infections (Ochsenbein and Zinkernagel 2000). NAbs are produced in the absence of exogenous antigenic stimulation and are supposedly unaffected by current infection (Ochsenbein and Zinkernagel 2000). NAb response is hypothesized to predict the strength of the acquired immune response, which is specific against a pathogen (Kohler et al. 2003). Complement is a group of proteins involved in inflammation that can be activated directly by pathogens or indirectly by antigen-bound antibodies (Müller-Eberhard 1988). The interaction between NAbs and complement is an important link between the innate and acquired parts of the humoral immune system (Ochsenbein and Zinkernagel 2000).

To measure serum concentrations of AIV-specific antibodies (i.e. antibodies that bind to the highly conserved nucleoprotein of AIV), we used 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. The absorbance (i.e. OD-values) of the bELISA can be used as a relative measure of AIV-specific antibody concentration, with lower OD-values reflecting higher relative AIV antibody concentrations (J.G.B. van Dijk unpublished data).

Hp concentrations (mg ml^{-1}) in serum were quantified using a commercially available assay kit (Tridelta Development Limited, Maynooth, County Kildare, Ireland), following manufacturer's instructions with several modifications (Matson et al. 2012a). Hp is an acute phase protein that binds free hemoglobin to prevent it from providing nutrients to pathogens. Concentrations of Hp typically increase in response to acute infection, inflammation or trauma (Delers et al. 1988). In birds Hp increases following simulated bacterial infection, but not following intense flight (Matson et al. 2012b).

The first 100 leukocytes per blood smear were classified and enumerated. Smears were evaluated blindly by an individual veterinary diagnostic laboratory technician (European Veterinary Laboratory, Woerden, the Netherlands). The ratio between

heterophils and lymphocytes (i.e. H:L) was calculated. Heterophils and lymphocytes are the two dominant leukocytes that mediate non-specific immunity and specific antibody responses, respectively (Campbell 1995). An increase in H:L ratio can reflect stress and susceptibility to infection (Davis et al. 2008). H:L is not affected by handling time (Davis 2005).

Stable isotope analysis

Stable hydrogen isotope ratios (δ^2H) in feathers were used to assess the moulting location of birds, and hereby their migratory strategy (i.e. migrant or resident). For full details, see van Dijk et al. (2014a). In short, feathers were cleaned with 2:1 chloroform:methanol solvent mixture to remove surface contaminants and oils, and air-dried overnight under a fume hood. Feather samples were placed into silver capsules, folded into tiny balls and stored in 96-well trays. Trays were shipped to the Colorado Plateau Stable Isotope Laboratory (Northern Arizona University, Flagstaff, Arizona, USA), and stable hydrogen isotope analyses were performed on a Delta Plus XL isotope ratio mass spectrometry (IRMS) equipped with a 1400 C TC/EA pyrolysis furnace. Feather δ^2H are reported in units per mil (‰) relative to the Vienna Standard Mean Ocean Water-Standard Light Antarctic Precipitation (VSMOW-SLAP) standard scale.

Signatures of stable isotopes in animal tissue, including feathers, reflect those of local food webs (Peterson and Fry 1987). Precipitated water moves up the food chain and is eventually incorporated into feathers during their growth (Hobson 1999). There is a close correlation between feather δ^2H and amount-weighted growing-season δ^2H in precipitation (Hobson and Wassenaar 1997), the latter of which exhibits a gradient across Europe and North America (Bowen et al. 2005). For Europe, a similar gradient has been found in δ^2H of mallard feathers, enabling assessment of the geographic location where individuals moulted their feathers (van Dijk et al. 2014a).

Statistical analysis

The dataset contained individuals that were captured and sampled once (n = 266) and multiple times (n = 19; Table 5.1). A bird was considered LPAIV positive when either its cloacal or its oropharyngeal sample tested positive.

All response variables were log10-transformed to meet the assumption of normality. Collinearity between the response variables was tested with Pearson correlation (r) (Appendix 5.1 Table S5.1). Body mass and Hp concentration (r = -0.19), and lysis score and H:L ratio (r = 0.13) were significantly correlated. However, because of their low r^2 (respectively 0.03 and 0.01) they were used separately as response variables to test the association with

LPAIV infection. Agglutination and lysis scores were highly correlated (r = 0.56, r^2 = 0.31) and combined in a single measure by taking the average of both variables (called lysis-agglutination score). As an index of body size, we used the first principal component (PC1) of a PC analysis of tarsus, head+bill and wing lengths. PC1 explained 79% of the variance.

Linear mixed models (LMMs) were used to test the association between LPAIV infection and body mass and the four immunological indices (i.e. lysis-agglutination score, AIV-specific antibody concentration, Hp concentration and H:L ratio). The models included LPAIV infection status, age (i.e. juvenile, adult), sex, migratory strategy (i.e. resident, local migrant, distant migrant) and month as fixed factors, as well as three two-way interactions between infection status and age, sex and migratory strategy. The model of body mass included PC1 to correct for bird size, and the model of Hp concentration included sample redness to correct for hemolysis, which can affect the Hp assay (Matson et al. 2012a). A Tukey's post hoc test was performed to detect differences in body mass and immune status between birds with different migratory strategy and month. The significance level (α) equalled 0.05. The reported mean values and SE were back-transformed. All analyses were conducted using R 2.14.1 (R Development Core Team 2012). Package lme4 was used to fit the LMMs (Bates et al. 2012).

Table 5.1 Number of samples collected from primary captures and recaptures of residents (*RES*), local migrants (i.e. coming from a short distance, *LM*) and distant migrants (i.e. coming from a long distance, *DM*), by age (i.e. juvenile: <1 year, adult: >1 year) and sex from August until December 2010.

		RES		LM	DM
Age	Sex	Primary	Recapture		
Juvenile	Male	6	3	26	25
	Female	6	1	8	9
Adult	Male	31	14	30	30
	Female	22	3	44	29
Total		65	21	108	93

Results

Body mass

Body mass did not differ between LPAIV infected and non-infected birds, when corrected for bird size (Table 5.2, Table 5.3). The relationship between LPAIV infection and body mass did not depend on (or differ by) age class, sex or migratory strategy (Table 5.2).

However, body mass was negatively associated with migratory status: local and distant migrants weighed less than residents (Table 5.2, Fig. 5.1). There was also a difference in body mass between juveniles and adults (respectively, 1092 ± 13 g and 1141 ± 9 g; Table 5.2). Body mass differed between months (Table 5.2), with birds having a lower body mass in August and September compared to the following months (all $P > 0.05$).

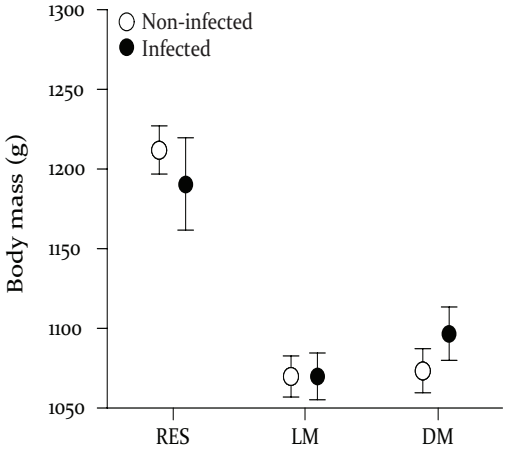

Figure 5.1 Body mass (mean ± upper/lower SE) of non-infected and LPAIV infected residents (*RES*), local migrants (*LM*) and distant migrants (*DM*).

Immunological indices

There were no differences in lysis-agglutination scores, AIV-specific antibody concentrations, Hp concentrations and H:L ratios between LPAIV infected and non-infected birds (Table 5.2, Table 5.3).

In females but not males, lysis-agglutination scores were lower in LPAIV infected birds compared to non-infected ones (Table 5.2, Fig. 5.2a). Anyway, lysis-agglutination scores were lower in females compared to males (respectively, 5.9 ± 0.1 and 6.2 ± 0.1; Table 5.2). Lysis-agglutination scores also varied in a manner that depended on the interaction between LPAIV status and migratory strategy (Table 5.2), i.e. infected local migrants had higher scores ($P = 0.048$), but there was no association with infection in residents and distant migrants (Fig. 5.2b). For lysis-agglutination scores there was no interaction between LPAIV infection and age class (Table 5.2).

There was no interaction between LPAIV infection and age class, sex and migratory strategy for concentrations of AIV-specific antibodies (Table 5.2). AIV-specific antibody concentrations differed between months (Table 5.2): birds had higher AIV-specific antibody concentrations in August than in September ($P = 0.023$), and lower concentrations in December compared to the previous three months (all $P < 0.001$).

Table 5.2 Model output produced by the LMMs used to test the association between LPAIV infection and body mass and immune status in mallards. The LMM of body mass included bird size ($df = 1, X^2 = 89.95, P < 0.001$), and the LMM of haptoglobin included sample redness ($df = 1, X^2 = 20.33, P < 0.001$). The *df* is applicable for each model ($n = 287$). Significant correlations are in bold.

		Body mass		Lysis-agglutination		AIV-specific antibodies		Haptoglobin		H:L ratio	
Variable	*df*	X^2	*P*-value	X^2	*P*-value	X^2	*P*-value	X^2	*P*-value	X^2	*P*-value
Infection status	1	0.06	0.800	0.00	0.955	0.07	0.795	1.07	0.300	2.99	0.084
Age	1	12.49	**<0.001**	0.46	0.498	0.23	0.631	0.42	0.515	0.58	0.444
Sex	1	1.23	0.268	6.76	**0.009**	0.36	0.550	0.82	0.366	2.07	0.150
Migratory strategy	2	69.13	**<0.001**	3.93	0.141	5.09	0.079	8.10	**0.018**	4.43	0.109
Month	4	93.85	**<0.001**	6.53	0.163	55.33	**<0.001**	4.42	0.352	21.94	**<0.001**
Infection status*age	1	0.26	0.608	0.15	0.701	1.94	0.164	6.78	**0.009**	0.05	0.825
Infection status*sex	1	0.47	0.494	5.92	**0.015**	2.04	0.154	4.58	**0.032**	0.04	0.846
Infection status*migratory strategy	2	1.75	0.417	6.24	**0.044**	3.00	0.223	3.16	0.205	0.21	0.900

Table 5.3 Average values (± upper/lower SE) of body condition and four indices of immune status produced by the LMMs of mallards that were non-infected ($n = 188$) and infected with LPAIV ($n = 99$). Body mass and immune status was measured of all sampled birds. Lysis-agglutination score is the average of agglutination score and lysis score. The reported mean values and SE were back-transformed. Note: lower AIV-specific antibodies (i.e. OD-value) reflect higher relative AIV-specific antibody concentrations.

	Non-infected			Infected		
Variable	mean	SE upper	SE lower	mean	SE upper	SE lower
Body mass (g)	1115	9	9	1117	14	13
Lysis-agglutination score	6.1	0.1	0.1	6.0	0.1	0.1
AIV-specific antibodies	0.74	0.03	0.03	0.68	0.04	0.04
Haptoglobin (mg ml^{-1})	0.04	0.01	0.01	0.03	0.01	0.01
H:L ratio	0.59	0.03	0.03	0.50	0.04	0.04

Hp concentrations in LPAIV infected and non-infected birds differed according to sex (Table 5.2): males had higher Hp concentrations when LPAIV infected than when uninfected ($P < 0.001$; Fig. 5.3). There was also an interaction effect between LPAIV virus and age; however there was no significant difference in Hp concentrations between infected and non-infected juveniles and adults. Hp concentrations differed between birds of different migratory strategy (Table 5.2), with higher concentrations in local migrants (0.06 ± 0.01 mg m^{-1}) than in residents (0.02 ± 0.01 mg m^{-1}; $P = 0.014$).

There was no interaction between LPAIV infection and age class, sex and migratory strategy for H:L ratios (Table 5.2). H:L ratios differed between months (Table 5.2): birds had lower H:L ratios in August and December compared to October and November (all $P < 0.05$).

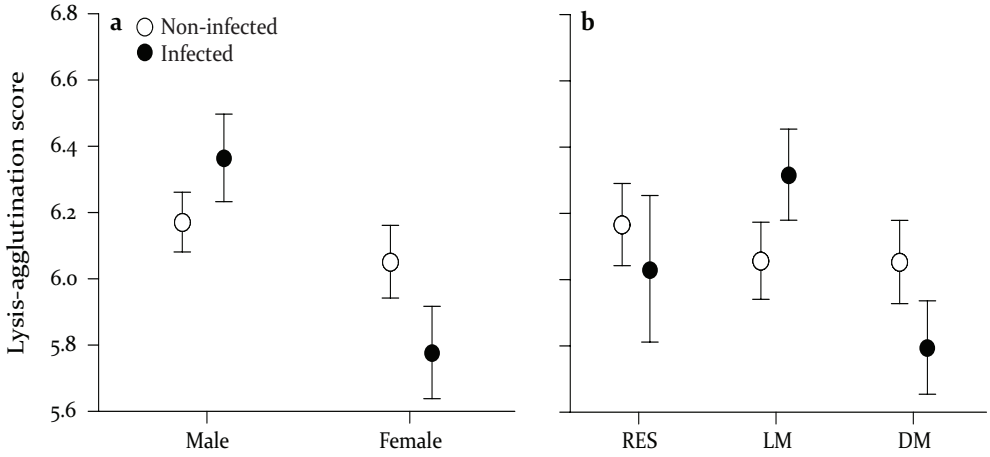

Figure 5.2 Lysis-agglutination scores (mean ± upper/lower SE) of non-infected and LPAIV infected (a) males and females and (b) resident (*RES*), local migrants (*LM*) and distant migrants (*DM*). Lysis-agglutination score is the average of agglutination score and lysis score.

Discussion

Body mass

During the autumn LPAIV infection peak, infected mallards did not differ in body mass from non-infected birds, when corrected for age, sex and migratory strategy. Irrespective of LPAIV infection, local and distant migrants had a lower body mass than residents.

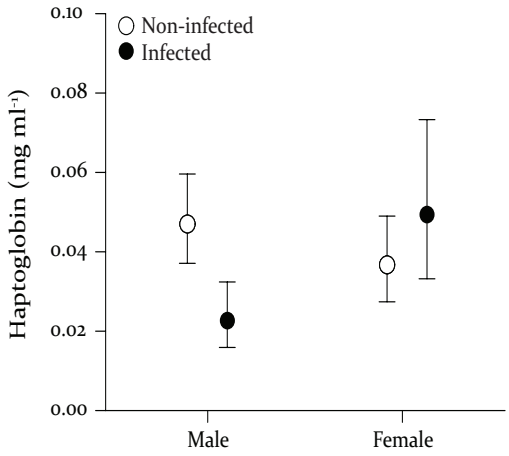

Figure 5.3 Haptoglobin concentrations (mean ± upper/lower SE) of non-infected and LPAIV infected males and females.

Our results contradict the findings of Latorre-Margalef et al. (2009) who found a lower body mass (almost 20 g) in migratory mallards infected with LPAIV compared to non-infected counterparts when sampled during autumn migration. However in contrast to our study, migratory strategy was not explicitly included as a factor in their study. An association between migratory strategy and body mass is demonstrated by our data. If we excluded migratory strategy from the current body mass analysis, we found an association between LPAIV infection and body mass (LMM: $X^2 = 4.78$, $P = 0.029$): LPAIV infected mallards had a lower body mass than non-infected individuals (respectively, 1081 ± 13 g and 1107 ± 10). This result suggests that differences in body mass were likely not explained by LPAIV infection, but merely by birds' migratory strategy. This could also be applicable in the study of Latorre-Margalef et al. (2009). In this study, mallards were sampled during autumn migration at a major staging site of migrating mallards and other European waterfowl (i.e. Ottenby, Sweden), hence all sampled birds were assumed to be migrants. Still, it cannot be fully excluded that resident mallards were also part of their sample size. Mallards are known to breed in southern Sweden, which is <200 km from Ottenby (Gunnarsson and Elmberg 2008). Additionally, feather δ^2H of hatch-year mallards captured at Ottenby (Gunnarsson et al. 2012) showed that birds may originate from locations ranging from southern to northern Europe (van Dijk et al. 2014a).

Immunological indices

There were no differences in any of the immunological variables between LPAIV infected and non-infected mallards. This suggests the absence of large immunological effects of infection with LPAIV in mallards.

NAbs and complement are part of the first line of defence against infections and generally show low sensitivity to current infections (Ochsenbein and Zinkernagel 2000). High levels of NAbs and complement indicate a high humoral immune status (Whiteman et al. 2006, Parejo and Silva 2009). In our study, LPAIV infected females had lower titres of NAbs and complement than non-infected females suggesting infected females had a lower immune status. While LPAIV infected local migrants had a higher immune status (i.e. higher titres of NAbs and complement) than non-infected counterparts. We found no differences in NAb and complement levels with infection status in distant migrants and residents. Hp concentrations, an acute phase protein that increases in response to infection and inflammation (Delers et al. 1988), were higher in LPAIV infected than in non-infected males. However, this difference was not shown in females, having similar concentrations of Hp when infected and non-infected with LPAIV. These Hp concentrations in females were comparable to the concentrations of Hp in infected males. This could suggest that females were capable of maintaining high concentrations of Hp also when uninfected.

We found sex differences in NAb and complement: males had higher titres of NAb and complement than females. Our findings are in line with a study on free-living skylarks (*Alauda arvensis*), in which males had higher complement levels than females (Hegemann et al. 2012). Sex differences in immune function may be attributed to effects of hormones (e.g. testosterone in males), resource allocation to immune function due to their differences in behaviour and physiology or exposure to pathogens (Møller et al. 1998). Only Hp concentrations differed between residents and migrants: local migrants had higher concentrations of Hp than residents. These elevated levels in local migrants could reflect previous (LPAIV) infection in which levels were still high even when uninfected.

In conclusion, studying the physiological effects of LPAIV infection in waterfowl is important for generating better perspectives on their potential role as asymptomatic carriers of this virus. We studied associations between LPAIV infection and body condition and immune status in free-living mallards during the autumn infection peak at the wintering grounds. We found no differences in body condition and only small differences in immune status that could potentially be attributed to LPAIV infection. The weak association between LPAIV infection and the physiological and immunological conditions of mallards likely supports their role as asymptomatic carriers of this virus. This raises the intriguing question whether the weak associations between LPAIV infection and mallard's fitness could be a consequence of co-evolution between mallards and LPAIV. Hosts and pathogens may co-evolve if their relationship is intimate and if strong selective pressures act on both the host and pathogen (Woolhouse et al. 2002). Susceptibility and virulence may depend on host-pathogen co-evolutionary processes, in which hosts have the ability to minimize

virulence (i.e. mild or no disease effects) without minimizing pathogen fitness (Little et al. 2010). Whether there is a tight mallard-LPAIV co-evolution is currently unknown and requires further investigation, for instance by assessing a gene-for-gene relationship (i.e. a single locus in the genome of both host and parasite; Woolhouse et al. 2002) to fully understand the role of mallards in LPAIV infection dynamics.

Acknowledgements

We express our gratitude to Teunis de Vaal, who caught all mallards and assisted when sampling the birds. Peter de Vries is also thanked for his help in the field. Furthermore, we thank Josanne Verhagen, Pascal Lexmond, Oanh Vuong, Judith Guldemeester and Peter van Veelen for their laboratory assistance. Robert Doucett and Melanie Caron of the Colorado Plateau Stable Isotope Laboratory are thanked for performing the stable hydrogen isotope analysis, and the European Veterinary Laboratory for classification and enumeration of leukocytes. Funding was provided by the Netherlands Organization for Scientific Research (NWO; grant 820.01.018), the National Institute of Health (NIH; contract NIAID HHSN266200700010C) and the Australian Research Council. KDM was supported by NWO-Vidi (grant to B.I. Tieleman 864.10.012). Mallards were handled under approval from the Animal Experiment Committee (protocol 122-10-20). Flora and Fauna permit FF/75A/2009/067 was issued by the Dutch Ministry of Economic Affairs to capture free-living mallards outside the hunting season in August. All efforts were made to minimize any animal suffering throughout the study.

6 Transfer of maternal antibodies against avian influenza virus in mallards (*Anas platyrhynchos*)

Jacintha G.B. van Dijk, A. Christa Mateman & Marcel Klaassen

Maternal antibodies protect chicks from infection with pathogens early in life and may impact pathogen dynamics due to the alteration of the proportion of susceptible individuals in a population. We investigated the transfer of maternal antibodies against avian influenza virus (AIV) in a key AIV host species, the mallard (*Anas platyrhynchos*). Combining observations in both the field and in mallards kept in captivity, we connected maternal AIV antibody concentrations in eggs to (i) female body condition, (ii) female AIV antibody concentration, (iii) egg laying order, (iv) egg size and (v) embryo sex. We applied maternity analysis to the eggs collected in the field to account for intraspecific nest parasitism, which is reportedly high in *Anseriformes*, detecting parasitic eggs in one out of eight clutches. AIV antibody prevalence in free-living and captive females was respectively 48% and 56%, with 43% and 24% of the eggs receiving these antibodies maternally. In both field and captive study, maternal AIV antibody concentrations in egg yolk correlated positively with circulating AIV antibody concentrations in females. In the captive study, yolk AIV antibody concentrations correlated positively with egg laying order. Female body mass and egg size from the field and captive study, and embryos sex from the field study were not associated with maternal AIV antibody concentrations in eggs. Our study indicates that maternal AIV antibody transfer may potentially play an important role in shaping AIV infection dynamics in mallards.

Plos One (in revision)

Introduction

Chicks are very vulnerable to pathogens in the early stages of their life, as they lack a fully developed immune system. Mothers are potentially able to protect their offspring in this crucial phase by transferring maternal antibodies (i.e. mainly immunoglobulin IgY) to the egg yolk, which is passed through the yolk sac membrane into the embryonic bloodstream (Boulinier and Staszewski 2008). These antibodies provide protection against pathogens to which the mother was previously exposed and mounted an immune response (Heller et al. 1990, Lemke et al. 2003). Maternal antibodies are relatively short-lived (from a few days to more than a month), with the duration of their presence in a chick's blood plasma being dependent on species (King et al. 2010, Garnier et al. 2012). After this period, a chick's own antibody production needs to take over (Grindstaff et al. 2006, Staszewski et al. 2007). The fitness benefits of maternal antibodies may be considerable for the receiving neonates. As shown by a range of studies, maternal antibodies can be of crucial importance for a chick's initial humoral defence and growth rate, increasing its survival during the early stages of its life (for review see Hasselquist and Nilsson 2009). Moreover, these maternal antibodies can have considerable impact on pathogen dynamics at the population level, reducing the proportion of susceptible individuals in a host population (Boulinier and Staszewski 2008). Hence, studying maternal antibodies in any host-pathogen system is important to evaluate their potential role in shaping pathogen dynamics in the host population(s).

Low pathogenic avian influenza virus (LPAIV), a pathogen that circulates naturally in wild birds, is highly studied, though the role of maternal antibodies in LPAIV infection dynamics in wild birds has been largely neglected. The limited studies on maternal antibodies against avian influenza virus (AIV) in wild birds are conducted in gulls, a family of birds that acts as a reservoir host for LPAIV subtypes H13 and H16 (Hinshaw et al. 1982, Fouchier et al. 2005). Up to 92% of adult gulls had antibodies against AIV (Velarde et al. 2010, Hammouda et al. 2011) and 14 to 51% of eggs received these antibodies maternally (Pearce-Duvet et al. 2009, Hammouda et al. 2011). In yellow-legged gulls (*Larus michahellis*) it was furthermore shown that maternal AIV antibodies in eggs reflected the circulating AIV antibodies of the laying females and that the first-laid eggs received highest levels of maternal antibodies (Hammouda et al. 2012). These findings in gulls indicate a considerable transfer of maternal AIV antibodies to eggs, raising the possibility that maternal antibody transfer might also play a vital role in LPAIV infection dynamics in other host species.

Even though LPAIV predominantly infects birds inhabiting wetlands and aquatic environments (order *Anseriformes* and *Charadriiformes*; Webster et al. 1992), mallards (*Anas platyrhynchos*) are frequently infected with this virus and generally considered to be one of the major reservoirs of LPAIV (Olsen et al. 2006). LPAIV infection dynamics

in free-living mallard populations in the northern hemisphere show peak infections in late summer and early autumn, with some studies reporting a small infection peak in spring or early summer (Munster et al. 2007, Latorre-Margalef et al. 2014, van Dijk et al. 2014b). The influx of immunologically naïve juveniles in summer and susceptible migrants in autumn are the likely drivers of these seasonal dynamics of LPAIV infections in mallards (van Dijk et al. 2014b). But whether transfer of maternal AIV antibodies plays a significant role in shaping this summer LPAIV infection peak in mallards, and which factors may potentially impact the process of maternal AIV transfer to eggs is unclear.

A range of factors may determine the quantity of maternal antibodies transferred to eggs. Importantly, maternal antibody transfer to offspring is strongly correlated with the mother's antibody levels (Gasparini et al. 2002, Grindstaff et al. 2005, Staszewski et al. 2007, Grindstaff 2010). Female body condition may also positively impact maternal antibody transfer (Hargitai et al. 2006, Karell et al. 2008), which may, at least in part, be due to the positive effect of body condition on female antibody levels (Karell et al. 2008). Additional correlations between maternal antibody levels in eggs have been shown with female age and colour (Grindstaff et al. 2003, Jacquin et al. 2013), breeding density and sexual attractiveness of male mates (Saino et al. 2002, Müller et al. 2004), egg colour (Morales et al. 2006, Holveck et al. 2012), laying order (Groothuis et al. 2006, Pihlaja et al. 2006) and the sex of offspring (Saino et al. 2003, Martyka et al. 2011, Abad-Gómez et al. 2012).

The aim of our study was to investigate maternal AIV antibody transfer in free-living mallards, and to assess which individual factors correlate with the concentration of maternal AIV antibodies in eggs. We sampled free-living females together with their eggs, connecting yolk maternal AIV antibody concentrations to (i) female body condition, (ii) female AIV antibody concentration, (iii) egg size and (iv) embryo sex. As potentially correlated factors are difficult to disentangle in a field study, we investigated the same factors, except embryo sex, also in a study with captive females and their eggs. Additionally, we examined the correlation between yolk maternal AIV antibody concentrations and (v) egg laying order in the captive study. In the light of previous findings in other species, we hypothesized that good body condition and high AIV antibody levels in females correlate with high concentrations of maternal AIV antibodies in eggs. We hypothesized that egg size would not correlate with yolk maternal AIV antibody concentration, since studies in other avian species showed no association between egg size and IgY concentrations in eggs and chicks (Grindstaff et al. 2005, Karell et al. 2008). We predicted that female offspring would receive higher concentrations of maternal AIV antibodies than male offspring, as female mallards are potentially the more sensitive sex (i.e. lower chick, juvenile and adult survival rate than males; Gunnarsson et al. 2008). Furthermore, we hypothesized that the laying

order of eggs would not correlate with maternal AIV antibody concentration in yolk, since hatching of mallard chicks is synchronous (Cramp and Simmons 1977).

Materials and methods

Field study

From April until June 2010, 67 free-living female mallards breeding in a woodland area in the Alblasserwaard (51°52'38"N, 4°43'26"E), the Netherlands, were caught from their nest with a sweep net. Females were caught during incubation to lower the chance of nest abandonment. To reduce the risk of including nests with eggs that had primarily been dumped there by other females, we only sampled females with a clutch of 13 eggs or less (Pehrsson 1991). Captured females were marked with a metal ring and categorized as juvenile (± 1 year; first reproduction) or adult (>1 year) based on plumage characteristics (Boyd et al. 1975). To index body size, we measured tarsus length (nearest 0.01 mm; Byers and Cary 1991), head+bill length (nearest 0.1 mm) and wing length (maximum wing chord, nearest 1 mm; Baker 1993). A digital balance was used to measure body mass (nearest 1 g). Blood samples (<1 ml, 2% of the circulating blood volume) were collected from the brachial vein for detection of antibodies to AIV. Blood was allowed to clot for approximately 6 h before centrifugation to separate serum from red blood cells (Hoye 2012). Ethanol (70%) was added to the red blood cells, and together with the sera samples stored at -20 °C until analysis.

Per clutch, two randomly chosen eggs were collected to assess maternal AIV antibody concentration in egg yolk. Of each egg, the length (nearest 0.01 mm) and breadth (two measurements as eggs are frequently not circular; nearest 0.01 mm) were taken. Egg yolks were separated on the day of collection. The size of each embryo was measured with a ruler (nearest 0.01 mm) to account for potential age differences affecting yolk AIV antibody concentration (Kowalczyk et al. 1985). Egg yolk and embryos were frozen at -20 °C until analysis.

Captive study

In the same period as the field study, we conducted a study with 16 adult female and 10 adult male mallards kept in captivity in an outdoor aviary at the Netherlands Institute of Ecology (NIOO-KNAW) in Heteren (51°57'26"N, 5°44'33"E), the Netherlands. All birds were captive bred and either originated from a waterfowl breeder (n = 16; P. Kooy & Sons, 't Zand, the Netherlands) or were bred at the NIOO-KNAW (n = 10). All birds had been kept in the outdoor aviary for at least a year prior to the study. The females were individually marked with colour rings to allow visual recognition.

The outdoor aviary was divided in five compartments: one large compartment (15 x 13 m) and four smaller compartments (6 x 13 m). In the large compartment, six females and three males were housed. The smaller compartments contained: two females and two males, three females and one male, three females and two males. Males were assigned to females according to pairs that had already formed before the start of the study. Each compartment was connected to a pond (34 x 1.5 m), with continuous flowing water for bathing and drinking. The outdoor aviary was surrounded by anti-bird nets and vermin proof mesh wire to prevent (egg) predation. To lower the chance that eggs were laid in a foreign nest, a surplus of nest boxes were provided in each compartment. Birds had access to shelter in the form of tall vegetation surrounding the aviary. Food was provided *ad libitum* and consisted of a mixture of commercial food pellets and seed-based mixed grains.

During egg laying, blood samples were collected from the brachial vein of females to measure concentrations of AIV antibodies. Analogous to the field study, serum was separated from red blood cells, and stored at -20 °C until analysis. Female body mass, tarsus and head+bill lengths were measured (wing length was not scored as primary feathers were clipped to prevent flying). Once females started laying eggs, freshly laid eggs were numbered with a nontoxic pen referring to the position within the laying order. Per clutch, we collected four eggs (one fresh egg and three eggs during incubation) to assess a potential change in yolk AIV antibody concentration during the course of incubation. At the day of collection, egg length and breadth measurements were taken, egg yolks separated, embryos measured and samples frozen at -20 °C until analysis.

Antibody detection

The protocol of Mohammed et al. (1986) was followed to prepare egg yolk samples. Once thawed, 0.93 g of egg yolk was diluted 1:1 in phosphate-buffered saline and homogenized using a vortex shaker. Of the diluted egg yolk suspension, 0.9 ml was placed in a 2 ml tube and an equal volume of chloroform was added and vortexed 20-30 sec. The mixture was incubated at room temperature for 30 min, centrifuged at 4 ^{0}C at 17,949 x g (Eppendorf, Nijmegen, the Netherlands) for 10 min, and the clear supernatant was used in the immunoassay. Of four eggs collected in the field, we were unable to collect sufficient yolk as the embryos were too large and had absorbed most of the yolk.

The presence of antibodies to the highly conserved nucleoprotein of AIV in female serum and egg yolk 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 (i.e. OD-value) was measured at 620 nm using an infinite M200 plate reader (Tecan Group Ltd, Männedorf, Switzerland). Female serum and egg yolk samples were considered AIV antibody positive if the signal to-noise ratio (i.e. mean OD-value of the sample divided by the mean OD-value of the negative control) was <0.5.

To validate the use of OD-values as a quantitative estimate of antibody concentration, we applied a serial dilution of 10 (randomly selected) AIV antibody positive egg yolks and female sera from the field study. On two bELISA plates, the AIV antibody positive yolks and sera were diluted 1/3, 1/10, 1/30, and 1/100, together with positive and negative controls for each dilution except 1/3. Samples were tested in duplicate and the OD-value measured. Dilution and OD-value were log10-transformed before tested in a linear mixed model (LMM), with individual sample as random factor. There was a strong correlation between the dilution and the OD-value of egg yolk (X^2 = 86.79, P < 0.001; linear model: y = -0.55x + 1.07, r^2 = 0.73) and female sera (X^2 = 68.22, P < 0.001; linear model: y = -0.47x + 1.23, r^2 = 0.78; Appendix 6.1 Fig. S6.1). This linear relation suggests that OD-values of yolk and sera can indeed be used as a relative measure of AIV antibody concentration (hereafter called AIV antibody concentration), with lower OD-values reflecting a higher relative AIV antibody concentration.

Intra-plate repeatability of the ELISA was assessed by calculating the coefficient of variation (CV%) of the OD-values of 50 replicates of positive and negative control samples, which was respectively 10.4% and 3.8%. Inter-plate reproducibility of the ELISA was evaluated using the CV of the OD-values of positive and negative controls on 62 different plates, which was respectively 10.9% and 4.3%. CVs <20% for raw OD-values indicate adequate repeatability of the assay (Jacobson 1998).

Maternity analysis

Given the provisioning of sufficient nest boxes and space, continued monitoring and the observation that no clutches exceeded 13 eggs, we were confident that the females that laid and incubated the eggs in the captive study were the biological females. In the wild, intraspecific nest parasitism, whereby females lay eggs in nests of other conspecifics, is very common among *Anseriformes* (Geffen and Yom-Tov 2001). In free-living mallards, 24% of clutches may contain parasitic eggs (Kreisinger et al. 2010). Therefore, maternity was assessed for all eggs collected in the field, minus the eggs that did not contain an embryo (n = 3) or had insufficient yolk (n = 4), i.e. 127 eggs in 67 clutches.

DNA was extracted from female red blood cells and embryonic tissue using a Gentra Puregene Kit (Qiagen, Venlo, the Netherlands). A small amount of blood or tissue was transferred into a 1.5 ml tube containing 1000 µl of Cell Lysis Solution and 10 µl Puregene

Proteinase K solution. After overnight incubation at 55 °C, the remainder of the extraction protocol was completed according to the manufacturer's instructions. At the final step DNA was dissolved in 100 µl DNA Hydration Solution (Qiagen, Venlo, the Netherlands).

Maternity was assigned using eight polymorphic microsatellite markers for mallards (Maak et al. 2003: APH 17, Denk et al. 2004: APL 2, APL 11, APL 12, APL 14, APL 23, APL 26, APL 36). PCR was performed in a 10 µl reaction mixture containing 5 µl Multiplex PCR mix (Qiagen, Venlo, the Netherlands), 40 ng DNA and 4 labelled primers. After amplification PCR products were analysed using an ABI 3130 automated capillary sequencer with a molecular size standard (Life Technologies, Bleiswijk, the Netherlands). Sizes of the amplification products were determined using commercial software (GeneMapper 4.0; Life Technologies, Bleiswijk, the Netherlands). Using CERVUS 3.0, the combined non-exclusionary probability of the first parent of the given set of markers was 0.99948, and of the second parent 0.99999. Matching females with offspring was based on maximum likelihood, as implemented in CERVUS 3.0 (Kalinowski et al. 2007). To estimate the 95% confidence interval for the differences in log-likelihood scores between the genetic and second-most likely mothers, based on known maternal genotype, a simulation (10,000 cycles) was performed using the known distribution of allele frequencies. The proportion of candidate mother samples was 98%. Maternity was assigned when the most likely mother matched the young at least at seven loci.

Embryo sexing

The sex of embryos was determined for eggs collected in the field. Embryo gender was identified using the W chromosome-linked CHD-1 gene. Primers P2 and P8 were used following Griffiths et al. (1998) and PCR amplifications was carried out in a total volume of 10 µl, containing 5 µl PCR-Mix (Promega, Leiden, the Netherlands), with an extra 0.2 µl $MgCl_2$ (25mM) and 40 ng genomic DNA. PCR was performed on a PTC200 (Biorad, Veenendaal, the Netherlands). PCR product was digested with 1 µl EcoRI restriction endonuclease (20 U/µl) in a total volume of 10 µl (Boutette et al. 2002). The mixture was incubated at 37 °C for 1.5 h, and 2 µl of loading buffer was added to each sample. PCR products were separated by electrophoresis for 120 min at 40 mA in a 2% agarose gel and stained with ethidium bromide.

Statistical analysis

The field dataset contained 64 biological females (of three clutches the female caught on the nest was not the biological mother) and their (non-parasitic) eggs (n = 115). Of 13 clutches only one egg was included in the analysis, as the other eggs contained no embryo

(n = 3), had insufficient yolk (n = 4) or the female caught on the nest was not the biological mother (n = 6). The dataset of the captive study contained eight egg laying females, where one female produced two clutches. A total of 33 eggs were collected. Of three clutches, one egg during incubation was missing.

As an index of body size, we used the first principal component (PC1) of a PC analysis of the biometric measurements. For the field study, PC1 explained 60% of the variance across tarsus, head+bill and wing lengths. Only tarsus and head+bill lengths were used in the PC analysis of the captive study, PC1 explaining 79% of the variance. To assess egg size, the volume (mm^3) was calculated following Hoyt (1979): 0.000515 x L x B_1 x B_2 (L: length, B_1, B_2: breadth).

All factors were log10-transformed to meet the assumption of normality. Collinearity between the various factors for the field and captive study were tested using Pearson correlation (r). In the field study, body mass and female AIV antibody concentration (r = -0.38, P < 0.001, r^2 = 0.14), and body mass and egg volume were correlated (r = 0.41, P < 0.001, r^2 = 0.16; Appendix 6.2 Table S6.1). All three variables were retained in the model to test the variance in yolk AIV antibody concentration, because of their relatively weak correlation and to allow comparison with the captive study results (where no collinearity existed between these three factors). There was no correlation between egg laying order and embryo size in the captive study (r = -0.21, t = -1.21, P = 0.237, r^2 = 0.01).

For both the field and the captive study, we used LMMs to test the association between yolk AIV antibody concentration and female body mass, female AIV antibody concentration, egg volume and all two-way interactions, with clutch as random factor. Additionally, embryo sex was included as an explanatory variable for the field study, whereas egg laying order was included in the model for the captive study. Female size and embryo size were included in the models as covariates, respectively to adjust body mass for structural size and to account for age differences potentially affecting yolk AIV antibody concentration. Female age was excluded from the analysis, since most of the females were adults (adult: 86%, juvenile: 3%, unknown: 11%). Full factorial models were tested and model selection was used to assess the better models on the basis of Akaike Information Criterion (AIC) corrected for small sample sizes (AICc; Burnham and Anderson 2002). The better models were defined as those models with the least number of parameter within a ΔAIC_c <2 relative to the best-supported model (i.e. the model with the lowest AIC_c). After model selection, the models without the interactions were defined as the better models (for model selection see Appendix 6.3 Table S6.2). The significance level (α) equalled 0.05. All analyses were conducted using R 2.14.1 (R Development Core Team 2012), where package lme4 was used to fit the LMMs (Bates et al. 2012).

Results

Field study

We identified 12 parasitic eggs out of 127 (9%) in nine out of 67 clutches (13%), with both eggs parasitic in three clutches. Prevalence of AIV antibodies in breeding females was 48% (31 out of 64), with 43% (50 out of 115) of the eggs receiving maternal AIV antibodies. Of all investigated factors, only female AIV antibody concentration was correlated with AIV antibody concentration in egg yolk (X^2 = 30.43, P < 0.001): high antibody concentrations in females corresponded with high concentrations in eggs (Fig. 6.1a). Female body mass, corrected for size (X^2 = 0.18, P = 0.670), was not associated with yolk AIV antibody concentration (X^2 = 0.01, P = 0.943). Also egg volume and embryo sex were not correlated with concentrations of AIV antibodies in egg yolk (respectively, X^2 = 0.07, P = 0.789 and X^2 = 1.35, P = 0.246). There was no effect of embryo size on yolk AIV antibody concentration (X^2 = 0.15, P = 0.700).

Captive study

AIV antibody prevalence in breeding females was 56% (5 out of 9) and in eggs 24% (8 out of 33). Similar to the field study, AIV antibody concentrations in egg yolk were positively correlated with female AIV antibody concentrations (X^2 = 16.65, P < 0.001, Fig. 6.1b).

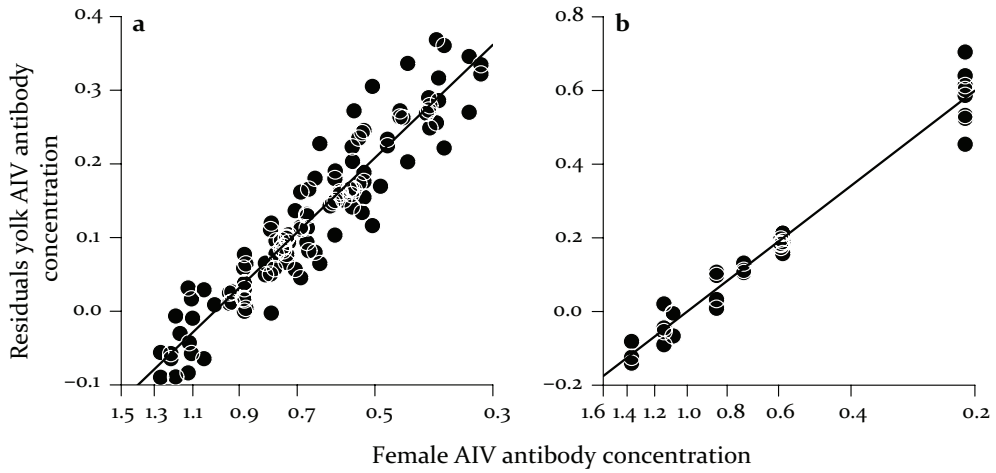

Figure 6.1 Association between the relative AIV antibody concentration in egg yolk and female serum from (a) the field study and (b) the captive study. The y-axis shows the partial residuals of yolk AIV antibody concentration in which the other factors used in the LMMs are also included. Note: low values reflect high relative AIV concentration; x-axes are log-scaled.

Egg laying order was correlated with yolk AIV antibody concentration (X^2 = 8.14, P = 0.004): eggs that were laid later in the laying sequence had higher antibody concentrations than first laid eggs (Fig. 6.2). Female body mass was not associated with AIV antibody concentration in egg yolk (X^2 = 1.23, P = 0.267), with no effect of female size (X^2 = 0.60, P = 0.439). There was also no correlation between egg volume and yolk AIV antibody concentration (X^2 = 1.22, P = 0.270), nor an effect of embryo size (X^2 = 2.08, P = 0.149).

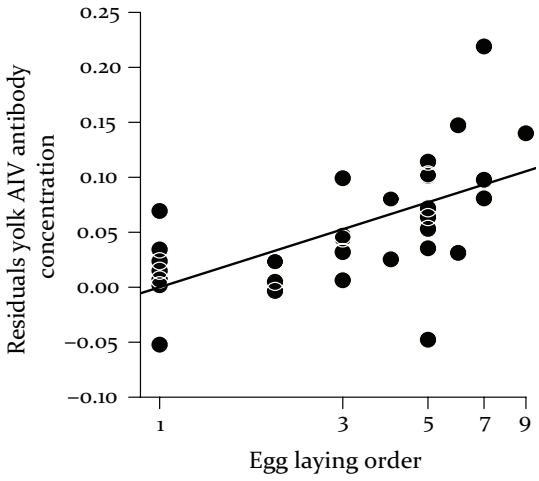

Figure 6.2 Association between the relative AIV antibody concentration in egg yolk and egg laying order from the captive study. The y-axis shows the partial residuals of yolk AIV antibody concentration in which the other factors used in the LMM are also included. Note: low values reflect high relative AIV antibody concentration; x-axis is log-scaled.

Discussion

In free-living mallards, nearly half of the eggs received maternal AIV antibodies. This suggests that a large proportion of newly hatched mallard chicks were protected against LPAIV during their first weeks by means of maternal AIV antibodies. In mallard chicks, maternal immunoglobulin IgY decreases after five days post-hatch, reaching minimum levels at about 14 days of age (Liu and Higgins 1990). The protective effects may also be longer than a few weeks due to the long-term effects maternal antibodies may have on offspring immunity (Hasselquist and Nilsson 2009). If a large proportion of mallard chicks receive maternal AIV antibodies, this likely reduces the number of susceptible individuals in the population, potentially affecting the dynamics of LPAIV infections in mallards. In the same year, LPAIV infection was measured in the mallard population under study, showing that 5% of the birds were infected in summer (van Dijk et al. 2014b). This peak might have been considerably higher, and could potentially have started already in spring when the first mallard chicks hatch, without this significant transfer of AIV antibodies from mothers to their otherwise immunologically naïve offspring. Whether these maternal AIV antibodies have prolonged

protective effects for juveniles and may influence LPAIV prevalence also in other seasons, for instance in the peak infection season autumn, is unclear.

Only a quarter of the mallard eggs received maternal AIV antibodies in the captive study. This may be considered surprising, since females in captivity have unlimited access to food and thus resources to support immune function. However, previous studies not only found positive effects of feeding conditions on maternal antibody transfer (Karell et al. 2008), but also negative effects, similar to our findings (Gasparini et al. 2007).

As hypothesized, the circulating AIV antibody concentrations in females correlated with the AIV antibody concentrations in egg yolk in both the field and the captive study. Our results are in accordance with earlier findings of maternal AIV antibody transfer in free-living gulls (Hammouda et al. 2012). An experimental study in breeder ducks also showed that AIV antibody titres in egg yolk and serum were highly correlated (Jeong et al. 2010). Without the captive study, it would have been difficult to assess whether concentrations of AIV antibodies in eggs from the field study were explained by the females' AIV antibody levels or their body mass, since circulating AIV antibody concentrations were positively correlated with body mass in free-living females. Even though we cannot completely rule out the role of female body condition, AIV antibody concentrations in mallard eggs were best explained by the circulating AIV antibody levels in females. Also in female gulls no correlation was found between their body mass and AIV antibody levels in their eggs (Hammouda et al. 2012). Yet, some other avian studies did find a positive correlation between the two (Pihlaja et al. 2006, Moreno et al. 2008). Transferring high levels of maternal antibodies to offspring likely increases their period of protection against pathogen infection (Al-Natour et al. 2004). Poultry chicks with high maternal antibody titres against chicken anaemia agent maintained antibodies one to three weeks longer compared to chicks with low maternal antibody titres (Otaki et al. 1992). Besides a protective role, maternal AIV antibodies may also affect the development of a chick's own immune system by blocking the immune response when chicks are exposed to pathogens (Boulinier and Staszewski 2008). Garnier et al. (2012) showed that three week old Cory's shearwater (*Calonectris diomedea*) chicks with maternal antibodies against Newcastle disease virus (NDV) did not increase their antibody levels when vaccinated with NDV, whereas chicks without maternal NDV-antibodies did.

Contrary to our prediction, we found a positive correlation between egg laying order and maternal AIV antibody concentration in our captive study. An increase in maternal antibodies over the laying sequence likely functions as a maternal tool to mitigate the negative consequences for the last-hatched chick(s) (Schwabl 1993). On the other hand, females may also enhance the levels of maternal antibodies in first-laid eggs to increase the

competitive disparity among siblings when food availability is insufficient to rear the entire brood (Schwabl et al. 1997). Within-clutch variation in maternal antibodies can be a flexible mechanism by which females can influence sibling competition. This functional interpretation is, however, strongly related to asynchronous hatching (Groothuis et al. 2005). In mallards, hatching of chicks is synchronous (Cramp and Simmons 1977), which likely reduces the need to deposit more maternal antibodies in last-laid eggs to increase chick survival. On the other hand, it would also be advantageous to increase the protective effects of last-laid chicks by means of maternal antibodies. Since these mallard chicks have reduced lipid reserves, due to investment in higher metabolic rates to increase embryo development and completion of the hatching process (MacCluskie et al. 1997), which may lower their body mass and influence survival (Rhymer 1988).

As hypothesized, we found no correlation between yolk maternal AIV antibody concentration and egg size in mallards. A similar non-significant correlation between these two is found in other bird studies (Hargitai et al. 2006). Grindstaff et al. (2005) showed that female Japanese quail (*Coturnix japonica*) that were fed low protein diets produced smaller eggs, but maternal antibody concentration in egg yolk was not affected. In contrast to our prediction, eggs with female embryos did not receive higher concentrations of maternal AIV antibodies than males. Despite lower survival in females (Gunnarsson et al. 2008), mallards seemingly do not favour a particular offspring sex by allocating higher concentrations of maternal AIV antibodies. A potential explanation could be that with each clutch mallards produce many offspring of both sexes as they lay large clutches (average clutch size 9-13 eggs; Cramp and Simmons 1977). In contrast to bird species that produce clutches half the size of that of mallards, and thus produce fewer offspring of each sex per clutch, making it profitable to allocate higher concentrations of maternal antibodies to a particular offspring sex in favour (Saino et al. 2003, Martyka et al. 2011). In both field and captive study, embryo size had no effect on the AIV antibody concentration in egg yolk. This indicates that the concentration of AIV antibodies in egg yolk does not change during incubation, which is similar as is found with IgY levels in chicken eggs (Kowalczyk et al. 1985).

In conclusion, by investigating transfer of maternal AIV antibodies in mallards as a key LPAIV host species, we demonstrated that in free-living mallards nearly half of the eggs received maternal AIV antibodies. Concentrations of maternal AIV antibodies in mallard eggs are positively correlated with circulating AIV antibody concentrations in females and laying order. With this study, we highlight the importance of studying maternal AIV antibody transfer in wild birds, which may play an important role in shaping LPAIV infection dynamics in host populations.

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7 Weak negative association between avian influenza virus infection and movement behaviour in a key host species

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Animal movement assists in the global spread of pathogens. Also in the case of some highly pathogenic avian influenza virus (HPAIV) strains (e.g. H5N1), and their low pathogenic (LPAIV) precursors circulating naturally in wild birds, (migratory) birds have been suggested to play a role in their spread. Recently with the H7N9 outbreak in China, which is caused by a LPAIV, the interest in the role of wild birds in spreading LPAIVs has intensified. For a better understanding of the emergence and spread of both HPAIV and LPAIV, the potential effects of LPAIVs on bird movement need to be evaluated. In a key host species, the mallard (*Anas platyrhynchos*), we compared the movement of LPAIV infected and non-infected individuals at both the (i) within and (ii) among individual level. Throughout the autumn LPAIV infection peak, we sampled 80 free-living adult males for current LPAIV infection and fitted them with GPS loggers. We distinguished between daily local movement (<100 m, excluding flights) and daily regional movement (>100 m). Within individuals, we found no association between LPAIV infection and daily local and regional movements. Among individuals, daily regional movements of LPAIV infected mallards were lower than those of non-infected birds. Moreover, these movements of LPAIV infected birds became increasingly lower when weather deteriorated (i.e. increased wind and/or precipitation, and lower temperatures). There was no association between LPAIV infection and daily local movements among individuals. Our study demonstrates that the dispersal of LPAIV, including LPAIV H7N9, which is causing high fatality rates in humans, may thus be lower on a regional scale than expected on the basis of the movement behaviour of non-infected birds. Our study underlines the importance of understanding the impact of pathogen infection on host movement in order to assess its potential role in the emergence and spread of infectious diseases.

Submitted manuscript

Introduction

Animal movements are an essential component of ecology, of critical importance for species survival and the provisioning of ecosystem functions (Bauer and Hoye 2014, Green and Elmberg 2014), including their assistance in the spread of pathogens (Altizer et al. 2011). The dispersal of pathogens by their animal host is a particularly interesting case, where pathogen impact on host movement behaviour is of critical importance. Some studies have shown that pathogens may reduce their hosts' capacity for locomotion (Bradley and Altizer 2005, Fellous et al. 2011). Pathogens may also reduce a host's food intake and therewith body condition (Delahay et al. 1995), which may in turn reduce the time spent in locomotion (Yorinks and Atkinson 2000). Hence, when studying the role of animal movement in the transmission of pathogens, it is vital to include potential effects of infection on the host's locomotion ability, since this may affect the local and global spread of pathogens.

In some cases, animal movements are associated with the transport of pathogens that can form a serious threat to animal and public health and the economy. Low pathogenic avian influenza virus (LPAIV) circulates globally in waterfowl populations. LPAIV subtypes H5 and H7 may become highly pathogenic (HPAIV) after spill-over into poultry (Alexander 2007). In high-density poultry areas, HPAIV may spread rapidly among poultry with massive damage to animal health and welfare, and the economy. In addition, HPAIV can also be of great concerns to public health. Since its emergence in 1996, HPAIV H5N1 has led to the culling of hundreds of millions of poultry and also resulted in 386 human deaths (24 January 2014; World Health Organization 2014a). Other HPAIV strains of concern have emerged since (e.g. H5N8 in South Korea; Lee et al. 2014). Several studies have correlated migration routes of wild waterfowl to HPAIV H5N1 outbreak events (e.g. Prosser et al. 2009, Takekawa et al. 2013). The potential relation between waterfowl migratory movements and HPAIV outbreaks has even led to propositions to cull all wild birds to stop the disease of getting transmitted (Yong et al. 2013). To accurately assess the risk of virus spread, information on the movement behaviour of infected wild host species is essential.

HPAIV, such as some H5N1 strains, are shown to occasionally spread into the wild bird reservoir, likely as a consequence of spill-over from infected poultry. Several orders of waterfowl species are susceptible to HPAIV H5N1 infection with high mortality, such as swans, diving ducks, mergansers and grebes (Hesterberg et al. 2009). Yet, other waterfowl species have rarely been reported to be affected with HPAIV H5N1, such as mallard ducks (*Anas platyrhynchos*) (Hesterberg et al. 2009). Experimental infection studies of HPAIV H5N1 in ducks showed that mallards excreted virus abundantly without clinical or pathologic signs of disease, suggesting mallards could act as potential vectors of HPAIV (Keawcharoen et al. 2008). Still, there is no evidence that mallards or other waterfowl are

indeed transporting HPAIV around the globe, even though they are occasionally infected. Waterfowl are also thought to transport the precursor of HPAIV, namely LPAIV, and they are claimed to do this asymptomatically over large distances, since infection causes only mild disease (Kuiken 2013). Just recently, the importance of studying movement behaviour of wild waterfowl infected with LPAIV became even more important with the emergence of LPAIV H7N9 in poultry in China. LPAIV H7N9 has a high fatality rate in humans despite being low pathogenic to poultry and other birds (Kreijtz et al. 2013). Since its emergence in February 2013, the H7N9 virus has led to more than hundred human deaths (World Health Organization 2014b). Due to the lack of observable clinical symptoms in poultry and wild waterfowl, LPAIV H7N9 is able to spread silently. Thus, a better understanding of effects of LPAIV infection on waterfowl movements is vital to incorporate in predictive models on the emergence and spread of both LPAIV and HPAIV.

Globally, birds of wetlands and aquatic environments (order *Anseriformes* and *Charadriiformes*) are often infected with LPAIV (Webster et al. 1992). LPAIV infection in waterfowl, and notably dabbling ducks, causes minor differences in body mass between infected and non-infected birds, with weak associations between infection and immune status (J.G.B. van Dijk unpublished data, Latorre-Margalef et al. 2009, Kleijn et al. 2010). Model studies aimed at estimating potential LPAIV virus spread by waterfowl are based on the assumption that birds' movements are not impaired by the infection (Lebarbenchon et al. 2009). Few studies investigated potential effects of LPAIV infection on waterfowl movements. A study in migratory Bewick's swans (*Cygnus columbianus bewickii*) fitted with GPS neck-collars showed that birds naturally infected with LPAIV were feeding at reduced rates and left one month later for spring migration (van Gils et al. 2007). A follow-up study in the same species using a much larger sample size yielded inconclusive results (Hoye 2011). Latorre-Margalef et al. (2009) using banding recoveries, found no effects of LPAIV infection on migration speed and distance of mallards. However, the spatiotemporal resolution of these studies is coarse, in part because they focus on rare long-distance movements.

LPAIV is transmitted primarily by the faecal-oral route, with virus particles in faeces shed into the surface water and being ingested with water during dabbling (Webster et al. 1992). LPAIV infections last approximately a week in wild waterfowl (Latorre-Margalef et al. 2009), providing opportunity for birds that undertake daily regional flights to spread the virus in the surrounding area, putting conspecifics and other bird species at risk of infection. In addition to migratory movements, regional waterfowl movements are therefore likely to play a role in the transmission of LPAIV to other wild birds and the potential introduction of LPAIV into poultry. The aim of our study was to investigate associations between LPAIV infection and the movement behaviour of wild birds. To this end, we recorded daily

local and regional movement patterns, at high temporal and spatial resolution using GPS loggers in LPAIV infected and non-infected individuals of a key LPAIV host species, the mallard, throughout the autumn infection peak.

Materials and methods

Study species and site

Mallards are one of the most common and numerous waterfowl species in the world (estimated population 19 million individuals; Delany and Scott 2006). Together with other dabbling ducks of the *Anas* genus, mallards are frequently infected with LPAIV and harbour most virus subtypes found in birds to date (Olsen et al. 2006). In the northern hemisphere, a dominant LPAIV infection peak in mallards occurs in autumn, with virus prevalence decreasing in winter, remaining low throughout spring and showing a minor infection peak in summer (Latorre-Margalef et al. 2014, van Dijk et al. 2014b). The autumn peak is likely driven by aggregation of birds, importantly involving susceptible migrants, while the summer peak coincides with the entrance of immunologically naïve juveniles into the population (van Dijk et al. 2014b). In north-western Europe the mallard population is estimated at approximately 5 million birds, consisting of migratory and resident birds. Migrants breed in northern Europe (i.e. Finland, Sweden, the Baltic, north-west Russia), and migrate in autumn to winter from Denmark to France and Britain. On the wintering grounds, migratory mallards congregate with residents that breed in western Europe (e.g. Netherlands) (Scott and Rose 1996). Regional movement behaviour of mallards on the wintering grounds is characterized by daily flights between roosts and foraging sites at distances of up to tens of kilometres, mostly at sunset and sunrise. However, short flights during the day or night are also observed (E. Kleyheeg unpublished data, Sauter et al. 2012).

Our study site was a duck decoy located near Oud Alblas (51°52′38″N, 4°43′26″E) in the Alblasserwaard, the Netherlands. A duck decoy is a landscape structure designed to capture free-living ducks with the use of swim-in traps that are connected to a large pond. In former times, ducks were caught for consumption, but nowadays only few duck decoys maintain this function and more duck decoys are used for scientific research. A pilot study at this location in March 2012, in which 20 mallards were equipped with similar GPS loggers, revealed that the duck decoy was used as a roosting site during the day, with morning and evening flights to feeding areas in the surrounding.

Sampling

We captured 80 free-living mallards, and equipped them with GPS loggers, during the peak

of the infection period (autumn and winter) in 2012. Mallards were captured throughout the end of summer, autumn and winter (August: 7, September: 13, October: 40, December: 20) to assess whether a potential effect of LPAIV infection on the birds' movements changed with a change in weather conditions.

Plumage characteristics were used to determine the age and sex of birds. Previously, no difference was observed in LPAIV prevalence between male and female mallards (Munster et al. 2007). Only adult male mallards (>1 year old) were used in order to avoid age and sex-related variance in movement patterns. Another reason for using only males in our study was the relatively high chance of capturing pairs (most pairs are formed by end October; Cramp and Simmons 1977); it was expected that pairs would have similar movement patterns. Each bird was fitted with a ring and three biometric measurements were taken to assess bird size: tarsus length (nearest 0.01 mm), head+bill length (nearest 0.1 mm) and wing length (maximum wing chord, nearest 1 mm). Body mass was measured with a digital balance (nearest 1 g) to represent each bird's energetic condition. Blood samples (0.5-1.0 ml, <2% of the circulating blood volume) were collected from the brachial vein for detection of antibodies to avian influenza virus (AIV). Blood was allowed to clot for approximately 6 h before centrifugation to separate serum from red blood cells (Hoye 2012), and was stored at -20 °C until analysis. Sterile cotton applicators were used to swab both the cloaca and the oropharynx for detection of current LPAIV infection. LPAIV may replicate in both the intestinal and respiratory tract of wild birds (Fouchier and Munster 2009). Swabs were stored individually in transport medium (Hank's balanced salt solution with supplements; Munster et al. 2007), preserved at 4 °C and transported to Erasmus MC for analysis. Cloacal and oropharyngeal samples were also collected from birds when recaptured to remove the GPS logger and read-out the movement data.

GPS loggers

Each bird was equipped with a CatTrack I GPS logger (45 × 25 × 15 mm; Perthold Engineering, Anderson, South Carolina, USA), fitted to the bird as a backpack with a Teflon harness (Roshier and Asmus 2009). Birds were recaptured to read-out the data. The weight of the GPS logger together with the harness was approximately 30 g, which is around 3% of the body mass of male mallards. The GPS loggers were programmed to record a bird's position at 15 min intervals resulting in 96 fixes per day. Based on these 15 min intervals, battery life was expected to be at least 14 days. We measured spatial accuracy of the GPS loggers at three locations, in which three loggers per location were fixed to a pole. Based on 24.081 positions and after excluding obvious misreadings exceeding 100 m (0.6% of the positions), average logger inaccuracy was 10 m.

Movement parameters

Logged GPS positions were used to calculate movements of individual birds for each recorded 24 h period, measured from 12:00 noon until 12:00 noon the next day. This period was chosen in order to fully cover the period that birds were foraging at night. Noon tends to be a period of inactivity (i.e. rest). Only days that covered a full 24 h period were used in data analyses. Moreover, to avoid potential handling effects on movement patterns, GPS locations recorded until noon on the day following GPS deployment were excluded.

Five movement parameters were calculated: (i) daily local movements (m d^{-1}), (ii) daily number of flights (d^{-1}), (iii) daily flight distance (m d^{-1}), (iv) daily home range (km^2 d^{-1}) and (v) daily time away from the roost (i.e. the duck decoy; min d^{-1}). Before parameter estimation, we first removed clearly erroneous GPS fixes. These were identified as sudden large apparent displacements (i.e. 100 m) followed by an immediate return to the original position. Local movements were considered to be within-patch movements of less than 100 m between two positions (readings 15 min apart). We defined *daily local movements* as the cumulative distance without flights covered within 24 h. Displacements were considered to involve flight if these exceeded 100 m between two positions. Per 24 h recording period the *daily number of flights* was the total number of such >100 m displacements, while *daily flight distance* was defined as the total accumulated distance birds covered during these events. *Daily home range* was likewise calculated over the 24 h recording period and based on the concept of utilization distribution (van Winkle 1975), in which an animal's range is described by a bivariate probability density function of location over a period of time (i.e. the probability of finding an animal in a defined area within its home range with a confidence region set at 90%). A bird's utilization distribution was calculated with the kernelUD function of the R package adehabitatHR (Calenge 2006). *Daily time away from the roost* was defined as the period that birds were >50 m from the duck decoy for at least two consecutive positions over the 24 h recording period.

Weather conditions

We included weather conditions in our study to account for potential weather effects on mallard movements (Sauter et al. 2012). We used hourly measurements of average wind speed (m/s) and temperature (°C), and sums of precipitation (mm) collected by the Royal Netherlands Meteorological Institute at Cabauw (51°57'55"N, 4°53'52"E), situated 16 km from the duck decoy. For each 24 h period, we calculated mean wind speed, mean temperature and total precipitation to serve as covariates in the analyses. For mean monthly averages of these weather parameters, see Appendix 7.1 Fig. S7.1.

Virus and antibody detection

Cloacal and oropharyngeal swabs were used to detect influenza A virus. For full details on RNA isolation and virus detection, see Munster et al. (2007). In short, a Magna Pure LC Total nucleic acid isolation kit with a Magna Pure LC system (Roche Diagnostics, Almere, the Netherlands) was used to isolate RNA. Influenza A virus was detected using a generic real-time reverse transcriptase PCR assay targeting the matrix gene. Birds were considered LPAIV positive when either cloacal or oropharyngeal samples were positive.

Serum AIV-specific antibodies (i.e. antibodies that bind to the highly conserved nucleoprotein of AIV) were measured 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 two positive and negative controls per plate, and the absorbance was measured at 620 nm using an infinite M200 plate reader (Tecan Group Ltd, Männedorf, Switzerland). Samples were considered AIV antibody positive, if the signal to-noise ratio (i.e. the absorbance of the samples divided by the mean absorbance of the negative control) was <0.5.

Statistical analysis

Birds that were infected with LPAIV on the day of GPS logger deployment (i.e. day 0) were assumed to be infected during day 0 to 3. This assumption was based on the fact that after LPAIV infection, approximately 90% of all LPAIV particles are shed via the cloaca and beak within 3 to 4 days (Hénaux and Samuel 2011). LPAIV infection lasts a bit over a week in free-living mallards (Latorre-Margalef et al. 2009). Similarly, birds infected at the day of GPS logger removal were assumed infected during the last four days of tracking. Non-infected individuals at day 0 were assumed to stay uninfected three days after logger deployment and prior to logger removal.

We tested whether LPAIV infection was associated with lower movement patterns within and among birds. For the analysis within individuals, we selected only those individuals of which LPAIV infection status was known both at day 0 and at the day of GPS logger removal (n = 22; for details see Appendix 7.2 Fig. S7.2). The recordings of the first three days after capture (day 1 to 3) and the last three days before removal of the logger were used in the analysis. For the analysis among birds, we used tracking data recorded (i) in the first three days after capture (n = 72) or (ii) in the last three days before removal of the GPS logger (n = 22; for details see Appendix 7.2 Fig. S7.3 and S7.4). Eight mallards were excluded from the analysis using recordings of the first three days after capture: four birds were not recaptured; of two birds no samples were collected; from one bird only the first two tracking

days were recorded; and of another bird the time spent away from the roost could not be calculated properly as there were no consecutive positions that the individual was >50 m from the roost. This was also the reason for disregarding day 1 of one individual bird from this analysis. Unfortunately, the number of individuals that could be used for the period preceding the day of logger removal was low, since in most recaptured individuals the batteries of the GPS loggers had run out of power in the days before logger removal.

The five movement parameters, body mass and the weather parameters were log10-transformed to meet the assumption of normality. Collinearity between the five movement parameters and the three weather parameters was tested using Pearson correlation (r). Daily number of flights, daily flight distance, daily home range and daily time away from the roost were highly correlated (Appendix 7.3 Table S7.1). There were no correlations between daily local movements and the other movement parameters, except home range (r = 0.14). However due to the low r^2 (0.02), daily local movements were retained in the models to test the variance in movement patterns in relation to LPAIV infection status. A principal component (PC) analysis was performed on the four highly correlated movement parameters to create a first PC (PC1) to use as an index of daily regional movements (PC1 explained 66% of the variance, PC2 only 16%, eigenvalue = 2.64). For the weather parameters, collinearity only existed between wind speed and precipitation (Appendix 7.3 Table S7.2). A PC analysis was performed on wind speed and precipitation and the PC1 was used as index for wind-precipitation (PC1 explained 81% of the variance, PC2 only 19%, eigenvalue = 1.62). As an index of bird body size the PC1 of a PC analysis of tarsus, head+bill and wing lengths is commonly used. The PC1 of body size explained 49% of the variance (PC2 only 30%, eigenvalue = 1.47). Correlations between the PC1s and parameters (i.e. factor loadings) are shown in Table S7.3 (Appendix 7.3).

To test the associations between LPAIV infection and daily local and regional movements within individuals, we used linear mixed models (LMMs), with LPAIV infection status as fixed factor, and individual bird as random factor. As covariates we included tracking day, tracking period (i.e. three days following GPS deployment or three days prior to logger removal), wind-precipitation and temperature. Furthermore, we included the interactions between infection status and wind-precipitation, and infection status and temperature to test whether movements of LPAIV infected and non-infected individuals were influenced by weather conditions. We also used LMMs to test associations between LPAIV infection and daily local and regional movements between birds. The LMMs included LPAIV status as fixed factor, and tracking day, wind-precipitation and temperature as covariates, and two-way interactions between infection status and wind-precipitation, and infection status and temperature. The LMM for the first three days also included antibody status,

body mass and bird size as covariates, and the interaction between infection status and antibody status, and infection status and body mass. Antibody status was included, since more than half of the LPAIV infected mallards (6 out of 11, 55%) had AIV antibodies. AIV antibodies in infected mallards reflect previous infection, whereas infected birds without AIV antibodies are likely naïve, and potentially there is a difference in daily movements between birds with previous infections and individuals that were immunologically naïve. AIV antibodies were not correlated with LPAIV infection (X^2 = 0.66, P = 0.417). In order to correct for differences in body condition among individuals, body mass, and bird size, to adjust body mass for the structural size, were included in the model. The interaction between infection status and body mass was included, since body mass may differ between LPAIV infected and non-infected birds (Latorre-Margalef et al. 2009). Antibody status and body mass were not included in the LMMs using tracking data recorded in the last three days, since these measurements were collected at day 0 and cannot be extrapolated to the last days.

All analyses were conducted using R 2.14.1 (R Development Core Team 2012). Package lme4 was used to fit LMMs and package languageR to generate output of LMMs.

Results

At the day of logger deployment (i.e. day 0), 15% (11 out of 72) of mallards were infected with LPAIV and 65% (47 out of 72) had antibodies against AIV (Appendix 7.4 Table S7.4). At the day of logger removal, 59% (13 out of 22) of mallards were infected with LPAIV, with 9% (2 out of 22) of these individuals infected at both logger deployment and removal (Appendix 7.4 Table S7.4).

Associations between infection and daily movements within individuals

There was no association between LPAIV infection and local and regional movements within individuals (Table 7.1). There was a significant interaction effect between LPAIV infection status and wind-precipitation on local movements (Table 7.1). When uninfected, local movements were not associated with wind-precipitation (P = 0.547), but when infected with LPAIV their local movements tended to decrease with an increase in wind-precipitation (P = 0.058). Temperature was not correlated with individual's movements (Table 7.1).

Table 7.1 Differences in daily local and regional movements within individuals, as tested by a LMM. Significant *P*-values are in bold.

	Local movements		Regional movements	
Variable	*t*-value	*P*-value	*t*-value	*P*-value
Infection status	-0.21	0.832	0.12	0.909
Tracking day	-0.26	0.797	-0.09	0.932
Tracking period	0.38	0.703	0.21	0.831
Wind-precipitation	0.28	0.778	-0.25	0.807
Temperature	-0.16	0.876	-1.33	0.187
Infection status*wind-precipitation	-2.15	**0.033**	-1.15	0.253
Infection status*temperature	1.21	0.229	0.52	0.604

Associations between infection and daily movements among individuals

Local movements were not associated with LPAIV infection (Table 7.2, Fig. 7.1a). Also regional movements recorded during the first days of tracking were not associated with LPAIV infection (Table 7.2). However, regional movements recorded over the last days of tracking were lower in LPAIV infected than in non-infected mallards (Table 7.2, Fig. 7.1b). When ignoring the other variables in the statistical model, in order to get a feeling of the potential effect of LPAIV infection on daily regional movements, the mean ± SE of LPAIV infected and non-infected mallards per regional movement parameter were respectively: number of flights: 3.4 d^{-1} (± 0.3) and 3.9 d^{-1} (± 0.4), flight distance: 1314 m d^{-1} (± 162) and 1842 m d^{-1} (± 209), home range: 0.16 km^2 d^{-1} (± 0.02) and 0.20 km^2 d^{-1} (± 0.03), time away from the roost: 430 min d^{-1} (± 45) and 602 min d^{-1} (± 44) (see Appendix 7.2 Fig. S7.4).

There were no correlations between weather and local movements of individuals (Table 7.2). However, regional movements recorded over the first three days of tracking were associated with temperature (Table 7.2): mallards were flying less and shorter distances, had smaller home ranges and spent less time away from the roost when temperatures were lower. There was an interaction between LPAIV infection status and both wind-precipitation and temperature for regional movements recorded the last days of tracking (Table 7.2). LPAIV infected birds were flying less and shorter distances, had smaller home ranges and spent less time away from the roost, when there was more wind and precipitation ($P = 0.022$; Fig. 7.2a), and temperatures were lower ($P < 0.001$; Fig. 7.3a). Regional movements of non-infected birds were not associated with wind-precipitation ($P = 0.286$; Fig. 7.2b) and temperature ($P = 0.665$; Fig. 7.3b). Antibody status and body mass were not associated with local and regional movements of birds (Table 7.2).

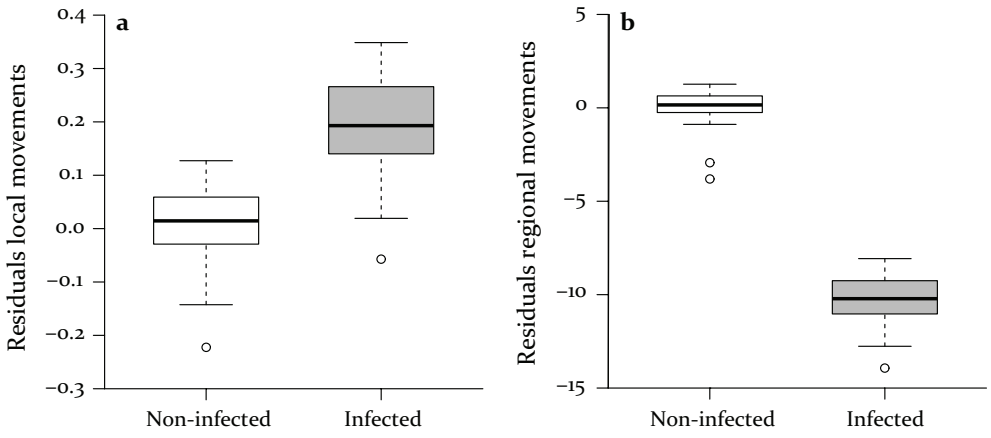

Figure 7.1 LPAIV infection status among individuals associated with (a) daily local movements (<100 m, excluding flights) and (b) daily regional movements (>100 m) in the last three days prior to logger removal. The y-axis shows the partial residuals of the local and regional movements in which the other fixed factors used in the LMMs are also included. Regional movements are positively correlated with number of flights, flight distance, home range and time away from the roost (Appendix 7.3 Table S7.3).

Table 7.2 Differences in daily local and regional movements among individuals, as tested by LMMs. Different tests were performed for the first three days and the last three days of logging. Significant *P*-values are in bold.

	Local movements				Regional movements			
	First three days		Last three days		First three days		Last three days	
Variable	*t*-value	*P*-value	*t*-value	*P*-value	*t*-value	*P*-value	*t*-value	*P*-value
Infection status	-0.07	0.944	1.61	0.112	-0.41	0.681	4.30	**<0.001**
Tracking day	1.54	0.125	-1.00	0.323	0.08	0.937	-0.51	0.613
Antibody	0.41	0.679			-0.18	0.855		
Body mass	0.02	0.987			-0.60	0.550		
Bird size	1.20	0.231			-0.96	0.337		
Wind-precipitation	0.73	0.468	0.19	0.852	0.39	0.695	-1.64	0.106
Temperature	-0.28	0.779	0.17	0.869	-4.43	**<0.001**	0.73	0.468
Infection status*antibody	-0.33	0.741			0.78	0.438		
Infection status*body mass	0.02	0.984			0.36	0.718		
Infection status*wind-precip.	-1.62	0.108	-0.14	0.893	-1.28	0.203	2.94	**0.005**
Infection status*temperature	1.13	0.259	-1.44	0.154	1.00	0.319	-3.95	**<0.001**

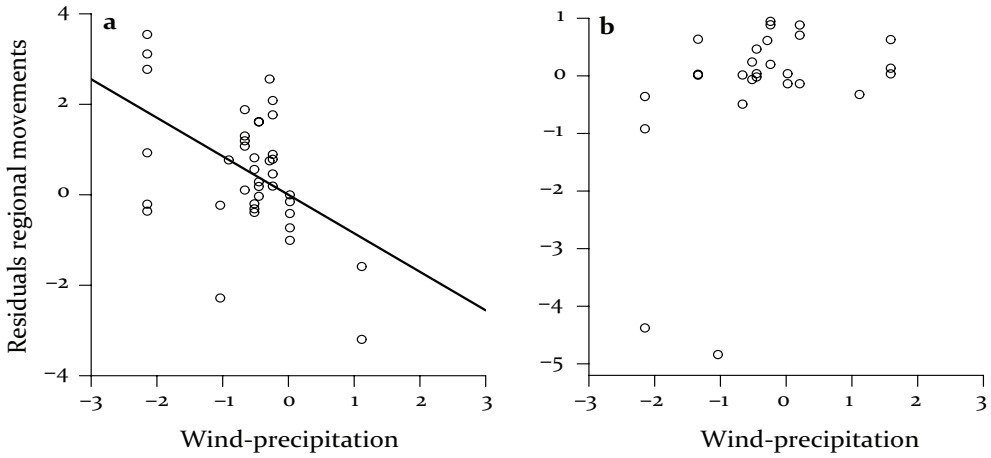

Figure 7.2 Daily regional movements (>100 m) recorded during the last three days of tracking between (a) LPAIV infected and (b) non-infected mallards and their relationship with wind-precipitation (note log-scale). The y-axes show the partial residuals of the regional movements in which the other fixed factors used in the LMM are also included. Regional movements are positively correlated with number of flights, flight distance, home range and time away from the roost. Wind-precipitation is positively correlated with wind speed and precipitation (Appendix 7.3 Table S7.3).

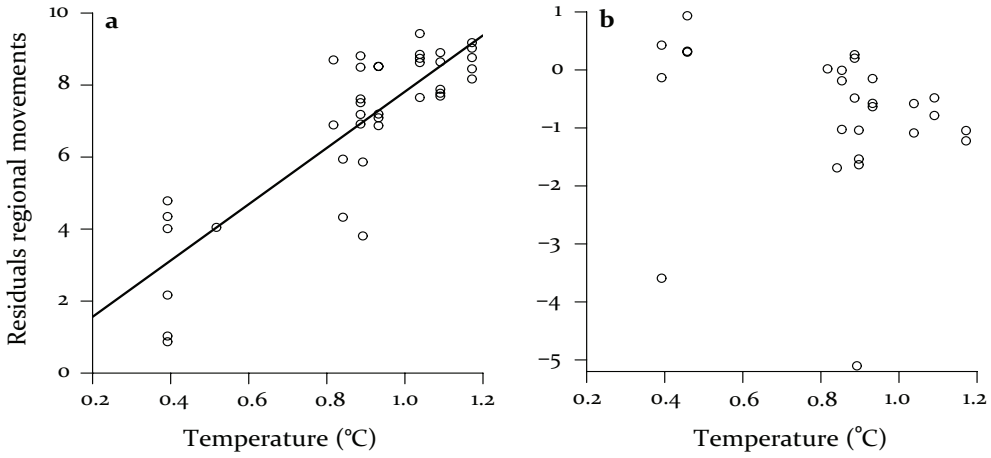

Figure 7.3 Daily regional movements (>100 m) recorded during the last three days of tracking between (a) LPAIV infected and (b) non-infected mallards and their relationship with temperature (note log-scale). The y-axes show the partial residuals of the regional movements in which the other fixed factors used in the LMM are also included. Regional movements are positively correlated with number of flights, flight distance, home range and time away from the roost (Appendix 7.3 Table S7.3).

Discussion

Daily regional movements (>100 m) of mallards were smaller in LPAIV infected individuals than non-infected individuals. Based on uncorrected values, LPAIV infected mallards were flying at least 10% less, had 20% smaller home ranges, and flight distance and time away from the roost (i.e. duck decoy) were almost 30% lower. This finding corresponds with the hampered pre-migratory movements found in Bewick's swans infected with the same virus, but in that study movements were based on resightings of individuals (i.e. displacement) (van Gils et al. 2007). Similar negative effects of pathogen infection on host movement using GPS loggers were found in other host-pathogen systems (Alasaad et al. 2013). For instance, bighorn sheep (*Ovis canadensis*) suffering from keratoconjunctivitis were moving less during the epizootic than non-infected animals (Jansen et al. 2007). Yet, many studies make inferences about the consequences of animal movements for disease transmission without knowing whether pathogen infection affects those movements (Wyckoff et al. 2009, Woodroffe and Donnelly 2011). This is also frequently the case in studies predicting HPAIV H5N1 dispersal extrapolating from GPS-tracking studies in non-infected individuals (Gaidet et al. 2010, Newman et al. 2012). Even though LPAIV infection was likely associated with mallard movements, infected individuals were probably still capable of transporting LPAIV to other areas: most LPAIV infected individuals still left the roosting site at night to fly to their foraging areas, making it plausible that they shed viral particles in the surface water when foraging, potentially infecting other birds.

Contrastingly, daily local movements (<100 m) of mallards were not negatively associated with LPAIV infection. Local movements were assumed to involve no flights, whereas regional movements did. Hence, a potential explanation for why we found an association between LPAIV infection and daily regional movements but not local movements could be the higher energetic (flight) costs of regional compared to local movements. Nudds and Bryant (2000) showed that particularly short flights in birds, also involving take-off, landing, ascent and descent, are extremely costly (nearly 28 times basal metabolic rate in their study object, the zebra finch, *Taenuipygia guttata*). Mounting and maintaining an immune response is believed to be energetically costly requiring substantial protein and nutrient supplies (Lochmiller and Deerenberg 2000). It is therefore possible that LPAIV infected birds were less capable of investing this extra energy in regional movements due to the costs of clearing the infection.

Weather was associated with the daily regional movements of LPAIV infected mallards that were recorded before logger removal. LPAIV infected mallards were flying less and shorter distances, had smaller home ranges and spent less time away from the roost when it was colder, windier and/or there was more rain. A similar trend was visible

in daily local movements when individuals were infected with LPAIV (within individual-analysis). Sauter et al. (2012) also showed that travelling distance of mallards decreased with an increase in wind speed and precipitation, but in our study the correlation was only shown in LPAIV infected mallards. There was no association between both wind speed and precipitation and regional movements of non-infected birds. Normally, ducks reduce feeding activity with more wind and precipitation (Paulus 1988), but increase foraging time when temperatures decrease (Sauter et al. 2010). Since foraging is likely related to regional movements, a decrease in foraging with more wind and rain coincides with the pattern found in LPAIV infected mallards. Possibly, the harsher weather conditions reduced foraging and negatively affected body condition of LPAIV infected individuals. Latorre-Margalef et al.(2009) showed that LPAIV infected mallards tend to have slightly less body stores than non-infected birds. Lower temperatures were negatively associated with daily regional movements of both LPAIV infected and non-infected birds recorded after logger deployment. Potentially, the negative effect of handling directly after deployment obscured any potential effect of cold temperature on regional movements of LPAIV infection of the kind found during the last days of tracking.

Differences in daily regional movements between LPAIV infected and non-infected mallards were only detected using the tracks immediately prior to GPS logger removal, and not after logger deployment. This may also have been caused by handling of the birds to fit the GPS loggers. We made an attempt to correct for such a handling effect by disregarding the GPS tracks recorded until noon on the day following GPS deployment. But possibly, this period was not long enough to account for the handling effect and for the birds to become accustomed to the logger. This handling effect could also be responsible for our failure to find differences in daily local and regional movements within individuals. Indeed, the movement differences we were able to detect between LPAIV infected and non-infected birds were anyway subtle. Its detection may therefore have been best guaranteed long after the birds were equipped with a GPS logger and had become fully accustomed to wearing it.

Overall, we found negative, although weak, associations between LPAIV infection and the movement behaviour in mallards. Together with findings of other studies that showed weak associations between LPAIV infection and mallard's body condition and immune status (J.G.B. van Dijk unpublished data, Latorre-Margalef et al. 2009), raises the question whether this is a consequence of a co-evolution between mallards and this pathogen. Hosts and pathogens may co-evolve (i.e. go through a process of reciprocal, adaptive genetic changes) if their relationship is intimate and strong selective pressures exist, acting on both the host and pathogen (Woolhouse et al. 2002). A co-evolution between mallards and LPAIV could likely explain the role of mallards as one of the key reservoirs of LPAIV.

In conclusion, by comparing movements of LPAIV infected and non-infected individuals of a key host species, we showed a negative association between LPAIV infection and daily regional movements of mallards, while there was no discernible association between infection and daily local movements. Particularly, LPAIV infected birds responded more strongly to adverse weather conditions, and flew less and over shorter distances, occupying a smaller home range when it was colder, more windy and/or rainy. Differences in daily regional movements were only found between LPAIV infected and non-infected individuals and could not be detected within individuals. Our study suggests that LPAIV infection may impair bird movements, although associations were small; however this does not fully hinder the potential transport of LPAIV by infected birds. These findings contribute to assessing the potential role birds play in the spread of LPAIV, as dispersal of LPAIV may be lower than what one would predict based on movements of non-infected subjects. With this study we highlight the importance of pathogen infection on animal movements, as a critical factor in assessing their potential role in the emergence and spread of pathogens.

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WETH VAN STRAFF

IV Synthesis

8 Synthesis: elucidating the ecological processes of avian influenza virus infection dynamics in wild birds

Avian influenza virus (AIV) is a zoonotic pathogen that originates from wild birds. Recently novel influenza viruses have been detected also in fruit bats that are distantly related to AIV (Tong et al. 2012, Tong et al. 2013), indicating that bats, alongside wild birds, harbour influenza viruses and might play a distinct role in the dynamics of this infectious disease. AIV in wild birds is generally of a low pathogenic phenotype (called low pathogenic avian influenza virus, LPAIV), and causes mild disease (Daoust et al. 2011, Höfle et al. 2012, Kuiken 2013). When LPAIV is introduced into poultry, it may mutate towards a highly pathogenic phenotype (called highly pathogenic avian influenza virus, HPAIV) which is lethal for poultry and may cause illness and occasional deaths in humans and wild birds (Alexander 2007). After the emergence of HPAIV H5N1 in Asia in 1996, many surveillance schemes were initiated around the globe to sample wild birds for LPAIV as an early warning system. Almost 15 years later, Hoye et al. (2010) critically reviewed these wild bird surveillance schemes concluding that in many aspects LPAIV research in wild birds was still in the exploratory phase. Wild birds were mostly sampled in an opportunistic way, by sampling a wide range of species at various locations throughout the year, often collecting insufficient samples from solely the cloaca (Hoye et al. 2010). To move forward, it was suggested to apply a multi-disciplinary approach in which scientists of various fields should work together to broaden our understanding of the ecology, epidemiology and evolution of LPAIV in wild birds. Since it was indeed the aim to address these issues in my studies, I took this advice to heart and worked together with virologists, ornithologists, (molecular) ecologists, immunologists and modellers to study the ecological processes underlying the epidemiology of LPAIVs in wild birds.

Sampling mallards at a local scale during an annual cycle

One of the first steps in any ecological study that investigates wildlife pathogens is to understand its dynamics in (key) host species. Our understanding of the dynamics of LPAIV infections in wild birds is still rather limited. For the seasonal and temporal regions of the northern hemisphere, our insights were largely based on compilations of separate studies in which multiple bird species were sampled at various locations in time (Stallknecht et al. 1990b, Krauss et al. 2004, Munster et al. 2007, Wallensten et al. 2007). Together these studies showed an annual LPAIV infection peak in late summer and early autumn, which was followed by low levels of infection during the winter period and a small increase during spring. In tropical regions and the southern hemisphere, LPAIV infection dynamics in wild birds are less well understood, but likely produce different infection patterns due to differences in host ecology, climate and seasonality (Klaassen et al. 2011, Gaidet et al. 2012). In order to describe LPAIV infection dynamics in wild birds, birds should ideally be sampled extensively for a prolonged period of time until a full cycle of LPAIV infections is covered. This idea was

the backbone of this study, and the reason why wild birds were comprehensively sampled for LPAIV for 17 consecutive months.

A single bird species (mallard, *Anas platyrhynchos*) was sampled at a local scale (a duck decoy) in the Netherlands in order to reduce (i) the variance between species in susceptibility to LPAIV infection and (ii) the spatial variance in geographical scale influencing apparent LPAIV infection dynamics. A thorough understanding of the spatial resolution of epidemic peaks is important in any host-pathogen system for instance to predict the magnitude and timing of peak incidence (Mills and Riley 2014). Variation in the spatial scale of pathogen dispersal, and thus the spatial scale of host-pathogen interactions, has likely consequences for disease dynamics and pathogen persistence (Real and McElhany 1996, Laine 2005). Thrall and Burdon (1999) suggested that disease persistence was highest when dispersal occurred at local scales resulting in more endemic disease dynamics. When dispersal occurred at larger spatial scales, disease persistence was lower with dynamics being more epidemic. Although a study on multiple spatial scales would have been favoured, it was also impractical. Since detailed studies at a small spatial scale have so far been lacking, possibly because birds are assumed to move all the time covering large distances from day to day, our study opted for a small spatial scale.

We focussed on studying the dynamics of LPAIV infections in free-living mallards, since they are a key host species of LPAIV (Olsen et al. 2006), and hardly show any clinical signs when infected experimentally with this virus (Kuiken 2013). Their ecology likely plays an important role in LPAIV infection dynamics (e.g. surface feeder, highly abundant, partial migrant; Cramp and Simmons 1977, Delany and Scott 2006). Also in the emergence and spread of HPAIV, mallards are suggested to play a role (e.g. related to domestic ducks, no clinical signs when infected experimentally; Keawcharoen et al. 2008). Mallards are relatively easily captured enabling relatively simple collection of samples for LPAIV. As mallards are a well-studied species in the field of LPAIV and HPAIV, it enabled us to place the findings of our studies into context and, assisted by a vast body of literature, it allowed us to better explain our results and hence increase the relevance of our work.

We showed that the annual LPAIV infection cycle in mallards lasted from March 2010 until February 2011. There were two distinct LPAIV infection peaks: a small LPAIV infection peak in summer (June-Aug) and a large, prominent peak in autumn (Sep-Nov). In spring (March-May), no mallards were infected with LPAIV (Chapter 3). We also collected blood samples to determine whether birds were previously infected with LPAIV based on the presence or absence of AIV-specific antibodies (i.e. antibodies to the highly conserved nucleoprotein of AIV; hereafter called AIV antibodies), as this has been largely neglected in LPAIV studies in wild birds (Hoye et al. 2011). We showed that during the LPAIV

infection peaks in summer and autumn, the proportion of birds with AIV antibodies was lower compared to winter and spring (Chapter 3). These results suggest that these AIV antibodies are probably present for a period of six to eight months, coinciding with experimental and modelling studies (Fereidouni et al. 2010, Hoye et al. 2011). Although our study was unique in its duration, and consistent temporal and spatial coverage of a single key host species, the seasonal patterns in LPAIV prevalence, specifically the autumn infection peak, are in accordance with similar LPAIV studies in wild birds in the northern hemisphere (Munster et al. 2007, Latorre-Margalef et al. 2014). However, by sampling mallards intensively during a full LPAIV infection cycle in combination with host demography and critical life-history events, such as breeding, moult and migration, we were also able to test suggested drivers of seasonal dynamics of LPAIV infections in wild birds.

The challenge is to extrapolate our findings and make inferences about LPAIV infection dynamics at larger spatial scales, like the Netherlands. A collaborative project with colleagues of the University of Utrecht, the Netherlands, provided the opportunity to study LPAIV prevalence in mallards at four distinct locations (including our study site) simultaneously in autumn and winter 2012. Here we could show that LPAIV prevalence and AIV antibody prevalence in mallards did not differ between the four locations and that the seasonal pattern in prevalence was consistent across the different locations (Box 8.1). The autumn and winter LPAIV prevalence patterns at the four locations in 2012 corresponded with the patterns described at our study site in 2010 (Chapter 3), suggesting that these LPAIV prevalence patterns are representative for the LPAIV infection dynamics in mallards in the Netherlands.

Drivers of seasonal dynamics of avian influenza virus infections in wild birds

Several mechanisms driving seasonal variation in LPAIV prevalence in wild birds have often been suggested, but never explicitly tested using empirical data. In this thesis, the validity of three drivers for the seasonal dynamics in LPAIV infections in wild birds was investigated: (i) host density, (ii) immunologically naïve young and (iii) increased susceptibility in migrants.

In order to test the third driver (increased susceptibility in migrants), migratory and resident mallards had to be distinguished, as they mix in autumn and winter at our study site. Mallards are partially migratory, which means that throughout Europe the population consists of migratory and resident individuals (Scott and Rose 1996). A way to distinguish the two mallard populations is by stable hydrogen isotope measurements (δ^2H) in feathers. Stable isotopes are based on the principle '*you are what you eat*', since

stable isotope signatures in animal tissue reflect those of local food webs (Peterson and Fry 1987). The hydrogen in water can occur in two different stable isotopic forms: as ^{1}H (light isotope) and ^{2}H (heavy isotope). When air masses saturated with water travel over land, water containing ^{2}H is lost faster in the form of precipitation than water containing ^{1}H. The ratio of ^{2}H to ^{1}H in precipitation thus decreases with time (West et al. 2010), resulting in a gradient across Europe with high ratios close to the Atlantic, ratios decreasing as one goes from the south-west to the north-east across the continent (Bowen et al. 2005). Water is a crucial resource for all life forms and its hydrogen is incorporated in all tissues. Also during the period of feather growth (moult) hydrogen (directly or indirectly from precipitation) is incorporated into the feathers of birds (Hobson 1999). Through this process, feather $\delta^{2}H$ is thus correlated with precipitation $\delta^{2}H$ (Hobson and Wassenaar 1997). A calibration curve is needed to convert feather $\delta^{2}H$ to precipitation $\delta^{2}H$. Since pre-existing calibration curves likely contained much isotopic variation, as they were based on feather samples across several species, age classes and years, we created a calibration curve, accounting for this variation, and a European feather $\delta^{2}H$ isoscape specifically for mallards (Chapter 2). Our study showed that indeed our mallard calibration curve yielded a higher coefficient of determination and was more accurate in assessing a bird's origin than pre-existing calibration curves. However, the relatively small gain in precision that is achieved by creating a species, age and year-specific calibration curve should be balanced against the work and costs of collecting and analysing feathers. $\delta^{2}H$ values provide a rough indication of the location where a bird moulted its feathers and this remains a rough indication also after creating a species-specific calibration curve. Therefore, for most studies, it would probably be recommended to select the calibration curve that most closely matches one's study species (i.e. diet), instead of creating a new curve.

In our study we found that the small summer LPAIV infection peak was likely driven by the entrance of unfledged naïve juveniles into the resident population: they were more likely to be infected, shed higher quantities of virus and were less likely to have AIV antibodies than adults (Chapter 3). Still, in this period nearly half of the eggs received maternal AIV antibodies (Chapter 6), suggesting that a large proportion of newly hatched mallard chicks were protected against LPAIV during their first two weeks by means of maternal antibodies. This could mean that without these maternal AIV antibodies the LPAIV infection peak might already start in spring and be higher. Not all eggs received maternal AIV antibodies, likely because the mother had not recently been infected with LPAIV, or had low concentrations of circulating AIV antibodies, as our study showed that maternal antibodies in eggs were positively correlated with circulating antibodies in the mother (Chapter 6). Besides we showed a negative correlation between maternal AIV antibodies in eggs and laying order (Chapter 6), which may also explain why not all eggs received

maternal antibodies. Our study is the first to investigate the levels of AIV antibodies in mothers and eggs in mallards, and correspond with findings of maternal AIV antibodies in gull eggs (Pearce-Duvet et al. 2009, Hammouda et al. 2011). Such maternal antibodies may have protective effects and may play an important role in LPAIV infection dynamics across a range of host species.

The large autumn LPAIV infection peak was likely driven by the arrival of susceptible migratory birds on the wintering ground. Both short and long distance migrants (i.e. local and distant migrants) were more likely to be infected than residents (Chapter 3). Despite these high levels of infection, AIV antibody levels in migrants remained low during the autumn infection peak, suggesting that migrants were transiting through the study site with each wave of migrants amplifying local LPAIV circulation. These low AIV antibody levels in migrants may reflect naivety to locally circulating LPAIV strains, reduced immunocompetence (as a consequence of their strenuous migratory journey in which they faced a trade-off between investment in immune function and flight; Altizer et al. 2011), loss of immunity or a combination of these processes. Using local and nationwide counts we also assessed whether or not mallard density could act as a potential driver of the two LPAIV infection peaks. Although the autumn LPAIV infection peak did not coincide with the peak in mallard numbers, it occurred indeed during the increase in numbers (Chapter 3). This increase in mallard density was likely caused by the other two drivers (influx of juveniles and migrants). Thus, despite the mismatch in peaks, we cannot rule host density out as an additional driver of the dynamics of LPAIV infections in wild birds.

To assess whether the above suggested drivers indeed play a role in explaining the LPAIV infection dynamics in wild birds (Chapter 3), comparative, experimental or theoretical studies are required. Using a susceptible-infected-recovered-susceptible (SIRS) model, we opted for the latter of the three methods to investigate the role of host-related drivers in explaining the LPAIV infection dynamics in mallards as observed in our study (Box 8.2). We suggest two non-mutually exclusive drivers that may explain the high and long-lasting LPAIV prevalence in mallards: (i) replacement (turnover) of migratory birds in autumn and (ii) short-term immunity. Furthermore, we suggest heterogeneity in transmission rates varying between age classes and birds with different migratory strategies. We distinguished three subpopulations, (i) migratory birds, (ii) local (i.e. resident) juveniles and (iii) local adults, for which we modelled LPAIV patterns separately. Using this epidemiological model we were able to simulate LPAIV infection dynamics that closely matched the observed patterns in migrants, local juveniles and local adults as observed in our study. Even though this modelling exercise provided us with encouraging confirmation for the potential importance of transient migrants, host short-term immunity and transmission

rates in explaining infection dynamics in wild birds, it does not provide us with a true test of our hypothesised drivers. But the results are encouraging and a stimulus to better study host demography, and LPAIV ecology and epidemiology in wild birds to further elucidate the mechanisms underlying the dynamics of LPAIV infections in wild birds.

In this modelling exercise we focussed on host factors only, ignoring the potential role of environmental factors (e.g. rainfall, temperature) as alternative or additional drivers of LPAIV infection dynamics. These factors can be of considerable importance with regard to (migratory) bird movements and density, and environmental survival of LPAIV (Nazir et al. 2010, Reside et al. 2010), and might therefore deserve to be included besides host factors in future LPAIV epidemiological studies.

Migratory and resident hosts and their impacts on avian influenza virus infection dynamics

Numerous species of ducks, waders and gulls that are considered key host species of LPAIV are partial migrants (Lack 1943). Nevertheless, in only few LPAIV studies it is being acknowledged that individuals within a species may differ in their migratory strategy and thus contribute differentially to LPAIV infection dynamics, in the same fashion that migratory and resident species contribute differentially to LPAIV dynamics (Stallknecht et al. 1990b). At least in part these differences may be due to partial migrants being more susceptible to LPAIV infection, possibly induced by migration or by host characteristics (e.g. hemagglutinin receptor binding affinity, body size, dominance status).

We studied the role of resident, local and distant migratory mallards during a local H3 LPAIV epizootic. This epizootic was initiated by a single introduction of H3 LPAIV in susceptible residents, which was followed by virus amplification, importantly associated with the influx of migratory mallards (Chapter 4). The local epizootic was thus not so much the result of new introductions of H3 LPAIV strains or other LPAIV subtypes, but predominantly by reinforcing local circulation of the initial H3 LPAIV strain by migratory mallards. We found that both migratory and resident mallards had low H3-specific antibody titres (Chapter 4), suggesting they were naïve for this H3 LPAIV strain prior to the epizootic. That residents were likely naïve for this virus was also shown by high H3 virus shedding at the start of the epizootic (Chapter 4). That migrants were susceptible to H3 LPAIV could be due to their reduced immunocompetence, as a result of the trade-off between immune investment and flight. Following up on this idea, we indeed found a lower body mass in migrants compared to residents (Chapter 5), suggesting they were in poorer body condition, which could have made them more susceptible to LPAIV infection. However, despite the considerable body mass differences, we found only minor differences in immune status

between migratory and resident mallards (Chapter 5). The fact that residents were in better body condition, albeit having low H3-specific antibody titres, could potentially explain why they were less frequently infected with H3 LPAIV (Chapter 4).

Surprisingly, we found almost no differences in virus prevalence, body condition and immune status between local and distant migrants (Chapter 3, 4 and 5). Local migrants may originate from several to hundreds of kilometres from our study site, whereas distant migrants originate from areas much further away, (e.g. northern Scandinavia or Russia). Since local migrants may potentially originate from locations relatively close to the study site, it was expected that these would more closely match residents in virus prevalence, body condition and immune status than distant migrants. Yet, our findings do not support this. Thus, given their similarity to distant migrants, the majority of local migrants may well have originated from areas that were actually more distant from the study site.

Mallards as asymptomatic carriers of avian influenza virus

Mallards are often assumed to carry LPAIV asymptomatically, since they show hardly any signs of disease when infected experimentally (Kuiken 2013). However, supportive evidence from free-living mallards is largely lacking (Daoust et al. 2011). Therefore we studied potential effects of LPAIV infection on body condition, immune status and movement behaviour in free-living mallards. As mentioned, we found only minor differences in immune status and no differences in body condition between LPAIV infected and non-infected mallards (Chapter 5). However, we did find a negative association between LPAIV infection and daily regional movements, albeit associations were weak, with no association between infection and birds' daily local movements (Chapter 7). Based on these findings it is not unlikely that mallards are able to carry LPAIV asymptomatically. LPAIV infected mallards are likely still capable of transporting viral particles to other areas, although the distance of spread might be lower than one might expect from the behaviour of non-infected individuals.

To further determine whether or not mallards are truly asymptomatic carriers of LPAIV, a better understanding of the potential effects of LPAIV infection on mallards' survival is required. Although our data collection was limited and not designed for this kind of study, we used a mark-recapture model to assess survival of resident mallards that were either infected or non-infected with LPAIV during the autumn infection peak (Box 8.3). Local (or apparent) survival is defined as survival within a defined study area (the probability that an individual that is in the population at time t is still alive and in the population at time $t+1$). We demonstrate that LPAIV infected mallards had similar local survival as non-infected individuals. This finding is in accordance with findings of a similar study

conducted in another LPAIV key host species, ruddy turnstones (*Arenaria interpres morinella*), migrating through Delaware Bay, USA, where apparent annual survival was not reduced by LPAIV infection at this stop-over site (Maxted et al. 2012). We found that recapture probability was higher in LPAIV infected mallards than in non-infected birds, suggesting that infected birds remained closer to the study site. This corresponds with our finding of a negative association between LPAIV infection and daily regional movements of mallards (Chapter 7).

The weak associations between LPAIV infection and body condition, immune status, movement behaviour (Chapter 5 and 7) and importantly, local survival of mallards (Box 8.3), raises the question if this could be a consequence of tight co-evolution between mallards and LPAIV. For mallards to co-evolve with LPAIV is challenging, since these viruses are continually evolving due to a high mutation rate (including antigenetic drift: changes in the structure of surface glycoproteins) and rapid reassortment of gene segments between multiple virus subtypes (Webster et al. 1992). Still, co-evolution is a long-term process that may take thousands of years, enabling hosts and pathogens to adapt to each other. Mallards are a major reservoir species of LPAIV, suggesting they have the ability to cope with infections (minimize virulence) without minimizing fitness of the virus. On the other hand, LPAIVs are possibly less virulent for mallards (and potentially other waterfowl) allowing LPAIVs to replicate and transmit efficiently, which may explain why this virus is very common and found all around the world.

Conclusion and recommendations

The aim of this thesis was to advance our knowledge of the ecological processes underlying the epidemiology of LPAIVs in wild birds, which could be used to undertake adequate control measures to prevent their emergence and spread into poultry and humans. Our study showed that (i) autumn is the most critical time period of the year, since the highest proportion of mallards were infected with LPAIV in this season; (ii) LPAIV epizootics in wild birds likely occur at local scales, since there is little influx of novel LPAIVs; (iii) migratory hosts play an important role in LPAIV infection dynamics, as a (a) potential driver and (b) reinforcer of the autumn LPAIV epizootic, probably mostly because of their increased susceptibility due to locally circulating viruses rather than by introducing new LPAIV strains from afar; (iv) mallards are likely able to carry LPAIV asymptomatically, since there are only weak associations between infection and body condition and immune status and (v) mallards are likely capable of spreading LPAIVs locally and regionally, due to the weak negative associations between infection and movement behaviour, but the spread of this virus is probably lower than expected on the basis of movements of non-infected birds.

Summarizing all findings, it suggests that the highest probability for poultry (and subsequently humans) to get infected with LPAIV from mallards is in autumn, at least in the northern hemisphere temperate zone, when LPAIV epizootics in wild birds emerge at local scales. Since LPAIV can also be detrimental for humans before it has mutated in poultry into a HPAIV, as was recently discovered with H7N9 LPAIV (Kreijtz et al. 2013), biosecurity at poultry farms should be high at all times, but particularly in autumn. There are several control measures opted by Swayne (2008), which may lower the chance that LPAIV will be introduced into poultry. These measures are for instance temporary confinement of poultry from outdoor access, no use of surface water sources, temporary closure of life bird markets and vaccination before autumn to decrease the population of susceptibles (Swayne 2008). The findings of this thesis suggests that these measures are most needed in (and can possibly be confined to) autumn.

This thesis illustrates the importance of studying zoonotic pathogens in wildlife hosts. I hope that these findings contribute to a better understanding of the dynamics of LPAIV infections in wild birds and the role of migratory birds in shaping these dynamics, and that they can be helpful in defining adequate control measures to manage and control this zoonotic pathogen in the future.

Box 8.1 Spatial variation of avian influenza virus infection dynamics in mallards

Jacintha G.B. van Dijk, Erik Kleyheeg, Merel B. Soons, Bart A. Nolet, Ron A.M. Fouchier & Marcel Klaassen

In a previous study, we comprehensively sampled mallards throughout a complete annual cycle on a small spatial scale (i.e. in the duck decoy near Oud Alblas, the Netherlands, in 2010), measuring both current and past infection with low pathogenic avian influenza virus (LPAIV) (Chapter 3). The temporal pattern in LPAIV prevalence detected at our study site was in accordance with seasonal patterns of LPAIV infections in free-living mallards described in other studies conducted in the northern hemisphere: a dominant peak in prevalence at the end of summer or in autumn (Hanson et al. 2003, Munster et al. 2007, Runstadler et al. 2007, Latorre-Margalef et al. 2014). As these studies were conducted in other years (i.e. 1998, 1999, 2000, 2005), or combined multiple years (i.e. 1998-2006, 2002-2010), making a comparison with the seasonal patterns in LPAIV prevalence in our study less solid. Therefore, we sampled mallards at four distinct locations in the Netherlands, including our study site, during the same period in autumn and winter 2012.

Mallards were caught in (i) the duck decoy located near Oud Alblas (*OA*) (51°52'38"N, 4°43'26"E), and in custom made traps at (ii) Terra Nova (*TN*), Loenen (52°12'55"N, 5°02'26"E), (iii) Juliusput (*JP*), Hoevelaken (52°09'35"N, 5°28'43"E) and (iv) Enterveen (*EV*), Enter (51°16'41"N, 6°33'33"E). Based on total water surface (km^2), Terra Nova and Oud Alblas were assigned as wet areas (respectively, 9.8 and 2.3 km^2), while Juliusput and Enterveen were drier areas (respectively, 0.7 and 0.5 km^2). Of each mallard, cloacal and oropharyngeal samples were collected (n = 387 samples from 317 mallards) to assess current LPAIV infection (i.e. viral prevalence). Blood samples (0.5-1.0 ml, up to 2% of the circulating blood volume) were collected from the brachial vein from 160 mallards to detect antibodies to avian influenza virus (AIV) (i.e. seroprevalence). Similar sampling methods and virus and antibody detection techniques were used as described in Chapter 3. We used the northern hemisphere meteorological seasons to assign months: *autumn*: September–November, *winter*: December–February. Differences in viral and seroprevalence were determined using a generalized linear (mixed) model, with location, season and their two-way interaction as fixed factors (and individual bird as random factor). After model selection, based on Akaike Information Criterion (AIC) corrected for small sample sizes (AICc; Burnham and Anderson 2002), the models without the interaction were defined as the better models ($\Delta AIC_c < 2$).

There was no difference in viral prevalence between the four locations (X^2 = 1.88, P = 0.597; Fig. 8.1a). However, there was a difference between the seasons (X^2 = 15.72, P < 0.001),

with a higher viral prevalence in autumn (15%) compared to winter (5%; Fig. 8.1a). In mallards sampled at Oud Alblas in 2010, viral prevalence levels were higher, but a similar three-fold difference between autumn and winter was found (respectively, 38% and 10%; Chapter 3). Seroprevalence was similar between the four locations (X^2 = 3.82, P = 0.281) and between the two seasons (X^2 = 1.44, P = 0.230; Fig. 8.1b). In the study of 2010 at Oud Alblas, we found an almost two-fold higher seroprevalence in winter (48%) compared to autumn (28%; Chapter 3). This could potentially be due to higher viral prevalence in 2010 than in 2012, resulting in a higher number of mallards with antibodies against AIV in the following season.

The results of this study demonstrate that viral and seroprevalence in mallard populations did not differ much across sites situated in different landscapes, at least on small geographical scales (up to 135 km as the crow flies). Seasonal differences in viral and seroprevalence were consistent across sites. In addition, it demonstrates that the autumn and winter LPAIV prevalence patterns in mallards sampled in 2010 at our study site corresponded to the prevalence patterns detected in 2012 at this site and three other locations. These findings suggest that the patterns in LPAIV prevalence in mallards found in the duck decoy in autumn and winter 2010 (Chapter 3) were representative for the LPAIV infection dynamics in mallards in the Netherlands.

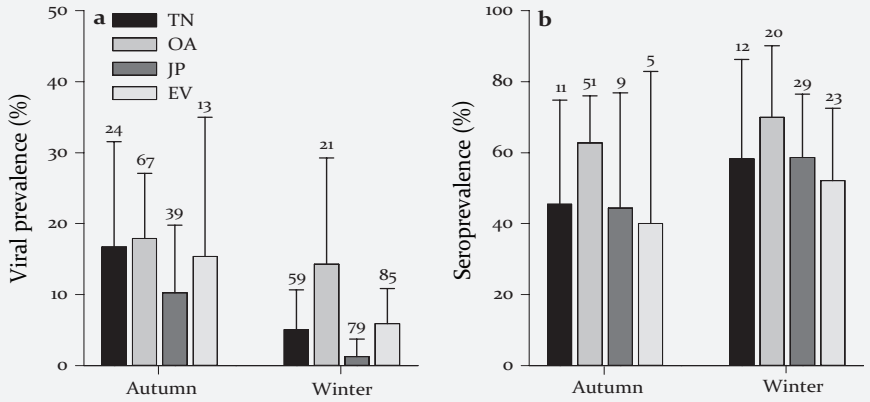


Figure 8.1 Autumn and winter prevalence (± 95% CI) of mallards sampled in 2012 at four locations in the Netherlands. Prevalence of (a) LPAIV infection (i.e. current infection) and (b) AIV antibodies (i.e. past infection). For each location per season the number of samples is shown.

Box 8.2 A quantitative evaluation of short-term immunity and transient migrants as potential drivers of observed avian influenza virus infection dynamics

Simeon Lisovski, Jacintha G.B. van Dijk, Don Klinkenberg, Bart A. Nolet, Ron A.M. Fouchier & Marcel Klaassen

In the study described in this thesis, in which mallards were comprehensively sampled for low pathogenic avian influenza virus (LPAIV) at a local scale for a complete annual cycle, we identified two mechanisms that were likely driving the summer and autumn LPAIV infection peaks in wild birds. These drivers are the influx of immunologically naïve (resident) juveniles and the arrival of susceptible migrants. There was less compelling evidence for an exclusive role of host density as driver of seasonal LPAIV infection dynamics (Chapter 3). It was furthermore shown that the autumn LPAIV infection peak was extremely high and long-lasting (Chapter 3), which was especially apparent in migrants, but also in the local (i.e. resident) population (Fig. 8.3).

Although the arrival of migrants and the influx of juveniles potentially explain an LPAIV infection peak in the population, the observed high prevalence over such a long period requires additional drivers. One could be an extremely long infectious period (>1 month), but this would be in sharp contrast to earlier findings of LPAIV infections in free-living mallards, indicating infectious periods are generally up to a week (Latorre-Margalef et al. 2009). We therefore suggest two additional non-mutually exclusive drivers. (i) A replacement (turnover) of migratory birds in autumn, resulting in a constant influx of susceptible migrants. This hypothesis is corroborated by the observation that AIV antibody levels in migrants remained low throughout the autumn infection peak (Chapter 3), indeed suggesting that migrants were transiting through the study site with each wave of migrants amplifying local LPAIV circulation. (ii) Short-term immunity resulting in multiple LPAIV infections in individual birds within a relatively short period of time (i.e. several weeks up to two months; Latorre-Margalef et al. 2013). Experimental infection studies have indeed shown that antibodies against specific LPAIV subtypes are short-lived (Curran et al. 2013). In addition we suggest heterogeneity in transmission rates (determined by contact rate and transmission probability per contact), which might differ between age classes and birds with different migratory strategies. Immune status may indeed vary between juveniles and adults (Sol et al. 2003) and in our study it is not unlikely that (many) juveniles are immunologically naïve to LPAIV. Furthermore, migrants may have a reduced immunocompetence compared to residents due to the trade-off between immune investment and long-distance flight (Altizer et al. 2011). These differences in immune status may translate into different levels of infectiousness or likelihoods of becoming infected

after contact with an infectious individual. The latter suggests a transition of juveniles into an adult epidemiological status after being infected once (or several times) with LPAIV. We incorporated these potentially explaining drivers of the LPAIV infection dynamics in mallards as observed in the field into a SIRS (Susceptible-Infectious-Recovered-Susceptible) model framework in which we distinguished between (i) migratory birds, (ii) local juveniles and (c) local adults, considering them as three subpopulations for which we modelled LPAIV patterns separately. For each subpopulation we used the standard compartments: susceptible (S), infectious (I) and recovered (R) as shown in Fig. 8.2. Definitions and parameter values are presented in Table 8.1.

Model terms related to demography are birth of local juveniles at rate $B(t)N_{hatch}$ per adult female, with $B(t)$ the rate of eggs hatching and N_{hatch} the mean number of hatchlings; background mortality of all birds (Schekkerman and Slaterus 2008), and additional mortalities of juveniles and by hunting (Hirschfeld and Heyd 2005), at per-capita rates μ, $\mu_{juv}(t)$ and $\mu_h(t)$ respectively; net immigration of susceptible migrants at rate $m_{in}(t)N_a(t)$, and continuous migration of migrants leaving and arriving, resulting in a per-capita replacement rate $m_r(t)$. Except for background mortality, all demographic rates change during the season (for more details see Appendix 8.1 Fig. S8.1).

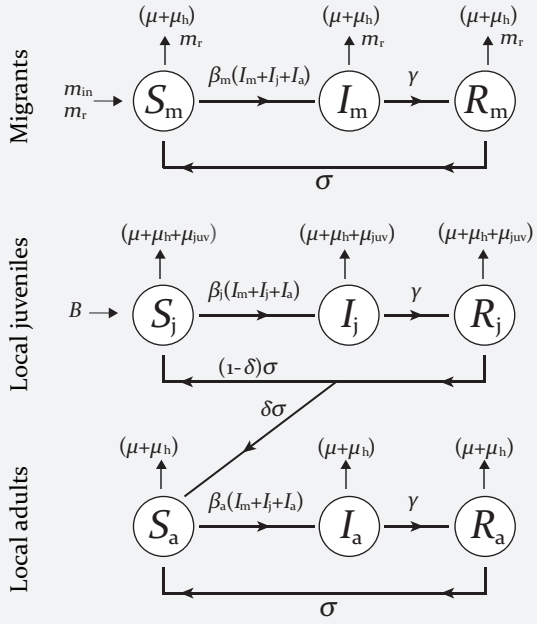


Figure 8.2 Epidemiological and demographic flowchart of the three mallard subpopulations. Departure of migrants and transition of remaining juveniles at day 60 (day of the year) into adults is realised using a step function (at day 60).

Table 8.1 List of model parameters and values. Parameters in bold were estimated by the MCMC simulation.

Symbol	Definition	Units	Values
β_m, β_j, β_a	Transmission rates for migrants, local juveniles and local adults, respectively	bird^{-1} day^{-1}	**0.23 x 10^{-2}, 0.88 x 10^{-2}, 0.66 x 10^{-4}**
η	Environmental transmission rate	day	10^{-5}
γ	Recovery rate	day	log(2)/6
σ	Immune rate	bird^{-1} day^{-1}	**0.17 x 10**
δ	Juvenile-adult transition	proportion	**0.26**
μ	Natural mortality rate	day	0.315/365
μ_h	Hunting mortality rate**	day	0.320/365
μ_{juv}	Juvenile mortality rate*, **	day	0.1064687, mean = 207, sd = 20
N_{pop}	Population size*	individuals	600
N_{hatch}	Number of hatchlings*	individuals	6
B	Birth rate*, **	bird^{-1} day^{-1}	Shape: mean = 187, sd = 20
m_{in}	Migration rate*, **	day	Shape: mean = 273, sd = 15
m_r	Replacement of migrants**	1/time	Shape: **mean = 238, amp = 0.365, slope = 67.9, kurt = 2.95**

* Estimated prior to simulation

** Parameters vary throughout the year, see Appendix 8.1 Fig. S8.1

The following four model terms are related to infection: (i) density-dependent transmission of the infection at rate β_m, β_j, β_a, for migrants, juveniles and adults, respectively. Arriving migrants and hatched local juveniles were expected to be susceptible (arriving and birth entirely in S (susceptible) compartments), and even more susceptible than susceptible local adults, reflecting reduced immunocompetence due to flight in migrants and naivety to locally circulating LPAIV strains in migrants and juveniles. (ii) In each of the three subpopulations a very low background transmission rate η (i.e. 'environmental transmission rate') was included (Galsworthy et al. 2011). (iii) Recovery occurred at per-capita rate γ (Latorre-Margalef et al. 2009) and (iv) immunity was lost at per capita rate σ. A proportion δ of juveniles losing immunity moves to the compartment of susceptible adults (i.e. moves from R_j to S_a) reflecting reduced naivety to locally circulating strains after having experienced an LPAIV infection.

To allow demographic patterns to stabilise, the model was always run for a period covering five annual cycles and the last year was used for comparison with the patterns observed in the field. The model was integrated using the ode method of the R Package deSolve (Soetaert et al. 2010). For estimation of parameter values and uncertainties, we fitted data of number of infected and uninfected individuals per month for each subpopulation in our study site (for details see Chapter 3) with maximum likelihood, using Markov Chain Monte Carlo (MCMC) simulation with an adaptive Metropolis algorithm (implemented in MCMCmod from the R Package FME; Soetaert and Petzoldt 2010). The metropolis algorithm optimizes the parameter settings by minimizing the negative sum of the log-binomial densities for each observation with the respective model output as the probability. The differential equations for the three mallard subpopulations are given in Appendix 8.1. A sensitivity analysis was conducted: the effect of parameters on the log-likelihood (model fit) was estimated by sampling parameters within a range of best estimate plus/minus 40%.

The model was able to reasonably fit the LPAIV infection patterns in migrants, local juveniles and local adults as observed in our study (Fig. 8.3). Sensitivity analysis revealed that the model was most sensitive to the immune rate σ, i.e. the average individual transition from R (recovered) to S (susceptible), which was estimated at 41.7 days. This result agrees with the

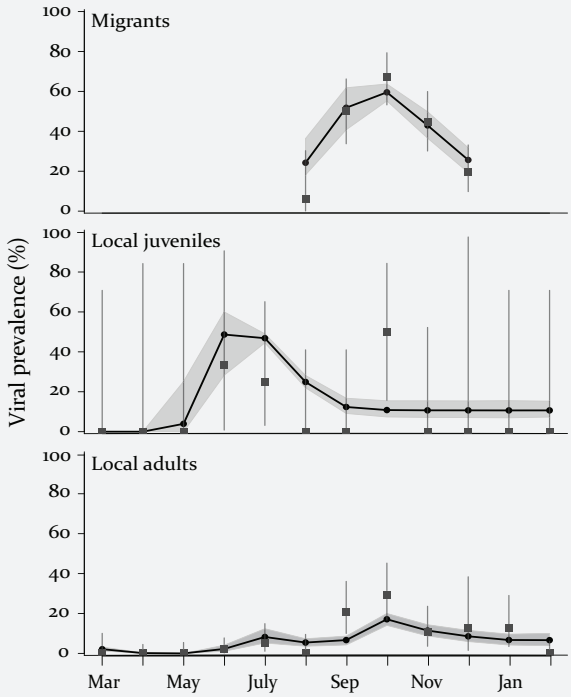


Figure 8.3 Monthly viral prevalence of free-living mallards as observed in our study (black squares with grey error bars indicating observed prevalence with 95% CI) and simulated by the SIRS model (black symbols and lines with grey shaded area indicating best fit and sensitivity range 95% quartiles).

findings of Curran et al. (2013), who infected ducks experimentally with LPAIV subtype H6N2, which subsequently remained seropositive against this subtype (i.e. specific antibodies against H6N2) for up to day 42 post-infection. Moreover, a recent study by Latorre-Margalef et al. (2013) showed that in free-living mallards the time between initial infection and reinfection with a similar or different LPAIV subtype is probably relatively short and in the range from several weeks up to two months.

The model also proved sensitive to m_r (mean), describing the mean date of the Gaussian distributed replacement of migrants (i.e. 27 August 2010; Table 8.1). The modelling yielded the best fit when the proportion of transient migrants was relatively high during the initial phase of autumn migration (Fig. 8.4). This suggests that when migratory mallards first arrive at the wintering grounds they might show some exploratory behaviour before settling in a specific area. However, empirical data supporting this hypothesis is lacking.

The high LPAIV infection peak in local juveniles in early summer was realised by a very high transmission rate β_j 0.88 x 10^{-2}. Juveniles have no or low cross-protective immunity due to prior infections resulting in a high force of infection. After LPAIV infection, the force of infection is lower due to acquired and potentially cross-protective immunity making juveniles

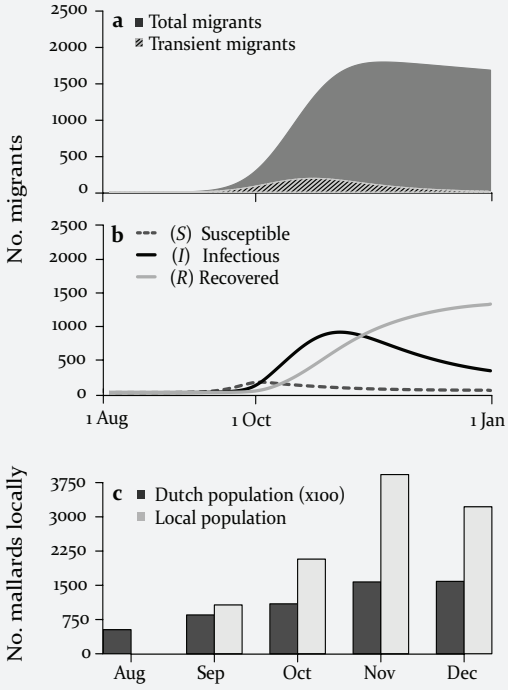


Figure 8.4 (a) Number of migratory mallards, together with the proportion of migrants that are daily replaced by new individuals, (b) SIR pattern in migratory mallards as predicted by the SIRS model and (c) mallard counts in the area of our study site (i.e. Alblasserwaard) and across the Netherlands as a whole, from August until December 2010.

probably more immunologically similar to adults. Juvenile-adult transition δ was estimated at 26% of juveniles transiting into adults after a LPAIV infection. The autumn LPAIV infection peak in migrants was realised with a high transmission rate β_m of 0.23 x 10^{-2}, contrasting sharply with the estimated transmission rate for local adults β_a which was 0.66 x 10^{-4}. Migrants are expected to have a higher force of infection than local adults due to the costs of migration potentially impairing their immune system (Altizer et al. 2011).

By using a SIRS model in which we included transient migratory mallards and estimated, using an optimization algorithm, separate transmission rates for migrants, local juveniles and local adults, overall immune rate and a proportion of juveniles transiting into adults, we were able to find parameter settings to simulate the LPAIV infection dynamics in these three subpopulations like observed in our study (Chapter 3). This modelling exercise thus lends credit to the hypothesised roles of juveniles and migrants as important factors in the epidemiology of LPAIV at our study site. However, confirmation of these findings can only be achieved by further empirical research; the modelling highlights the need for specific information on host demography, such as timing and extent of reproduction, arrival and departure of migrants, replacement of migrants, number of juveniles, adults and migrants in a population.

Box 8.3 Avian influenza virus infection and local survival of mallards

Tamar Lok, Jacintha G.B. van Dijk, Ron A.M. Fouchier & Marcel Klaassen

In two previous studies, we demonstrated weak associations between low pathogenic avian influenza virus (LPAIV) infection and mallards' body condition, immune response (Chapter 5) and movement (Chapter 7). To determine whether mallards were truly asymptomatic carriers of LPAIV, as is frequently suggested, a better understanding of the potential effects of LPAIV infection on mallards' survival is required.

Whether LPAIV infected mallards differed in survival to non-infected birds was tested during the period that LPAIV prevalence in free-living mallards at our study site was at its highest: from September 2010 until March 2011. On the basis of monthly captured and recaptured individually ringed mallards at our study site, the duck decoy near Oud Alblas, we estimated local survival (Φ). Local (or apparent) survival is defined as survival within a defined study area: the probability that an individual that is in the population at time t is still alive and

in the population at time t+1. This implies that true mortality and permanent emigration are confounded. For this reason, we selected resident mallards only, since migratory birds transited through the study site in autumn, herewith having a large effect on local survival estimates. Resident mallards were identified based on their feather stable hydrogen isotope ratio (for details see Chapter 3) or the fact they had been captured during the breeding season of 2010. Mallards breeding in western Europe (e.g. the Netherlands) are generally sedentary (Scott and Rose 1996). The data used in the survival analysis included both mallards that had been sampled for our own study (Chapter 3) and for the national wild bird surveillance programme executed by the Department of Viroscience of Erasmus MC in Rotterdam, the Netherlands. We pooled all (re)captures per month resulting in seven recapture occasions (i.e. September, October, … , March) and six monthly intervals (mid-September to mid-October, …, mid-February to mid-March) over which local survival was estimated.

To estimate a potential effect of LPAIV infection on local survival we used Cormack-Jolly-Seber (CJS) models allowing separate estimates of local survival (Φ) and recapture probabilities (p) (Lebreton et al. 1992). Recapture probability is defined as the probability of sighting a marked individual that is alive and in the population at time t. Ideally, the effect of LPAIV infection on local survival is estimated using multi-state mark-recapture models, as there is uncertainty about the infection status of mallards in the months that they were not (re)captured. These models not only estimate survival and recapture probabilities, but also transition probabilities between states (LPAIV infected or non-infected). These transition probabilities are likely not constant over time and should therefore be modelled with monthly variation. To estimate all these parameters requires more data than is currently available, and therefore, as an alternative, we used CJS models where we compared survival between two groups: LPAIV infected birds (i.e. infected at least once during the seven-month period) and non-infected individuals. This classification might potentially result in a bias, since birds were only tested for LPAIV infection when (re)captured. Some birds may have been wrongly assigned to the non-infected group when they had been infected in months when they were not caught, which would result in biased high estimates of recapture probability of birds in the infected group. However, this bias is expected to be small. The percentage of (re)captures in the months of highest viral prevalence (September-November) was very similar for the non-infected and infected groups: 40% and 44% (see also Appendix 8.2 Table S8.1). In addition, selecting only the birds in the infected group that were infected upon first capture (59% of the birds) produced similar model outcomes.

In addition to LPAIV infection status (i: infected/non-infected), we investigated the explanatory power of birds' sex (s: male/female) and age class (a: juvenile (<1 year old) /adult (>1 year old)). Since data on juveniles were limited, we only modelled additive effects of age, except for the interaction effect between LPAIV infection status and age on local survival. It is

likely that juveniles suffer more from infection with LPAIV than adults, since they have a less developed acquired immune system (Hudson and Dobson 1997) and lower likelihood of cross-protective immunity due to prior infections. Juveniles and adults were pooled together to assess goodness-of-fit of the model $\Phi_{i*s*t}\, p_{i*s*t}$, where t represents time as a categorical variable (i.e. months) using program U-Care (Choquet et al. 2009). There was no evidence for capture heterogeneity (X^2 = 20.21, df = 26, P = 0.780), nor for transience effects (i.e. no excess of birds captured only once; X^2 = 30.77, df = 29, P = 0.380).

Model selection was performed in two steps. First, different parameterizations of the recapture probability were investigated (i.e. 'reduced' parameterizations with and without effects of i, s, a, t and biological meaningful interactions) after which the most parsimonious parameterization was selected. This parameterization of recapture probability was subsequently used to investigate different parameterizations of local survival (i.e. reduced parameterization with and without effects of i, a, t and interactions). Model selection was performed on the basis of Akaike Information Criterion (AIC) corrected for small sample sizes (AIC_c; Burnham and Anderson 2002). The most parsimonious model was defined as the model with the least number of parameter within a ΔAIC_c<2 relative to the best-supported model (i.e. the model with the lowest AIC_c). Only parameters contained in the most parsimonious model were considered to have substantial explanatory power. All models were built using R-package RMark (Laake 2013), and run with the algorithm of program MARK (White and Burnham 1999).

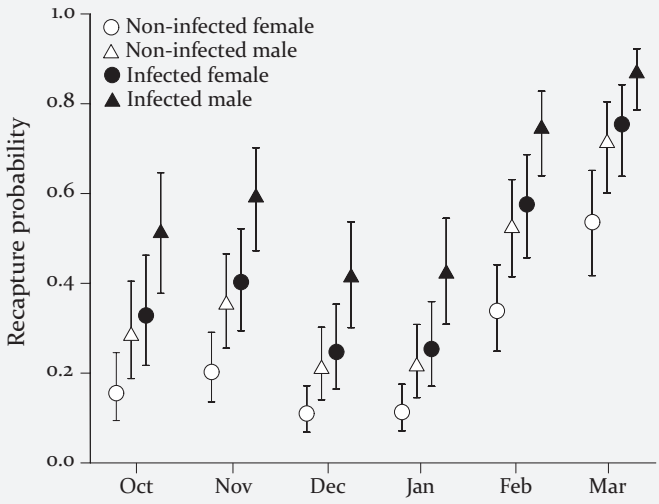


Figure 8.5 Monthly variation in recapture probability (p) between LPAIV infected and non-infected male and female mallards, as estimated using the most parsimonious model $\Phi_c p_{t+i+s}$ (see Appendix 8.2 Table S8.3).

The most parsimonious parameterization for recapture probability was p_{t+i+s} (Appendix 8.2 Table S8.2). Recapture probability differed between months with LPAIV infected birds and males having consistently higher recapture probabilities than non-infected birds and females (Fig. 8.5). Local survival neither differed between LPAIV infected and non-infected birds, nor between age classes (Appendix 8.2, Table S8.3). Monthly local survival was estimated at 0.954 (95% CI: 0.921-0.974), equal to an annual local survival estimate of 0.57 (95% CI: 0.37-0.73).

We demonstrate that mallards infected with LPAIV during an autumn infection peak had similar local survival to non-infected individuals. Yet, recapture probability was higher in LPAIV infected birds, which could suggest that infected birds remained closer to the study site (supported by the findings of Chapter 7).


Duck Hut
Please
mind
your head
Turn the ducks to learn about this hut

Appendices

Appendix 2.1

Table S2.1 Feather δ^2H (mean ± SE, ‰) per location and year of juvenile mallards (except*= adult feathers), collected throughout Europe in 2010 and 2011. Numbers correspond to the locations shown in the European mallard feather δ^2H (‰) isoscape (Fig. 2.1).

No.	Country	Year	Location	Latitude (°)	Longitude (°)	Altitude (m)	*n*	Feather δ^2H (‰)
1	Finland	2010	Maaninka	63.1	27.4	90	22	-135 ± 1.7
2	Norway	2010	Valle Hovin, Oslo	59.9	10.8	89	1	-114
3	Norway	2010	Østensjøvannet, Oslo	59.9	10.8	111	5	-118 ± 4.2
4	Latvia	2010	Engure	57.3	23.1	1	1	-101
5	Latvia	2010	Tukums	57.0	23.0	71	4	-113 ± 2.9
6	Russia	2010	Pokrov	55.9	39.2	109	9	-121 ± 4.1
7	Russia	2010	Dubrovka, Markovo	55.8	39.3	121	4	-128 ± 1.7
8	Poland	2011	Słupsk	54.5	17.0	18	1	-107
9	Poland	2010	Gdańsk	54.3	18.6	42	4	-118 ± 3.2
9	Poland	2011	Gdańsk	54.3	18.6	42	2	-117 ± 4.0
10	Poland	2011	Trzebiatów	54.1	15.3	4	1	-119
11	Poland	2010	Sztum	53.9	19.0	51	2	-107 ± 9.9
12	Poland	2011	Ostróda	53.7	20.0	99	3	-107 ± 6.6
13	Poland	2010	Iława	53.6	19.6	101	1	-113
14	Poland	2010	Lubawa	53.5	19.8	140	1	-103
15	Poland	2011	Przasnysz	53.0	20.9	118	1	-90
16	Poland	2011	Ozorków	52.0	19.3	121	1	-107
17	Poland	2011	Skierniewice	52.0	20.1	122	1	-108
18	Poland	2011	Żyrardów	52.1	20.4	118	3	-104 ± 4.0
19	Poland	2011	Warszawa	52.3	21.0	78	5	-102 ± 5.2
20	Poland	2011	Jarocin	52.0	17.5	119	1	-103
21	Netherlands	2011	Wieringen	52.9	4.9	3	18	-101 ± 2.5
22	Netherlands	2010	Oud Alblas	51.9	4.7	1	19	-96 ± 1.2
22	Netherlands	2010	Oud Alblas	51.9	4.7	1	94	-89 ± 0.9*
22	Netherlands	2011	Oud Alblas	51.9	4.7	1	20	-101 ± 2.3
23	UK	2011	Slimbridge	51.7	-2.4	9	1	-96
24	Belgium	2011	Bornem	51.2	4.3	0	5	-91 ± 1.7

No.	Country	Year	Location	Latitude (°)	Longitude (°)	Altitude (m)	*n*	Feather δ^2H (‰)
25	Belgium	2011	Londerzeel	51.0	4.3	12	4	-78 ± 3.7
26	Belgium	2011	Holsbeek	50.9	4.8	18	9	-88 ± 2.7
27	Belgium	2011	Oud-Heverlee	50.8	4.7	51	9	-84 ± 3.5
28	Belgium	2011	Kluisbergen	50.8	3.5	84	4	-98 ± 2.6
29	Belgium	2011	Overboelare	50.8	3.9	48	2	-97 ± 4.1
30	Germany	2010	Ismaning	48.2	11.7	497	1	-118
30	Germany	2011	Ismaning	48.2	11.7	497	3	-126 ± 5.2
31	Hungary	2011	Budapest	47.6	19.1	110	6	-104 ± 2.3
32	Hungary	2011	Alsóörs	47.0	18.0	101	1	-89
33	Switzerland	2010	Sempach	47.1	8.2	505	12	-103 ± 1.2
33	Switzerland	2011	Sempach	47.1	8.2	505	10	-116 ± 0.9
34	Italy	2011	Rastignano	44.4	11.3	168	2	-100 ± 8.2
35	Italy	2011	Fiumi Uniti, Ravenna	44.4	12.3	-1	1	-103
36	Spain	2011	Vic	41.9	2.3	497	5	-94 ± 4.2
37	Portugal	2010	São Jacinto	40.7	-8.7	0	2	-61 ± 0.8
38	Portugal	2010	R. do Taipal, Penalva do Castelo	40.7	-7.5	627	8	-80 ± 2.1

Appendix 2.2

Table S2.2 δ^2H (mean ± SD, ‰) values for each of the keratin standards used in the hydrogen stable isotope analysis of mallard feathers in 2011 and 2012, with mean δ^2H (‰) within runs and mean δ^2H (‰) ± SD among runs.

Keratin standard	Analysis	δ^2H mean ± SD (‰)	δ^2H mean (‰) within runs	δ^2H mean ± SD (‰) among runs
SC, Lot SJ (powdered)	2011	-117.0 ± 1.9	-117.2 to -116.7	-116.9 ± 0.4
BWB-II - new (baleen, powdered)	2011	-109.7 ± 1.8	-110.0 to -109.5	-109.7 ± 0.4
CHS (cow hoof, powdered)	2011	-187.9 ± 1.7	-188.6 to -187.2	-187.9 ± 1.0
SC, Lot SJ (powdered)	2012	-120.7 ± 2.1	-120.9 to -120.4	-120.7 ± 0.2
CBS (Caribou hoof, powdered)	2012	-198.4 ± 2.7	-199.9 to -196.9	-198.4 ± 1.5
KHS (Kudo horn, powdered)	2012	-55.8 ± 2.4	-56.4 to -55.2	-55.8 ± 0.6

Appendix 3.1

Table S3.1 Number of samples collected from primary captures (P) and recaptures (R) of mallards, by age (i.e. juvenile: <1 year, adult: >1 year) and sex, at a duck decoy located near Oud Alblas, the Netherlands, from March 2010 until February 2011. All individuals were sampled for LPAIV and antibodies to AIV, except four juveniles in summer of which no blood was taken (sample size in brackets).

		Spring		Summer		Autumn		Winter	
Age	Sex	P	R	P	R	P	R	P	R
Juvenile	Male	2	0	7 (5)	2	47	12	19	8
	Female	0	0	10 (8)	1	17	12	15	5
Adult	Male	66	8	74	32	41	73	63	64
	Female	116	4	93	45	67	77	42	87
Total		184	12	184 (175)	80	172	174	139	164

Appendix 3.2

Mallards sampled at a duck decoy located near Oud Alblas in the Alblasserwaard, the Netherlands, from August until December 2010 were assigned as (i) resident, (ii) local migrant (i.e. short distance) or (iii) distant migrant (i.e. long distance). In Europe, northerly breeding mallards are migratory, wintering south, while birds breeding in temperate regions (i.e. most in Western Europe) are sedentary (Scott and Rose 1996). Distant migrants and resident/local migrants can be differentiated based on stable hydrogen isotope ratios (δ^2H) in feathers, as δ^2H in precipitation differs between northern regions in Europe and mid-European regions (Hobson et al. 2004). It is more difficult to differentiate between residents and local migrants, as they both originate from mid-European regions containing similar δ^2H values in their feathers. In our study we assigned individuals to a certain group based on the time of capture, recapture rate and whether or not they were in moult, enabling us to differentiate between residents, local migrants and distant migrants. A resident bird had grown its feathers near the duck decoy (i.e. unfledged juvenile or moulting adult), and had been recaptured multiple times throughout the sampling period. A local and distant migratory bird was seen and sampled once during the 12-month study, namely in August until December. As capture rate was reasonably high, we are quite confident that those individuals assigned as either local or distant migrants did not visit the duck decoy earlier during sampling. Incorrectly classifying residents as local migrants, or local migrants as residents is not likely to occur as they are two distinct groups: residents were caught during moult (i.e. juvenile or adult) in spring/summer, while local migrants, not in moult, were caught only once in August until December. Individuals that, using these criteria, could not be assigned to either the resident or the migratory group were disregarded from the analysis (n = 139).

Fitting a normal distribution, we determined the range of feather δ^2H of resident birds, which ranged from -103.5 to -73.7‰ (Fig. S3.1a). Distant migrants were differentiated from local migrants using the feather δ^2H range of resident birds, with the feather δ^2H of local migrants falling within this range of residents and δ^2H of distant migrants falling outside this range. The cut-off point between the two groups of migrants was -103.5‰, with local migrants ranging from -103.5 to -72.6‰ and distant migrants from -164.5 to -103.7‰ (Fig. S3.1b).

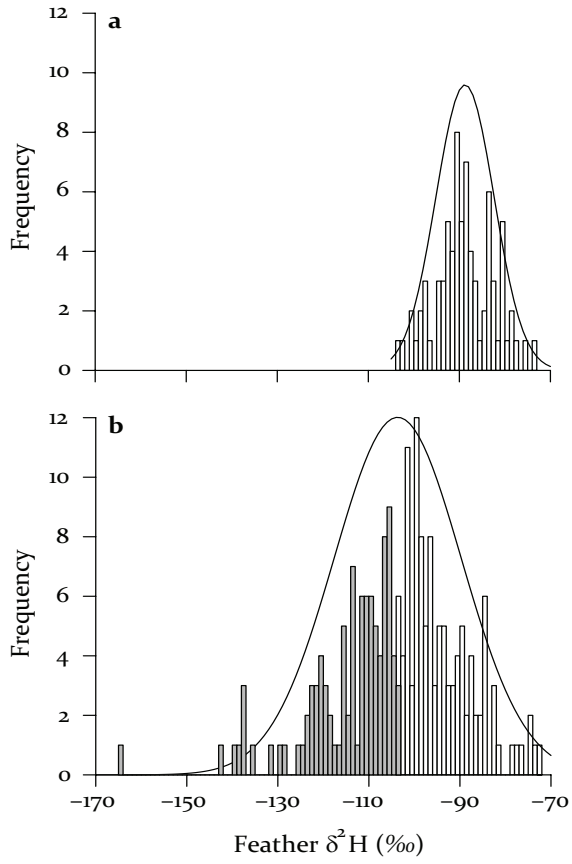


Figure S3.1 Stable hydrogen isotope ratio (δ^2H) in feathers of mallards sampled at a duck decoy located near Oud Alblas, the Netherlands, from August until December 2010, with the frequency of feather δ^2H of (a) resident birds and (b) local migrants (white bars) and distant migrants (grey bars).

Appendix 3.3

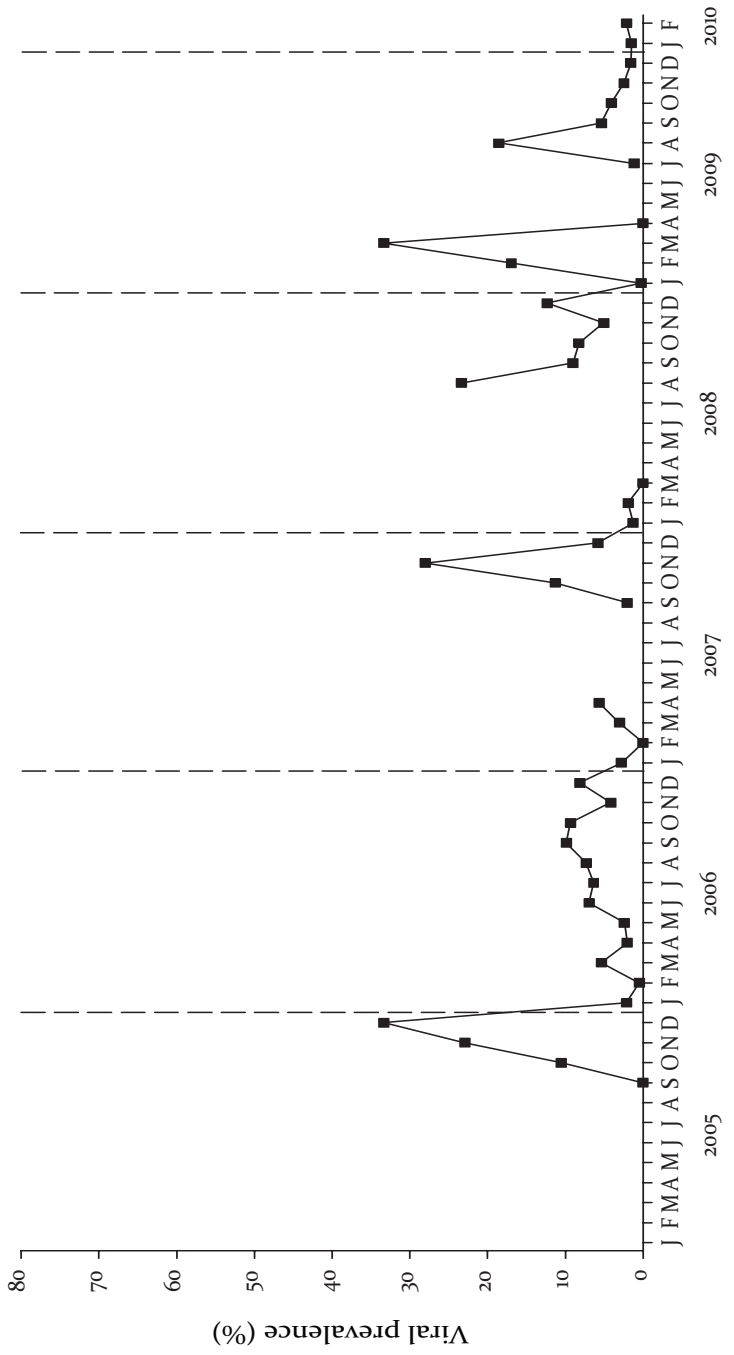


Figure S3.2 Monthly LPAIV prevalence of mallards sampled at a duck decoy located near Oud Alblas, the Netherlands, from January 2005 until February 2010. Samples collected in 2005 and (most of) 2006 consisted of cloacal samples to detect current LPAIV infection, while from 2007 onwards sampling was extended with oropharyngeal samples.

Appendix 4.1

Table S4.1 List of virus strain names and accession numbers of H3 influenza A viruses included in this study as listed in online databases IRD (http://www.fludb.org) and GISAID EpiFlu (http://platform.gisiad.org).

Virus strain name	Accession number
A/Bewick's Swan/Netherlands/4/2007(H3N6)	IRDAccession_1099728
A/Common Teal/Hungary-EMC/1/2009(H3N8)	IRDAccession_1099753
A/Common Teal/Hungary-EMC/5/2009(H3N8)	IRDAccession_1099754
A/Mallard/Netherlands/52/2010(H3N8)	IRDAccession_1099755
A/Common Teal/Netherlands/2/2011(H3N8)	IRDAccession_1099756
A/Mallard/Netherlands/6/2008(H3N6)	IRDAccession_1099759
A/Mallard/Netherlands/7/2008(H3N8)	IRDAccession_1099760
A/Mallard/Netherlands/10/2008(H3N6)	IRDAccession_1099761
A/Mallard/Netherlands/56/2008(H3N2)	IRDAccession_1099762
A/Mallard/Netherlands/39/2008(H3N2)	IRDAccession_1099763
A/Mallard/Netherlands/40/2008(H3N6)	IRDAccession_1099764
A/Mallard/Netherlands/41/2008(H3N8)	IRDAccession_1099765
A/Mallard/Netherlands/44/2008(H3N2)	IRDAccession_1099767
A/Mallard/Netherlands/19/2010(H3N8)	IRDAccession_1099768
A/Mallard/Netherlands/7/2010(H3N8)	IRDAccession_1099769
A/Mallard/Netherlands/13/2010(H3N8)	IRDAccession_1099770
A/Mallard/Netherlands/14/2010(H3N8)	IRDAccession_1099771
A/Mallard/Netherlands/16/2010(H3N8)	IRDAccession_1099772
A/Mallard/Netherlands/13/2009(H3N3)	IRDAccession_1099773
A/Mallard/Netherlands/9/2010(H3N8)	IRDAccession_1099774
A/Mallard/Netherlands/27/2010(H3N8)	IRDAccession_1099775
A/Mallard/Netherlands/30/2010(H3N8)	IRDAccession_1099776
A/Mallard/Netherlands/39/2010(H3N8)	IRDAccession_1099777
A/Mallard/Netherlands/24/2010(H3N6)	IRDAccession_1099778
A/Mallard/Netherlands/25/2010(H3N8)	IRDAccession_1099779
A/Mallard/Netherlands/34/2010(H3N2)	IRDAccession_1099780
A/Mallard/Netherlands/8/2010(H3N8)	IRDAccession_1099781

Virus strain name	Accession number
A/Mallard/Netherlands/33/2010(H3N8)	IRDAccession_1099782
A/Mallard/Netherlands/9/2011(H3N2)	IRDAccession_1099783
A/Mallard/Netherlands/10/2011(H3N2)	IRDAccession_1099784
A/Mallard/Netherlands/20/2011(H3N8)	IRDAccession_1099785
A/Mallard/Netherlands/37/2011(H3N8)	IRDAccession_1099786
A/Mallard/Netherlands/19/2008(H3N6)	IRDAccession_1099787
A/Mallard/Netherlands/20/2008(H3N6)	IRDAccession_1099788
A/Mallard/Netherlands/21/2008(H3N8)	IRDAccession_1099789
A/Mallard/Netherlands/22/2008(H3N6)	IRDAccession_1099790
A/Mallard/Netherlands/23/2008(H3N6)	IRDAccession_1099791
A/Mallard/Netherlands/24/2008(H3N8)	IRDAccession_1099792
A/Mallard/Netherlands/25/2008(H3N6)	IRDAccession_1099793
A/Mallard/Netherlands/26/2008(H3N6)	IRDAccession_1099794
A/Mallard/Netherlands/47/2008(H3N2)	IRDAccession_1099795
A/Mallard/Netherlands/48/2008(H3N2)	IRDAccession_1099796
A/Mallard/Netherlands/50/2008(H3N2)	IRDAccession_1099797
A/Mallard/Netherlands/20/2010(H3N8)	IRDAccession_1099798
A/Mallard/Netherlands/11/2010(H3N8)	IRDAccession_1099799
A/Mallard/Netherlands/6/2010(H3N8)	IRDAccession_1099800
A/Mallard/Netherlands/15/2010(H3N8)	IRDAccession_1099801
A/Mallard/Netherlands/35/2010(H3Nx)	IRDAccession_1099802
A/Mallard/Netherlands/32/2010(H3N8)	IRDAccession_1099803
A/Mallard/Netherlands/38/2010(H3N8)	IRDAccession_1099804
A/Mallard/Netherlands/36/2010(H3N8)	IRDAccession_1099805
A/Mallard/Netherlands/41/2010(H3N8)	IRDAccession_1099806
A/Mallard/Netherlands/42/2010(H3N8)	IRDAccession_1099807
A/Mallard/Netherlands/43/2010(H3N8)	IRDAccession_1099808
A/Mallard/Netherlands/44/2010(H3N8)	IRDAccession_1099809
A/Mallard/Netherlands/10/2010(H3N8)	IRDAccession_1099810
A/Mallard/Netherlands/37/2010(H3N8)	IRDAccession_1099811
A/Mallard/Netherlands/55/2010(H3N2)	IRDAccession_1099812
A/Anas_platyrhynchos/Belgium/12827/2007(H3N8)	EPI_ISL_26267
A/mallard/Germany-BW/SR872/2008(H3N8)	EPI_ISL_79643

Virus strain name	Accession number
A/mallard/Germany-BW/SR871/2008(H3N8)	EPI_ISL_79642
A/mallard/Germany-BW/SR632/2008(H3N2)	EPI_ISL_79640
A/mallard/Germany-BW/SR530/2007(H3N2)	EPI_ISL_79639
A/mallard/Germany-BW/SR520/2007(H3N2)	EPI_ISL_79638
A/mallard/Germany-BW/SR519/2007(H3N2)	EPI_ISL_79637
A/common_teal/Netherlands/7/2000(H3N8)	EPI_ISL_15008
A/mallard/Iceland/1007/2011(H3N6)	EPI_ISL_148200
A/mallard/Czech_Republic/14333-1K/2011(H3N8)	EPI_ISL_116136
A/mallard/Czech_Republic/14516/2007(H3N8)	EPI_ISL_63529
A/mallard/Sweden/50/2002(H3N8)	EPI_ISL_73381
A/mallard/Netherlands/5/2001(H3N6)	EPI_ISL_73371
A/mallard/Netherlands/2/1999(H3N5)	EPI_ISL_73370
A/common_teal/Sweden/1/2003(H3N3)	EPI_ISL_73363
A/mallard/Netherlands/1/2007(H3N2)	EPI_ISL_33850
A/mallard/Switzerland/WV4060167/2006(H3N5)	EPI_ISL_33832
A/turnstone/Netherlands/1/2007(H3N8)	EPI_ISL_30805
A/common_eider/Netherlands/1/2006(H3N8)	EPI_ISL_30804
A/mallard/Netherlands/3/2005(H3N8)	EPI_ISL_30793
A/teal/Chany/736/2008(H3N8)	EPI_ISL_97501
A/mallard/Czech_Republic/13577-24K/2010(H3N8)	EPI_ISL_89980
A/mallard/Netherlands/28/2006(H3N1)	EPI_ISL_84553
A/wigeon/Italy/3818-34/05(H3N8)	EPI_ISL_85911
A/mallard/Italy/4394-10/05(H3N8)	EPI_ISL_85910
A/chicken/Italy/3582-51/10(H3N8)	EPI_ISL_85902
A/duck/Italy/3139-2/06(H3N8)	EPI_ISL_85901
A/duck/Italy/6207/08(H3N6)	EPI_ISL_85900

Appendix 4.2

Table S4.2 Samples collected for virus and antibody detection from free-living mallards during the H3 LPAIV epizootic in 2010. Samples were collected from resident birds (*RES*) that were first captured (primary) and recaptured, local migrants (*LM*) and distant migrants (*DM*), and were specified by age (juvenile: <1 year, adult: >1 year) and sex.

			RES		LM	DM
	Age	Sex	Primary	Recapture		
Virology	Juvenile	Male	9	7	25	23
		Female	8	5	8	11
	Adult	Male	42	26	31	31
		Female	35	17	49	33
	Total		94	55	113	98
Serology	Juvenile	Male	8	5	27	25
		Female	8	4	8	11
	Adult	Male	32	14	27	30
		Female	31	7	44	30
	Total		79	30	106	96

Appendix 5.1

Table S5.1 Correlation coefficients between body mass and five immunological variables measured in free-living mallards. Variables that are significantly correlated are depicted in bold. Body mass* haptoglobin, and lysis score*H:L ratio (i.e. heterophil and lymphocyte ratio) were used separately as response variables due to the low r^2. Agglutination score*lysis score correlated strongly, hence a single measure (called lysis-agglutination score) was created by taking the average of both factors.

Correlation	*r*	*t*-value	*P*-value	r^2
Body mass*agglutination score	0.07	1.14	0.256	0.00
Body mass*lysis score	0.01	0.21	0.838	-0.00
Body mass*AIV-specific antibodies	-0.09	-1.42	0.156	0.00
Body mass*haptoglobin	**-0.19**	-3.15	**0.002**	0.03
Body mass*H:L ratio	-0.11	-1.72	0.086	0.01
Agglutination score*lysis score	**0.56**	10.98	**<0.001**	0.31
Agglutination score*AIV-specific antibodies	0.01	0.22	0.829	-0.00
Agglutination score*haptoglobin	-0.05	-0.74	0.462	-0.00
Agglutination score*H:L ratio	0.09	1.52	0.131	0.01
Lysis score*AIV-specific antibodies	0.06	1.04	0.298	0.00
Lysis score*haptoglobin	-0.00	-0.04	0.965	-0.00
Lysis score*H:L ratio	**0.13**	2.11	**0.036**	0.01
AIV-specific antibodies*haptoglobin	0.01	0.21	0.832	-0.00
AIV-specific antibodies*H:L ratio	0.04	0.73	0.464	-0.00
Haptoglobin*H:L ratio	0.04	0.57	0.569	-0.00

Appendix 6.1

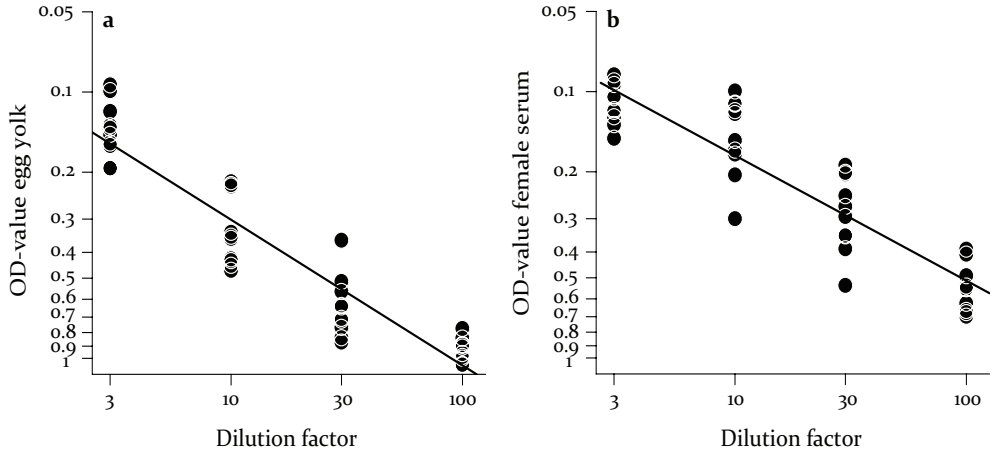


Figure S6.1 OD-values of antibodies against avian influenza virus as a function of dilution factor (i.e. 1/3, 1/10, 1/30, 1/100). (a) Egg yolk: $y = -0.55x + 1.07$, $r^2 = 0.73$ and (b) female serum: $y = -0.47x + 1.23$, $r^2 = 0.78$. Lines represent significant least square regression lines. Note: x-axes and y-axes are log-scaled.

Appendix 6.2

Table S6.1 Correlation coefficients between the four continuous factors of interest for the field and captive study separately. Factors that are significantly correlated are depicted in bold.

	Field study				Captive study			
Correlation	*r*	*t*-value	*P*-value	r^2	*r*	*t*-value	*P*-value	r^2
Body mass*female AIV antibody concentration	0.38	4.38	**<0.001**	0.14	0.14	0.79	0.433	0.05
Body mass*egg volume	0.43	5.12	**<0.001**	0.16	0.26	1.52	0.138	0.04
Body mass*egg laying order					0.13	0.69	0.496	-0.02
Female AIV antibody concentration*egg volume	0.07	0.75	0.455	0.00	-0.28	-1.65	0.109	0.05
Female AIV antibody concentration*egg laying order					0.28	1.65	0.110	0.05
Egg volume*egg laying order					-0.31	-1.80	0.082	0.07

Appendix 6.3

Table S6.2 Model selection on the basis of Akaike Information Criterion (AIC) corrected for small sample sizes (AIC_c) to assess the better models to test the relationship between maternal AIV antibody concentration in egg yolk and female body mass (m), female AIV antibody concentration (a), egg laying order (l), egg volume (v), embryo sex (es) and all two-way interactions for the field and captive study. Female size (s) and embryo size (esi) were included as covariates. Competing models were ranked according to ΔAIC_c. The better models were defined as those models with the least number of parameters (*df*) within a ΔAIC_c<2 relative to the best-supported model (i.e. the model with the lowest AIC_c). The better models are shown in black.

Study	Model	Variables	*df*	logLik	AIC_c	ΔAIC
Field study	6	m+s+a+v+es+esi + m*a	10	106.65	-193.3	0.00
	5	m+s+a+v+es+esi + m*a + m*v	11	107.52	-193.0	0.27
	4	m+s+a+v+es+esi + m*a + m*v + m*es	12	108.00	-192.0	1.30
	7	**m+s+a+v+es+esi**	**9**	**104.91**	**-191.8**	**1.49**
	3	m+s+a+v+es+esi + m*a + m*v + m*es + a*v	13	108.19	-190.4	2.92
	1	m+s+a+v+es+esi + m*a + m*v + m*es + a*v + a*es + v*es	15	109.48	-189.0	4.33
	2	m+s+a+v+es+esi + m*a + m*v + m*es + a*v + a*es	14	108.39	-188.8	4.52
Captive study	**7**	**m+s+a+v+l+esi**	**9**	**39.44**	**-60.9**	**0.00**
	6	m+s+a+v+l+esi + m*a	10	39.44	-58.9	2.00
	5	m+s+a+v+l+esi + m*a + m*v	11	40.07	-58.1	2.74
	4	m+s+a+v+l+esi + m*a + m*v + m*l	12	40.10	-56.2	4.67
	3	m+s+a+v+l+esi + m*a + m*v + m*l + a*v	13	40.12	-54.2	6.63
	2	m+s+a+v+l+esi + m*a + m*v + m*l + a*v + a*l	14	40.93	-53.9	7.02
	1	m+s+a+v+l+esi + m*a + m*v + m*l + a*v + a*l + v*l	15	40.93	-51.9	9.01

Appendix 7.1

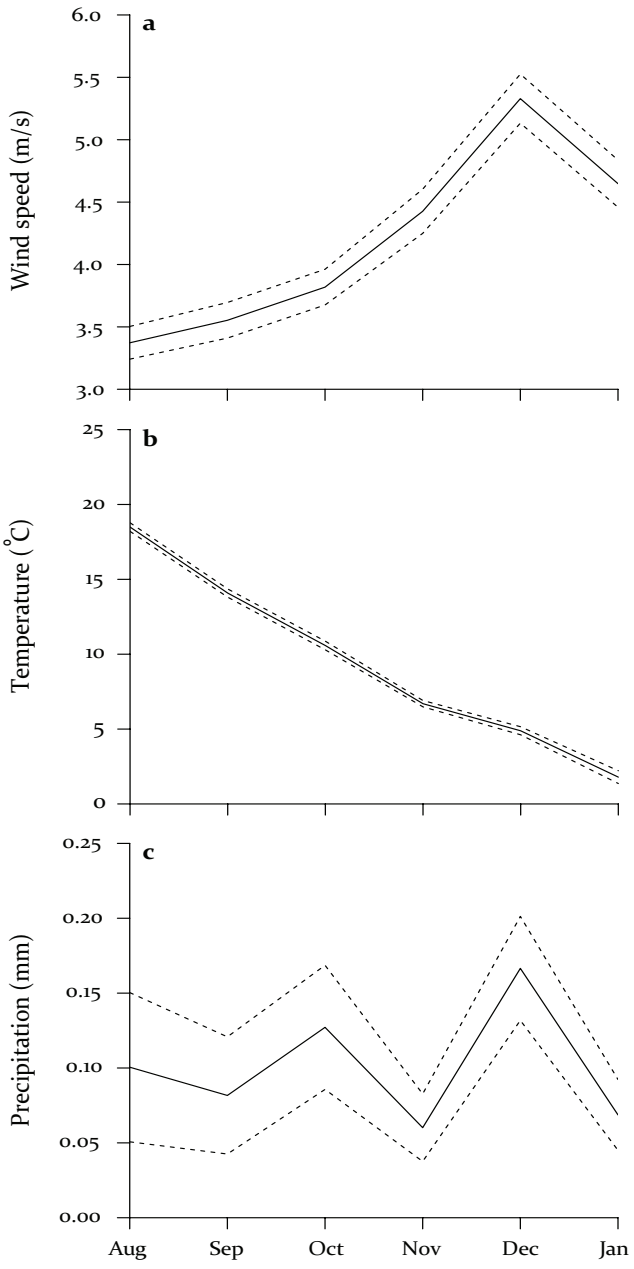
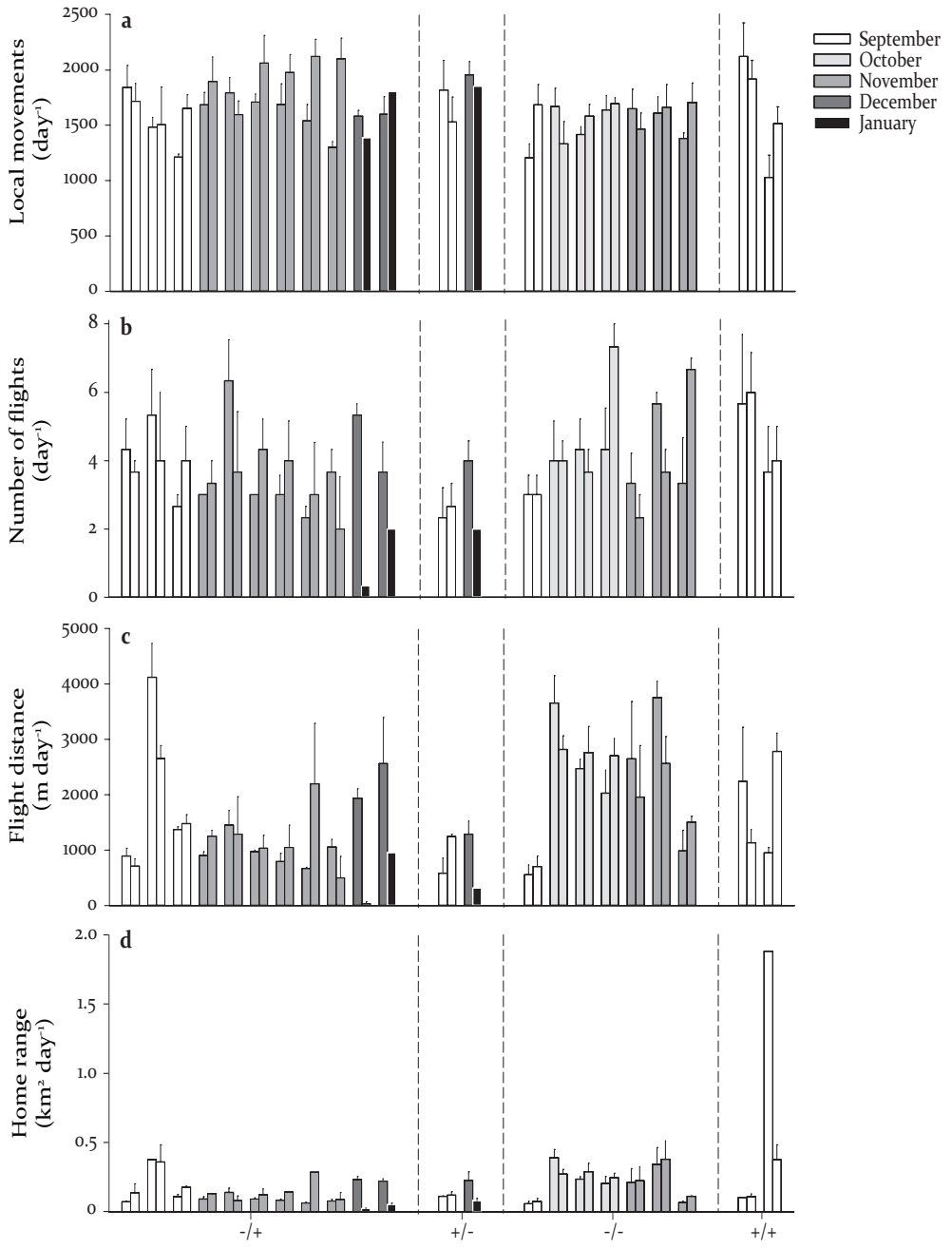


Figure S7.1 Monthly average weather parameters (± 95% CI of the mean, calculated over hour averages: wind speed, temperature, or sums: precipitation) collected at Cabauw (51°57'55"N, 4°53'52"E), the Netherlands, a station of the Royal Netherlands Meteorological Institute, situated 16 km from the study site (i.e. duck decoy near Oud Alblas). (a) Wind speed (m/s), (b) temperature (°C) and (c) precipitation (mm).

Appendix 7.2


a
b
c
d
Local movements (day⁻¹)
Number of flights (day⁻¹)
Flight distance (m day⁻¹)
Home range (km² day⁻¹)
2500
2000
1500
1000
500
0
8
6
4
2
5000
4000
3000
1.5
1.0
0.5
-/+
+/-
-/-
+/+
September
October
November
December
January

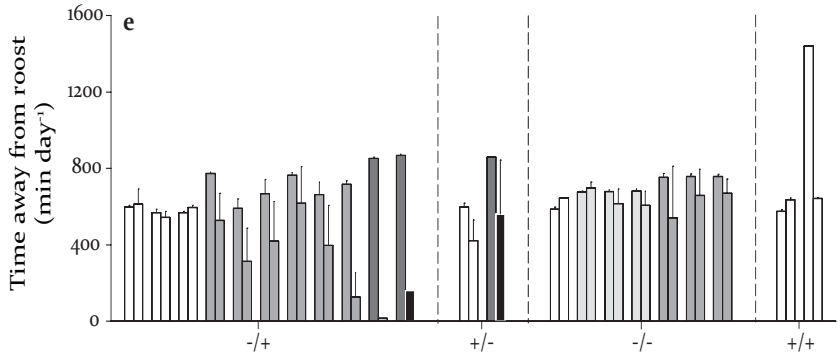
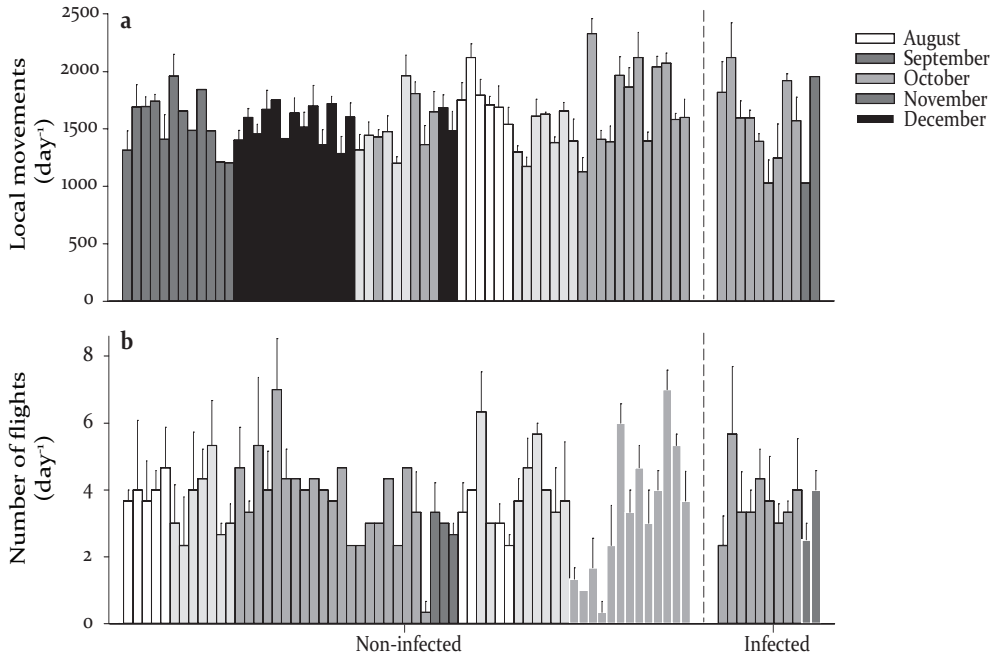


Figure S7.2 Basic data plot depicting for each individual mallard for which LPAIV infection status was known both at day 0 (i.e. GPS logger deployment) and at the day of GPS logger removal the averages of five movement parameters recorded three days after logger deployment (first bar) and three days before logger removal (second bar). The five movement parameters are (a) local movements, (b) number of flights, (c) flight distance, (d) home range and (e) time away from the roost (i.e. duck decoy). Individuals were either non-infected at logger deployment and infected with LPAIV at logger removal (-/+), infected at logger deployment and non-infected at logger removal (+/-), non-infected (-/-) or infected (+/+) at logger deployment and removal. De colour defines the month of logger deployment and removal.



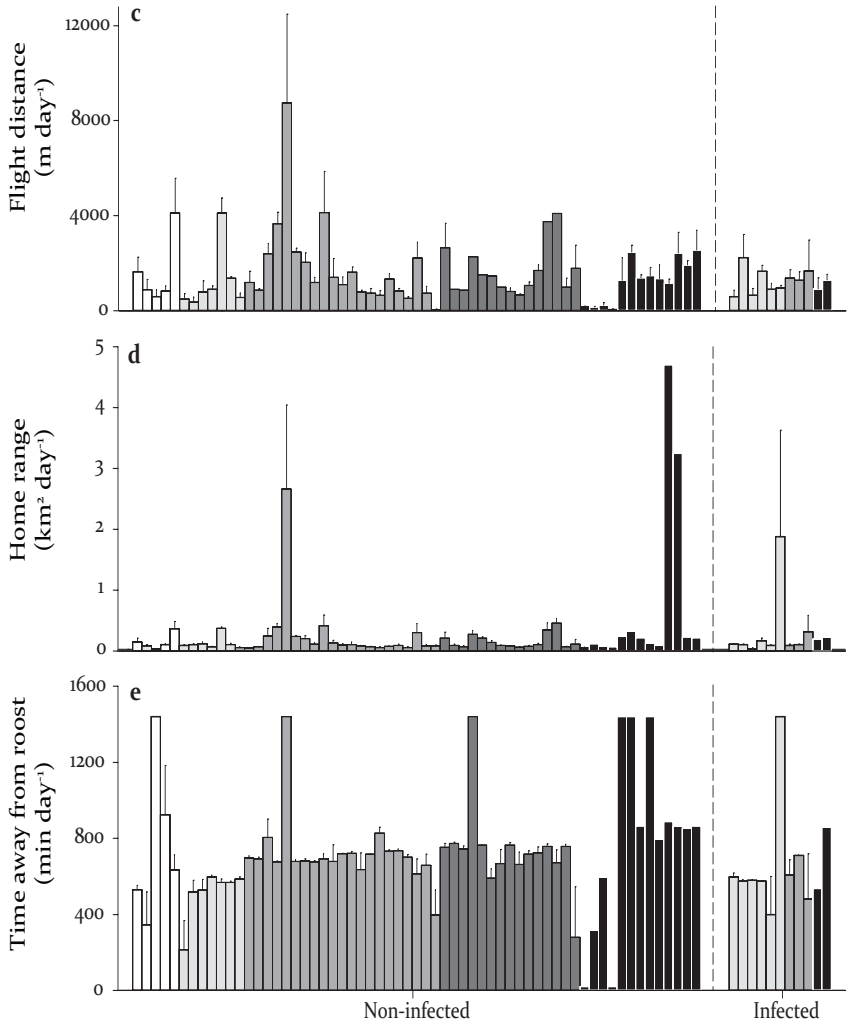
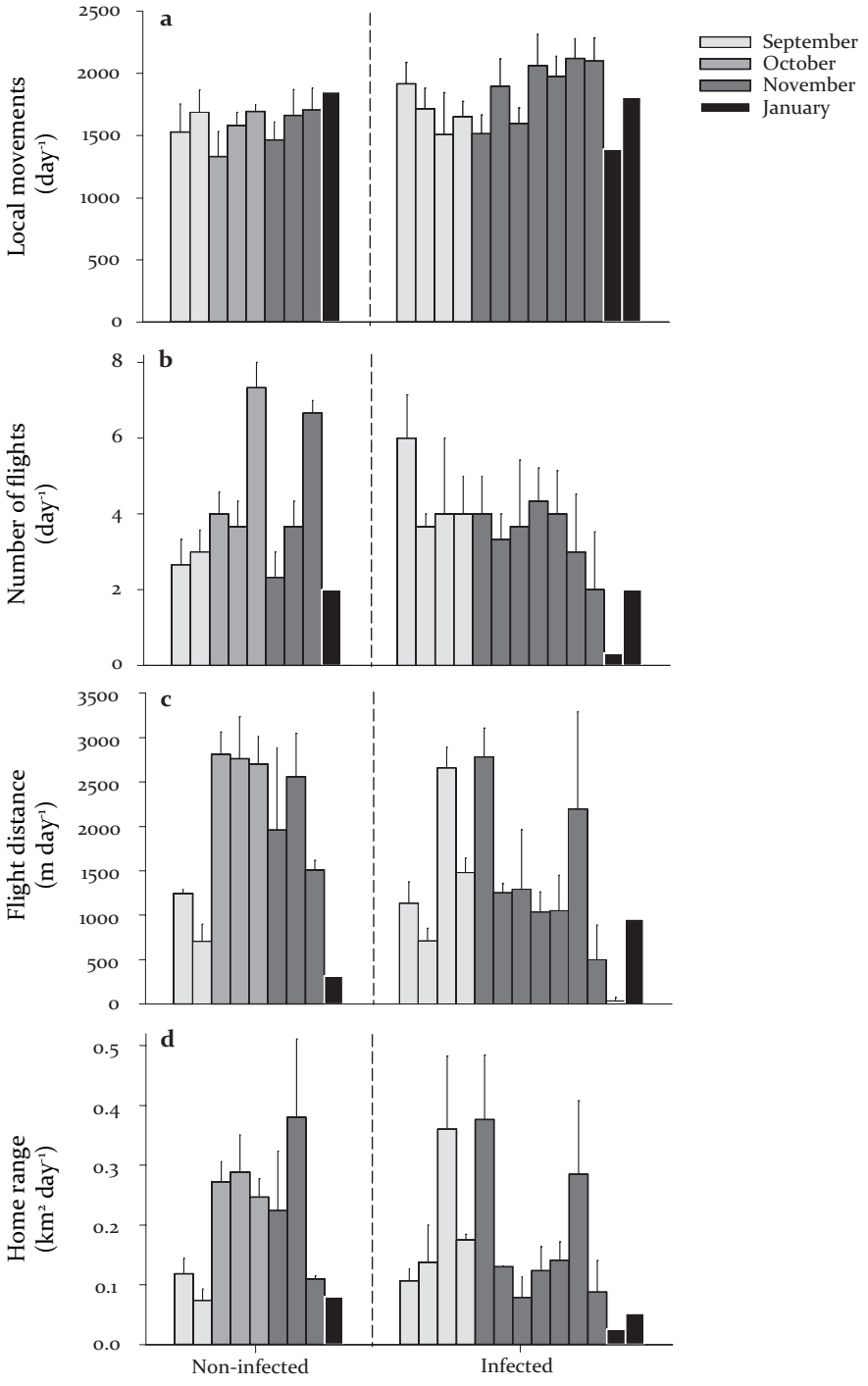


Figure S7.3 Basic data plot depicting for each individual mallard for which LPAIV infection status was known at day 0 (i.e. GPS logger deployment) the averages of five movement parameters recorded three days after logger deployment. The five movement parameters are (a) local movements, (b) number of flights, (c) flight distance, (d) home range and (e) time away from the roost (i.e. duck decoy). The colour defines the month of logger deployment.


a
b
c
d
Local movements (day⁻¹)
Number of flights (day⁻¹)
Flight distance (m day⁻¹)
Home range (km² day⁻¹)
2500
2000
1500
1000
500
0
8
6
4
2
0
3500
3000
2500
2000
1500
1000
500
0
0.5
0.4
0.3
0.2
0.1
0.0
September
October
November
January
Non-infected
Infected

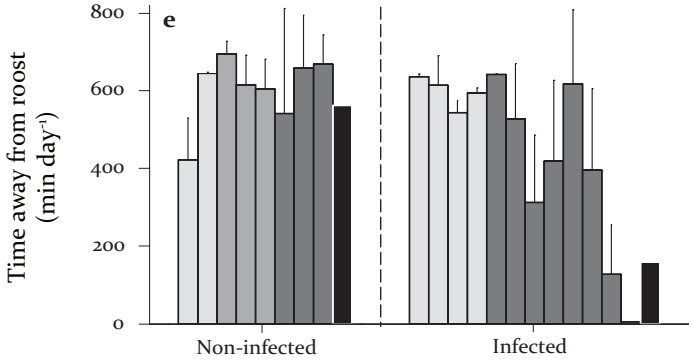


Figure S7.4 Basic data plot depicting for each individual mallard for which LPAIV infection status was known at day of GPS logger removal the averages of five movement parameters recorded three days before logger removal. The five movement parameters are (a) local movements, (b) number of flights, (c) flight distance, (d) home range and (e) time away from the roost (i.e. duck decoy). The colour defines the month of logger removal.

Appendix 7.3

Table S7.1 Correlation coefficients between the movement parameters calculated from tracking data of free-living adult male mallards fitted with GPS loggers. Parameters that are significantly correlated are depicted in bold. Local movements were retained in the statistical analyses, since the r^2 of the significant correlation with home range was low.

Correlation	*r*	*t*-value	*P*-value	r^2
Local movements*flight distance	-0.00	-0.02	0.985	-0.00
Local movements*number of flights	0.07	1.18	0.240	0.00
Local movements*home range	**0.14**	2.33	**0.021**	0.02
Local movements*time spent away from roost	0.02	0.33	0.741	-0.00
Flight distance*number of flights	**0.84**	26.03	**<0.001**	0.71
Flight distance*home range	**0.50**	9.61	**<0.001**	0.24
Flight distance*time spent away from roost	0.54	10.78	**<0.001**	0.29
Number of flights*home range	**0.45**	8.54	**<0.001**	0.20
Number of flights*time spent away from roost	**0.53**	10.45	**<0.001**	0.28
Home range*time spent away from roost	**0.37**	6.76	**<0.001**	0.14

Table S7.2 Correlation coefficients between the weather parameters measured at a station of the Royal Netherlands Meteorological Institute close to the roost (i.e. duck decoy). Parameters that are significantly correlated are depicted in bold.

Correlation	*r*	*t*-value	*P*-value	r^2
Wind speed*temperature	-0.08	-1.39	0.167	0.00
Wind speed*precipitation	**0.62**	13.25	**<0.001**	0.38
Temperature*precipitation	-0.08	-1.37	0.171	0.00

Table S7.3 Factor loadings (i.e. correlation between principal components and variables) of the first principal component (PC1) of three principal-component analyses directed at finding indices for daily regional movements, wind-precipitation and bird size.

PC1	Parameter	Factor loading
Regional movements	Number of flights	0.55
	Flight distance	0.56
	Home range	0.42
	Time away from roost	0.46
Wind-precipitation	Wind speed	0.71
	Precipitation	0.71
Bird size	Tarsus length	0.57
	Head+bill length	0.66
	Wing length	0.50

Appendix 7.4

Table S7.4 Number of mallards positive (+) or negative (-) for LPAIV or AIV-specific antibodies either at logger deployment (i.e. day 0), at logger removal or both at deployment and removal.

		LPAIV infection	AIV-antibodies
Logger deployment	+	11	47
	-	61	25
Logger removal	+	13	
	-	9	
Logger deployment/removal	-/+	11	
	+/-	2	
	-/-	7	
	+/+	2	

Appendix 8.1

Population dynamics were based on bird numbers observed at the study site during the study period. The demographic parameters and their distribution over the annual cycle are shown in Fig. S8.1. In the model, all mallards experience natural mortality at rate μ, and additional hunting mortality $\mu_h(t)$, which is fixed during the official local hunting period (end of August till end of January) and 0 otherwise (Conn and Kendall 2004, but see Sedinger and Herzog 2012). During a fixed post-fledging period, juveniles experience a higher mortality, at rate $\mu_{juv}(t)$, of which the time course follows a sigmoidal curve, described by a cumulative normal distribution. Mortality rates were estimated based on the average life expectancy of mallards (Schekkerman and Slaterus 2008) and a 30% contribution of hunting during the official hunting period (Hirschfeld and Heyd 2005). Juvenile mortality was estimated to maintain a stable population size of 600 individuals N_{pop}, i.e. the size of the estimated local mallard population at the study site. Birth occurs at rate $B(t)$ (following a normal density curve) multiplied by a fixed number of hatchlings (6) and half of the local adult population size (300). Mallards are more solitary whilst breeding and during the first weeks after the chicks hatch, therefore the mean for B is set to reflect the mean date at which juveniles fledge and effectively enter the population (in an epidemiological sense) (i.e. 7 July 2010). The sd of B reflects temporal distribution of birth in the observed population. Similarly to birth, the arrival of migrants is modelled such as to obtain a normally distributed arrival pattern: the stable local population size is multiplied by a normal distribution density function (m_{in}) times three (in autumn the population constitutes of 1/3rd locals and 2/3rd migrants). The replacement of migrants m_r was modelled using a symmetric double logistic function with mean, amplitude, slope and kurtosis as shaping parameters. Since a proportion δ of juveniles transits into adults after LPAIV infection, in which they move from R_j (recovered) to S_a (susceptible), this transition has an effect on the population dynamics. At day 59 (1st of March) all remaining juveniles enter the compartments S_a, I_a or R_a dependent on their compartments (S_j, I_j or R_j). At the same day (1st of March) all migrants depart.

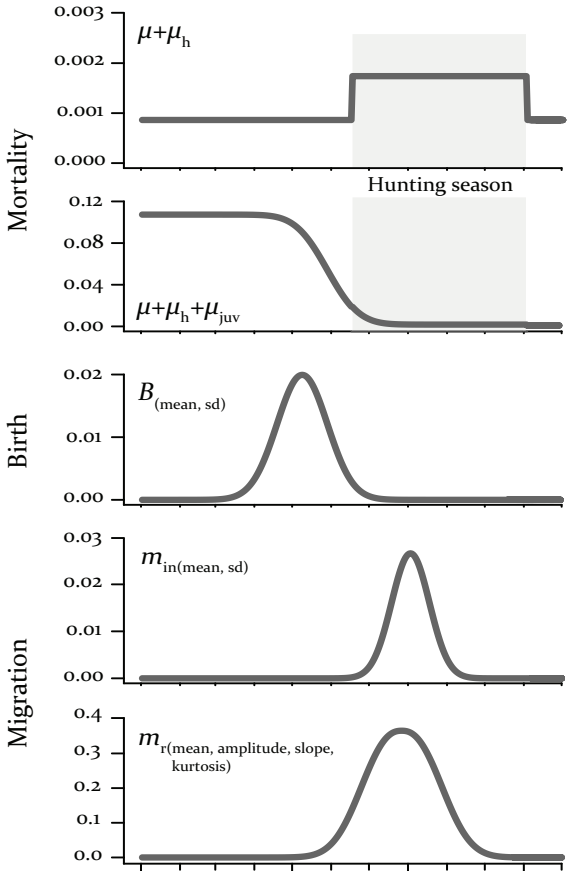


Figure S8.1 Dynamics of the demographic parameters over the annual cycle.

Infection dynamics equations

To improve readability of the equations, we did not explicitly include the time-dependence of the parameters describing birth, death and migration (see Table 8.1 and Fig. S8.1).

Migrants

$$(dS_m(t))/dt = -\beta_m(I_m+I_j+I_a)S_m + \sigma R_m - (\mu+\mu_h)S_m + (S_a+I_a+R_a)m_{in} + (I_m+R_m)m_r - \eta S_m$$

$$(dI_m(t))/dt = \beta_m(I_m+I_j+I_a)S_m - (\gamma+\mu+\mu_h)I_m - m_r I_m + \eta S_m$$

$$(dR_m(t))/dt = \gamma I_m - (\sigma+\mu+\mu_h)R_m - m_r R_m$$

Local juveniles

$$(dS_j(t))/dt = -\beta_j (I_m+I_j+I_a) S_j + (1-\delta)\sigma R_j - (\mu+\mu_h+\mu_{juv})S_j + B(N_{pop}N_{hatch}/2) - \eta S_j$$

$$(dI_j(t))/dt = \beta_j(I_m+I_j+I_a)S_j - (\gamma+\mu+\mu_h+\mu_{juv})I_j + \eta S_j$$

$$(dR_j(t))/dt = \gamma I_j - \sigma R_j - (\mu+\mu_h+\mu_{juv})R_j$$

Local adults

$$(dS_a(t))/dt = -\beta_a(I_m+I_j+I_a)S_a + (\delta R_j+\sigma)R_a - (\mu+\mu_h)S_a - \eta S_a$$

$$(dI_a(t))/dt = \beta_a(I_m+I_j+I_a)S_a - (\gamma+\mu+\mu_h)I_a + \eta S_a$$

$$(dR_a(t))/dt = \gamma I_a - \sigma R_a - (\mu+\mu_h)R_a$$

Table S8.1 Monthly number of individual mallards that were primary captured (P) and recaptured (R) per non-infected and LPAIV infected group, divided per age and sex.

	Non-infected								Infected							
	Adult				Juvenile				Adult				Juvenile			
	Female		Male		Female		Male		Female		Male		Female		Male	
Month	P	R	P	R	P	R	P	R	P	R	P	R	P	R	P	R
September	23		24		0		4		11		16		5		2	
October	15	2	6	4	1	0	0	1	10	4	4	10	1	3	2	1
November	7	13	11	10	0	0	0	1	1	3	1	9	0	4	0	2
December	5	4	1	8	0	0	1	1	4	3	3	7	0	2	0	4
January	8	4	3	7	0	0	0	2	0	7	0	9	0	1	0	1
February	18	13	12	21	0	0	0	1	0	17	0	16	0	2	0	1
March		33		37		1		2		12		17		5		2
Total	76	69	57	87	1	1	5	8	26	46	24	68	6	17	4	11

Table S8.2 Reduced parameterizations of recapture probability (*p*), using the full parameterization of local survival ($\Phi_{t\cdot i\cdot s+i\cdot a}$). K is the number of parameters, deviance is the difference in likelihood (-2logL) with the saturated model, and AIC_c is the Akaike Information Criterion corrected for small sample sizes.

p	K	ΔDeviance	ΔAIC_c
t·i+s+a	38	0.00	0.00
t+i+s+a	34	10.03	0.33
t+i+s	33	12.77	0.67
t·s+i+a	38	7.87	7.87
t+i	32	22.81	8.33
t+i+a	33	21.57	9.47
t·i	36	15.22	10.34
t+s+a	33	29.83	17.73
t+s	32	39.14	24.67
t+a	32	43.26	28.79
s+i	29	115.68	94.14

Deviance = 345.69

AIC_c = 1048.58

t = time (months)

i = infection status (infected/non-infected)

s = sex (male/female)

a = age (juvenile/adult)

Table S8.3 Reduced parameterizations of local apparent survival (Φ), where c represents constant survival. Recapture probability (p) is modelled as in the most parsimonious model in Table S8.2: p_{t+i+s}. Results were similar when p was modelled as in the best-supported parameterization of Table S8.2: $p_{t\cdot i+s+a}$. K is the number of parameters, deviance is the difference in likelihood (-2logL) with the saturated model, and AIC_c is the Akaike Information Criterion corrected for small sample sizes.

Φ	K	ΔDeviance	ΔAIC_c
c	9	23.47	0.00
T	13	16.24	1.25
a	10	23.06	1.69
I	10	23.35	1.98
t+i	14	16.02	3.18
t+a	14	16.10	3.26
t+i+a	15	15.54	4.87
i·a	12	22.6	5.47
t+i·a	16	15.51	7.00
t·i + a	20	14.34	14.63
t·i+i·a	21	14.33	16.86
t·i·s + i·a	33	0.00	30.24

Deviance = 358.46

AIC_c = 1019.01

t = time (months)

i = infection status (infected/non-infected)

s = sex (male/female)

a = age (juvenile/adult)

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Summary
Nederlandse samenvatting

Summary

A large proportion of infectious diseases in man originate in wildlife, for example HIV/AIDS, SARS, Ebola haemorrhagic fever and just recently H7N9 influenza. These zoonotic pathogens may pose a serious threat for humans, requiring a better understanding of the ecology and transmission of these pathogens in their natural (wildlife) hosts. Unravelling the ecological processes underlying the dynamics of pathogens in wildlife can be important to develop adequate control measures, for example to prevent disease emergence and spread to other hosts.

The zoonotic pathogen studied in this thesis is avian influenza virus (AIV). The low pathogenic phenotype of this pathogen (called low pathogenic avian influenza virus, LPAIV) circulates naturally in wild birds. Birds of wetlands and aquatic environments (orders *Anseriformes*: ducks, swans, geese and *Charadriiformes*: gulls, terns, waders) are considered the main LPAIV reservoir. Nearly all LPAIV subtypes, i.e. combinations of the hemagglutinin (H1-H16) and neuraminidase (N1-N9) surface proteins, are found in waterfowl. LPAIV causes transient and mainly intestinal infections in waterfowl, with no or limited signs of disease. Two LPAIV subtypes, i.e. H5 and H7, may become highly pathogenic (called highly pathogenic avian influenza virus, HPAIV) after introduction into poultry, resulting in disease outbreaks. These disease outbreaks, previously known as 'fowl plague' can cause 100% mortality in unprotected poultry flocks, and may cause illness and occasional deaths in humans and wild birds. One of the most well-known HPAIV outbreaks in recent history is caused by the H5N1 virus lineage that was first detected in Asia in 1996, which led to the worldwide culling of hundreds of millions of poultry and also caused almost 400 human deaths. In the last 10 years, more than 150 outbreaks of HPAIV in poultry were reported in various countries around the world. Just recently it became clear that not only HPAIV, but also LPAIV may pose a serious threat to man. The outbreak of LPAIV H7N9 in China has led to more than 100 human deaths since its first detection in February 2013, while wild birds and poultry show no clear symptoms of disease. The role of wild birds in the global spread of HPAIV, and to some extent also LPAIV, is still largely unclear, although their contribution to the spread of this infectious disease has often been suggested. Hence, it is of great societal interest to clarify the role of wild birds in the dynamics of LPAIV, which represents one of the greatest concerns for public and animal health that has emerged from the animal reservoir.

The aim of this study was to advance our knowledge of the ecological processes underlying the epidemiology of LPAIVs in wild birds, by investigating the host-pathogen interaction between a free-living key LPAIV host species, the mallard *(Anas platyrhynchos),*

and LPAIV at a local scale in the Netherlands. Mallards are frequently infected with LPAIV and harbour nearly all LPAIV subtypes found in birds to date. Just like other waterfowl species, mallards show little to no signs of disease when experimentally infected with LPAIV. For HPAIVs, mallards are also an important study species, since they are closely related to domestic ducks (Pekin duck, *Anas platyrhynchos domesticus*) and may respond asymptomatically when experimentally infected with this virus. Most of the work of this study was conducted at a duck decoy (i.e. swim-in traps connected to a large pond), which was located near Oud Alblas in the Alblasserwaard (51°52′38″N, 4°43′26″E), the Netherlands. Throughout a complete annual cycle, mallards were caught and sampled to investigate the underlying mechanisms of seasonal dynamics of LPAIV infections in wild birds, the role of migratory and resident hosts in LPAIV epizootics (i.e. epidemic in animals), and associations between LPAIV infection and host condition, immune status and behaviour. Throughout Europe mallards are partially migratory, meaning that the population consist of both migratory and resident birds. In autumn and winter, migratory and resident mallard populations mix at the wintering grounds, like the Netherlands, enabling studies on the role of both these host populations in LPAIV infection dynamics.

A frequently used technique to assess the origin or geographic location where individual birds moulted their feathers is by analysing hydrogen stable isotopes (δ^2H) in these feathers. This technique is based on the principle that feather δ^2H is closely correlated with precipitation δ^2H. During the period of feather growth, precipitated water is passed through the food web and incorporated into feathers. A calibration curve is needed to convert feather δ^2H to precipitation δ^2H in order to generate a feather δ^2H isoscape (i.e. isotope landscape) to identify the moulting location of individual birds. We collected juvenile mallard feathers of natal origin from 38 locations across 14 European countries and analysed these to create a calibration curve and a European feather δ^2H isoscape specifically for mallards (Chapter 2). We compared our mallard calibration curve with a pre-existing calibration curve that was based on feather samples across several species, age classes and years. Factors like species, age and year might generate isotopic variation in a calibration curve and herewith may affect provenance accuracy. As might be expected, our mallard calibration curve yielded a higher coefficient of determination and was more accurate in assessing the origin of mallards than the pre-existing calibration curve. However, we found that only a relatively small gain in precision can be achieved by creating a species, age, and year specific calibration curve, rather than by resorting to more general calibration curves.

After the emergence of HPAIV H5N1, many surveillance schemes were initiated around the globe to sample wild birds as an early warning system for this pathogen, often extended to include other AIV subtypes. Through these schemes it was found that LPAIV

infection in free-living ducks exhibits marked seasonal variation at the northern hemisphere. However, by the nature of the underlying surveillance schemes these seasonal LPAIV infection dynamics are primarily based on compilations of data from separate studies, which are often focussed on a single season of the annual cycle in which a wide range of duck species was sampled in a highly opportunistic manner. To study the temporal dynamics of LPAIV infections in a key host species (mallard) in a rigorous fashion, avoid the aforementioned shortcomings, we comprehensively sampled mallards on a small spatial scale (duck decoy) throughout a complete annual cycle, measuring both current and past infection (i.e. LPAIV prevalence and AIV antibody prevalence, respectively). We demonstrated a minor LPAIV infection peak in summer and a dominant peak in autumn, whereas prevalence of antibodies against AIV peaked in winter and spring (Chapter 3).

The summer LPAIV infection peak was likely driven by the entrance of unfledged naïve juveniles into the resident population, as they were more likely to be infected, shed higher quantities of virus and were less likely to have AIV antibodies than adults (Chapter 3). Still, we discovered that in this period nearly half of the mallard eggs received maternal AIV antibodies (Chapter 6). Maternal antibodies are transferred from mothers to their offspring to grant neonates protection against infection with pathogens early in life. In addition, we found that maternal AIV antibody concentrations in egg yolk correlated positively with circulating AIV antibody concentrations in females and correlated positively with egg laying order. Female body mass, egg size, and embryo sex were not associated with maternal AIV antibody concentrations in eggs. The large proportion of mallard eggs receiving maternal AIV antibodies suggests that these antibodies may play an important role in shaping LPAIV infection dynamics in free-living mallards.

The large autumn LPAIV infection peak was likely driven by the arrival of susceptible migratory birds on the wintering grounds, since both short and long distance migrants were more likely to be infected than residents and showed low AIV antibody prevalence (Chapter 3). Both LPAIV infection peaks did not correspond with the highest density of mallards, which was found in winter, although it cannot be ruled out that host density is an additional driver of LPAIV infection dynamics in wild birds. Even though this study was conducted at a single location on a small spatial scale, we were able to show, using the results of an additional study conducted two years later, that the autumn and winter LPAIV prevalence patterns observed in our study were representative for the LPAIV infection dynamics in free-living mallards in the Netherlands (Box 8.1).

To assess whether the above suggested drivers indeed play a role in explaining the LPAIV infection dynamics in wild birds, we used a susceptible-infected-recovered-susceptible (SIRS) model to determine whether host-related drivers could explain the LPAIV

infection dynamics as observed in migrants, resident juveniles and adults in our study (Box 8.2). We were able to find parameter settings to simulate the dynamics of LPAIV infections in these three subpopulations, when including transient migrants, host short-term immunity, and separate transmission rates for migrants, resident juveniles and adults. Even though this modelling exercise lends credit to the hypothesised roles of juveniles and migrants as important factors in the epidemiology of LPAIV at our study site, it does not provide us with a true test of our hypothesised drivers. However it is a stimulus for further empirical research on host demography and LPAIV ecology and epidemiology in wild birds.

A multifaceted interdisciplinary approach was used to study the role of migratory and resident hosts in the introduction and maintenance of the autumn LPAIV infection peak (Chapter 4). This infection peak was caused by an H3 LPAIV subtype, which most likely originated from a single virus introduction, followed by local clonal expansion. During the LPAIV epizootic, short and long distance migratory mallards were more often infected with H3 LPAIV than residents, whereas residents shed more H3 virus at the start of the epizootic. H3-specific antibodies were detected in only a few migrants and residents. These results suggest that the autumn LPAIV epizootic was likely initiated by a single introduction of H3 LPAIV in susceptible residents, which was followed by virus amplification that was importantly associated with the influx of migratory mallards. Migratory birds are thus suggested to drive the autumn LPAIV epizootic (Chapter 3) and act as local amplifiers to maintain this epizootic (Chapter 4). But whether migratory birds also acted as a vector, importing novel LPAIV strains from afar, is less clear.

Mallards are often assumed to carry LPAIV asymptomatically, since they show hardly any signs of disease when infected experimentally, albeit supportive evidence from free-living birds is largely lacking. Therefore we investigated potential effects of LPAIV infection on body mass and immune status of free-living mallards throughout the annual autumn LPAIV infection peak (Chapter 5). Immune status was evaluated using five immunological indices. Body mass did not differ between LPAIV infected and non-infected individuals. However, irrespective of LPAIV infection, short and long distance migrants had a lower body mass than residents. Although there were specific differences across the sexes and migratory strategies, immunological indices did not differ or differed only marginally between LPAIV infected and non-infected individuals. These weak associations between LPAIV infection and body condition and immune status of free-living mallards may be considered a support to their role as asymptomatic carriers of LPAIV; it raises the intriguing notion that this may be a consequence of host-pathogen co-evolution, explaining the alleged role of mallards as a key reservoir of LPAIV.

To further investigate whether mallards are truly asymptomatic carriers of LPAIV, we used a mark-recapture model to assess local (or apparent) survival of resident mallards that were either infected or non-infected with LPAIV during the autumn infection peak (Box 8.3). There was no difference in local survival between LPAIV infected and non-infected mallards. Recapture probability was higher in LPAIV infected mallards than in non-infected individuals, suggesting that infected birds remained closer to the study site, which is in line with the findings of Chapter 7 (see below).

For an additional increase in our understanding of the role of wild birds in the spread of LPAIV, we investigated potential effects of LPAIV infection on the movement behaviour of free-living mallards (Chapter 7). Throughout the autumn LPAIV infection peak, we recorded daily local and regional movements of LPAIV infected and non-infected individuals fitted with GPS loggers and compared these movements at both the within and among individual level. Within individuals, there were no associations between LPAIV infection and daily local and regional movements. However among individuals, daily regional movements of LPAIV infected mallards were lower than those of non-infected individuals, which became increasingly lower when weather conditions worsened (i.e. increased wind and/or precipitation, and lower temperatures). Daily local movements did not differ between LPAIV infected and non-infected individuals. These findings suggest that LPAIV infected mallards are probably still capable of transporting viral particles to other areas, although the distance of spread might be lower than one might expect from the behaviour of non-infected individuals.

To conclude, our study showed that (i) autumn is the most critical time period of the year having the highest proportion of mallards infected with LPAIV, (ii) LPAIV epizootics in wild birds likely occur at local scales, (iii) migratory hosts play an important role in LPAIV infection dynamics as a potential driver and reinforcer of the autumn LPAIV epizootic, probably mostly because of their increased susceptibility due to locally circulating viruses rather than by introducing new LPAIV strains from afar, (iv) mallards likely carry LPAIV asymptomatically, and (v) mallards are likely capable of spreading LPAIVs locally and regionally.

Nederlandse samenvatting

Een groot deel van de infectieziekten die in mensen voorkomt, is afkomstig uit het dierenrijk, zoals HIV/AIDS, SARS, Ebola en recentelijk H7N9 influenza. Omdat deze zogenaamde zoönosen een serieuze bedreiging kunnen vormen voor de mens, is het belangrijk om meer inzicht te krijgen in de ecologie en de overdracht van deze ziekteverwekkers in hun natuurlijke (in het wild levende) gastheren. Het ontrafelen van de ecologische processen die ten grondslag liggen aan de dynamiek van ziekteverwekkers in wilde dieren kan namelijk belangrijk zijn om adequate controlerende maatregelen te ontwikkelen, bijvoorbeeld om te voorkomen dat nieuwe ziekten ontstaan en zich verspreiden naar andere gastheren.

De zoönotische ziekteverwekkers bestudeerd in dit proefschrift zijn aviaire influenza virussen (AIV), ofwel vogelgriepvirussen. Het laag pathogene fenotype van deze ziekteverwekker (laag pathogene aviaire influenza virussen, LPAIV, genoemd) circuleert van nature in wilde vogels. Watervogels behorend tot de orde *Anseriformes* (eenden, zwanen en ganzen) en *Charadriiformes* (meeuwen, sternen en steltlopers) vormen het natuurlijke reservoir van LPAIV. Bijna alle LPAIV subtypen, d.w.z. alle combinaties van twee belangrijke oppervlakte-eiwitten die het genetisch materiaal van het virus omhullen, hemagglutinine (H1-H16) en neuraminidase (N1-N9), zijn gevonden in watervogels. LPAIV veroorzaakt kortstondige infecties in watervogels, vooral in de darmen, met geen of weinig ziekteverschijnselen. Na introductie in pluimvee kunnen LPAIV subtypen H5 en H7 hoog pathogeen worden (hoog pathogene aviaire influenza virussen, HPAIV, genoemd) hetgeen dan resulteert in ziekte-uitbraken. Deze ziekte-uitbraken, voorheen bekend als 'vogelpest', kunnen 100% sterfte veroorzaken in onbeschermde groepen pluimvee en kunnen ook ziekte en incidentele sterfgevallen bij mensen en wilde vogels teweegbrengen. Eén van de meest bekende HPAIV uitbraken in de recente geschiedenis is veroorzaakt door een variant van het H5N1-virus welke voor het eerst werd ontdekt in Azië in 1996. Deze uitbraken leidde tot op heden tot het wereldwijd ruimen van honderden miljoenen pluimvee en kostte bijna 400 mensen het leven. In de laatste 10 jaar zijn er meer dan 150 uitbraken van HPAIV in pluimvee gemeld uit diverse landen over de gehele wereld.

Onlangs werd duidelijk dat niet alleen HPAIV, maar ook LPAIV een ernstige bedreiging kan vormen voor de mens. De uitbraak van LPAIV H7N9 in China heeft geleid tot meer dan 100 sterfgevallen sinds de eerste ontdekking in februari 2013, terwijl wilde vogels en pluimvee geen duidelijke ziektesymptomen laten zien. De rol van wilde vogels in de wereldwijde verspreiding van HPAIV en, tot op zekere hoogte, ook LPAIV, is nog grotendeels onduidelijk, hoewel hun bijdrage aan de verspreiding van deze besmettelijke ziekte al vaak is gesuggereerd. Ook gezien de potentie van

vogelgriepvirussen om zich te ontwikkelen tot epidemische ziekteverwekkers in mens en pluimvee is het van groot maatschappelijk belang om de rol van wilde vogels in de dynamiek van deze ziekteverwekkers te verduidelijken.

Om onze kennis te vergroten van de ecologische processen die ten grondslag liggen aan de epidemiologie van LPAIV in wilde vogels hebben we de gastheer-ziekteverwekker interactie onderzocht tussen een belangrijke vrijlevende LPAIV gastheer, de wilde eend *(Anas platyrhynchos)*, en LPAIV. Wilde eenden zijn vaak geïnfecteerd met LPAIV en herbergen bijna alle LPAIV subtypen die tot op heden in vogels zijn gevonden. Net als andere watervogels, vertonen wilde eenden weinig tot geen tekenen van ziekte als ze experimenteel worden geïnfecteerd met LPAIV. Ook vanuit het oogpunt van HPAIV zijn wilde eenden belangrijke studieobjecten omdat ze nauw verwant zijn aan de gedomesticeerde Pekingeend (*Anas platyrhynchos domesticus*). Tevens kunnen wilde eenden asymptomatisch reageren als ze experimenteel worden geïnfecteerd met HPAIV. Ook vanuit praktisch oogpunt zijn wilde eenden een ideaal studioeobject, omdat ze traditioneel worden gevangen in zogenaamde eendenkooien (een stelsel van vangpijpen aangesloten op een grote vijver).

In een eendenkooi in de buurt van Oud Alblas in de Alblasserwaard (51°52′38″N, 4°43′26″E) in Nederland werden gedurende een volledige jaarcyclus wilde eenden gevangen en bemonsterd om de onderliggende mechanismen van de seizoens-dynamiek van LPAIV infecties in wilde vogels te onderzoeken. Ook de rol van migrerende (trekkende) en niet-migrerende (residente) wilde eenden in een LPAIV epidemie werd zo onderzocht, als ook het testen van verbanden tussen LPAIV infectie en lichaamsgewicht, immuun status en gedrag van wilde eenden. Wilde eenden in Europa zijn deeltrekkers wat betekent dat de populatie bestaat uit trekkende en residente individuen. In het najaar en winter mengen deze twee populaties in de overwinteringsgebieden zoals Nederland, wat het mogelijk maakt om de rol van trekkende en residente gastheer populaties in de dynamiek van LPAIV infecties te bestuderen.

Om de herkomst van de wilde eenden te bepalen hebben we isotopenratio's in hun veren gemeten. De geografische locatie waar individuele vogels hun veren hebben geruid kan worden afgeleid van de isotopenratio van waterstof (δ^2H) in deze veren. Deze techniek is gebaseerd op het principe dat δ^2H in veren nauw gecorreleerd is met δ^2H in neerslag. Gedurende de periode van veergroei wordt regenwater doorgegeven via de voedselketen en opgenomen in veren. Een kalibratiecurve is nodig om δ^2H in veren te converteren naar δ^2H in neerslag om zo een δ^2H veren isoscape (d.w.z. een landkaart met isotopenwaardes) te maken om daarmee de ruilocatie van individuele vogels te bepalen. Op 38 verschillende locaties in 14 Europese landen hebben we veren van jonge wilde eenden verzameld en deze geanalyseerd op δ^2H om daarmee een Europese δ^2H veren isoscape specifiek voor wilde

eenden te maken (Hoofdstuk 2). We vergeleken onze wilde eenden kalibratiecurve met een bestaande kalibratiecurve welke gebaseerd was op veermonsters van verschillende soorten, leeftijdscategorieën en jaren. Factoren zoals soort, leeftijd en jaar kunnen mogelijke variatie in een kalibratiecurve en een isoscape veroorzaken en daarmee de nauwkeurigheid van de bepaling van de herkomst van individuele dieren. Zoals te verwachten leverde onze wilde eend kalibratiecurve een hogere coëfficiënt van herkomst op en was deze nauwkeuriger in het vaststellen van de herkomst van wilde eenden dan de bestaande kalibratiecurve. De winst in nauwkeurigheid met het maken van een soort, leeftijd en jaar specifieke kalibratiecurve was echter gering en voor veel vervolgstudies aan andere vogelsoorten zijn de bestaande kalibratiecurven van voldoende kwaliteit.

Na de ontdekking van HPAIV H5N1 zijn er, als een soort vroegtijdig waarschuwingssysteem, over de hele wereld surveillance programma's geïnitieerd waarbij wilde vogels voor dit virus werden bemonsterd. Vaak werden de vogels niet alleen voor H5N1 maar ook op andere subtypen getest. Door middel van deze surveillances werd vastgesteld dat op het noordelijk halfrond LPAIV infecties in vrijlevende eenden een opvallende seizoensvariatie vertonen. Echter, door de aard van deze surveillance programma's was dit patroon voornamelijk gebaseerd op een compilatie van gegevens uit afzonderlijke studies, die vaak gericht waren op slechts een enkel seizoen en waarbij een breed scala aan eendensoorten op tamelijk opportunistische wijze was bemonsterd. Om deze temporele dynamiek van LPAIV infecties in een belangrijke gastheer (wilde eend) zonder de eerder genoemde tekortkomingen te bestuderen, hebben we op uitvoerige wijze wilde eenden bemonsterd op een kleine ruimtelijke schaal (in één eendenkooi) gedurende een gehele jaarcyclus, waarbij zowel huidige infecties (d.w.z. LPAIV prevalentie) als ook historische infecties (d.w.z. de prevalentie van antistoffen tegen vogelgriepvirussen) werden geëvalueerd. Aldus toonden we aan dat er in de zomer een kleine LPAIV infectiepiek is en een dominante infectiepiek in het najaar, terwijl de prevalentie van antistoffen tegen vogelgriepvirussen piekt in de winter en in het voorjaar (Hoofdstuk 3).

De zomer LPAIV infectiepiek werd waarschijnlijk veroorzaakt door de aanwas van immunologisch naïeve, jonge eenden (Hoofdstuk 3). Deze jonge eenden waren vaker geïnfecteerd, scheden grotere hoeveelheden virus uit en hadden minder antistoffen tegen vogelgriepvirussen dan oudere vogels. Toch ontdekten wij dat in deze periode bijna de helft van de eieren van wilde eenden maternale antistoffen tegen vogelgriepvirussen ontvangen (Hoofdstuk 6). Maternale antistoffen worden overgedragen van moeders op hun nakomelingen om hun pasgeborenen bescherming te geven tegen infectie van ziekteverwekkers. Daarnaast vonden wij dat de concentratie van antistoffen tegen vogelgriepvirussen in het eigeel positief gecorreleerd waren met circulerende concentraties van antistoffen

tegen vogelgriepvirussen in de vrouwtjes waarvan de eieren afkomstig waren, en positief gecorreleerd waren met de volgorde waarin de eieren waren gelegd. Lichaamsgewicht van vrouwtjes, grootte van het ei en het geslacht van het embryo waren niet geassocieerd met de concentraties van maternale antistoffen tegen vogelgriepvirussen in de eieren. Dat een groot deel van de eieren van wilde eenden maternale antistoffen tegen vogelgriepvirussen had suggereert dat deze antistoffen een belangrijke rol kunnen spelen in de dynamiek van LPAIV infecties in vrijlevende wilde eenden.

De grote LPAIV infectiepiek die we in het najaar vonden werd waarschijnlijk veroorzaakt door de aankomst van vatbare, trekkende wilde eenden in het overwinteringsgebied (Hoofdstuk 3). Zowel trekvogels die van ver als ook van korte afstand kwamen waren vaker geïnfecteerd dan residente wilde eenden. Ook hadden trekkende individuen weinig antistoffen tegen vogelgriepvirussen. Hoewel deze studie is uitgevoerd op één locatie en op een kleine ruimtelijke schaal, hebben we, met behulp van resultaten van een aanvullend onderzoek twee jaar later, kunnen aantonen dat de waargenomen patronen van LPAIV prevalentie in het najaar en in de winter in onze eendenkooi representatief waren voor de dynamiek van LPAIV infecties in vrijlevende wilde eenden elders in Nederland (Box 8.1).

Om te beoordelen of de hierboven voorgestelde mechanismen inderdaad een rol spelen in het verklaren van de dynamiek van LPAIV infecties in wilde vogels, gebruikten we een epidemiologisch model waarbij we onderscheid maakten tussen trekvogels, residente jongen en residente oudere vogels (Box 8.2). Dit model stelde ons in staat om parameter instellingen te vinden die de dynamiek van LPAIV infecties in deze drie subpopulaties konden nabootsen. Een dergelijk resultaat werd alleen behaald indien we in het model de volgende aspecten opnamen: (i) een constante doortrek (verversing) van trekvogels, (ii) korte termijn immuniteit van de gastheer en (iii) aparte virus transmissiesnelheden voor trekvogels, residente jongen en residente oudere vogels. Het model onderschrijft dus de veronderstelde rol van jongen en trekvogels als belangrijke factoren in de epidemiologie van LPAIV in ons studiegebied en ofschoon deze oefening geen daadwerkelijke test van deze factoren is, is het echter wel een stimulans voor verder empirisch onderzoek naar gastheerdemografie en LPAIV ecologie en epidemiologie in wilde vogels.

Voortbordurend op de hierboven vermelde bevindingen gebruikten we een veelzijdige en interdisciplinaire benadering om de rol van trekkende en residente gastheren in de introductie en handhaving van een specifieke LPAIV epidemie in het najaar verder onder de loep te nemen (Hoofdstuk 4). Deze epidemie werd veroorzaakt door een H3 LPAIV subtype. Tijdens de LPAIV epidemie werden korte- en lange-afstand trekkers van wilde eenden vaker geïnfecteerd met H3 LPAIV dan residente individuen, terwijl residenten meer virus uitscheden aan het begin van de epidemie. H3-specifieke antistoffen

werden gevonden in slechts een paar trekkende en residente wilde eenden. Deze resultaten suggereren dat de LPAIV epidemie in het najaar waarschijnlijk was geïnitieerd door een enkele introductie van H3 LPAIV in vatbare residenten, gevolgd door virusreplicatie die voor een belangrijke mate gecorreleerd was met de toestroom van trekkende wilde eenden. Deze bevindingen suggereren dat trekkende individuen de LPAIV epidemie in het najaar aandrijven (Hoofdstuk 3) en fungeren als lokale versterkers van het virus door het handhaven van deze epidemie (Hoofdstuk 4). Maar of trekkende individuen ook fungeerden als vector, door het introduceren van nieuwe LPAIV lijnen is minder duidelijk.

Het wordt vaak verondersteld dat wilde eenden asymptomatische dragers zijn van LPAIV, omdat ze nauwelijks ziekteverschijnselen vertonen als ze experimenteel geïnfecteerd worden. Echter, of dit ook het geval is in vrijlevende wilde eenden is grotendeels onbekend. Om deze reden onderzochten we mogelijke effecten van LPAIV infectie op lichaamsgewicht en immuun status van vrijlevende wilde eenden gedurende de jaarlijkse LPAIV infectiepiek in het najaar (Hoofdstuk 5). Immuun status werd bepaald met behulp van vijf immunologische indexen. Lichaamsgewicht verschilde niet tussen LPAIV geïnfecteerde en niet-geïnfecteerde individuen. Ongeacht of ze nu geïnfecteerd waren of niet, hadden korte- en lange- afstand trekkers een lager gewicht dan residenten. Hoewel er specifieke verschillen tussen de seksen en migratie strategieën waren, verschilden immunologische indexen niet of slechts marginaal tussen LPAIV geïnfecteerde en niet-geïnfecteerde individuen. Deze zwakke associaties tussen LPAIV infectie en lichaamsgewicht en immuun status van vrijlevende wilde eenden kan worden beschouwd als onderbouwing van hun rol als asymptomatische dragers van LPAIV; dit werpt het intrigerende idee op dat ze het gevolg kunnen zijn van gastheer-pathogeen co-evolutie, hetgeen de vermeende rol van wilde eenden als een belangrijk reservoir van LPAIV zou kunnen verklaren.

Om verder te onderzoeken of wilde eenden echt asymptomatische dragers zijn van LPAIV hebben we een model gebruikt om de (lokale) overleving van LPAIV geïnfecteerde en niet-geïnfecteerde residente wilde eenden te bepalen tijdens de infectiepiek in het najaar (Box 8.3). We vonden geen verschil in overleving tussen LPAIV geïnfecteerde en niet-geïnfecteerde wilde eenden. Echter, de kans om de eenden terug te vangen was wel hoger voor LPAIV geïnfecteerde wilde eenden, wat suggereert dat geïnfecteerde vogels dichter bij het studiegebied bleven, wat in overeenstemming is met de bevindingen van Hoofdstuk 7 (zie onder).

Om nog meer inzicht te krijgen in de rol van wilde vogels in de verspreiding van LPAIV onderzochten we mogelijke effecten van LPAIV infectie op het bewegingsgedrag van vrijlevende wilde eenden (Hoofdstuk 7). Gedurende de LPAIV infectiepiek in het najaar registreerden we de dagelijkse lokale en regionale bewegingen van met GPS loggers

uitgeruste LPAIV geïnfecteerde en niet-geïnfecteerde individuen op. Bij een vergelijking binnen individuen, individuen dus waarvan de infectiestatus tijdens de registratieperiode veranderde, was er geen verband tussen LPAIV infectie en dagelijkse lokale en regionale bewegingen. Echter, bij een vergelijking tussen individuen lagen de dagelijkse regionale bewegingen van LPAIV geïnfecteerde wilde eenden lager dan die van niet-geïnfecteerde individuen, welke steeds lager werd naarmate de weersomstandigheden slechter werden (d.w.z. meer wind, neerslag en lagere temperaturen leidden tot een sterkere afname in de regionale bewegingsintensiteit van geïnfecteerde vogels). De dagelijkse lokale bewegingspatronen verschilde niet tussen LPAIV geïnfecteerde en niet-geïnfecteerde individuen. Deze bevindingen suggereren dat LPAIV geïnfecteerde wilde eenden waarschijnlijk nog wel in staat zijn om virusdeeltjes te transporteren naar andere gebieden, maar dat de afstand van dergelijke verspreidingen waarschijnlijk lager ligt dan men zou verwachten op basis van het gedrag van niet-geïnfecteerde individuen.

Samenvattend heeft onze studie aangetoond dat (i) het najaar de meest kritische periode is van het jaar met het hoogste percentage aan LPAIV geïnfecteerde wilde eenden, (ii) LPAIV epidemieën in wilde vogels waarschijnlijk voornamelijk plaatsvinden op lokale schaal, (iii) trekkende gastheren een belangrijke rol spelen in de dynamiek van LPAIV infecties als potentiele veroorzakers en bekrachtigers van de najaar LPAIV epidemie en, dan waarschijnlijk vooral vanwege hun verhoogde vatbaarheid voor lokaal circulerende virussen in plaats van hun rol als vector van nieuw geïntroduceerde LPAIV lijnen van verre, (iv) wilde eenden waarschijnlijk in staat zijn om LPAIV asymptomatisch te dragen en (v) wilde eenden waarschijnlijk in staat zijn om LPAIV lokaal en zelfs regionaal te verspreiden.















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Curriculum Vitae

Jacintha was born on the 25th of October 1980 in Oosterhout, the Netherlands. She was intrigued by animals already at a young age. During her secondary education she was a volunteer at a bird shelter and went along with rides of the animal ambulance. Even though Jacintha first pursued a career in social law (for two years she studied in Leiden and Utrecht), in 2000 she went to Leeuwarden to study Animal Management at the Van Hall Institute in Leeuwarden. For her first internship she participated in a field study on the effects of introduced mammals on the native bird population of the remote island of New Caledonia. Her second internship was at Alertis - Fund for bear and nature conservation in Rhenen. For her BSc thesis she analysed the breeding success of black-headed gulls in the Netherlands, data collected by SOVON. During her BSc she worked as a volunteer at Stichting AAP in Almere. After Jacintha finished her BSc in 2004 she worked for a year in the insurance business. Since this was no future for her, and being inspired by the work of her first internship, in 2006 she decided to study Forest and Nature Conservation at Wageningen University. During her MSc she performed a research project at the Royal Netherlands Institute for Sea Research (NIOZ), where she focussed on the foraging behaviour of bar-tailed godwits during spring migration at the Dutch Wadden Sea island Terschelling. Her second MSc project was funded by Birdlife Netherlands in which she studied the relation between body condition and roost size of barn swallows in Zambia. Her third MSc project was an experimental study, where she studied interference competition in mallards at the Netherlands Institute of Ecology (NIOO-KNAW) in Nieuwersluis. At the same institute at the Department of Plant-Animal Interactions, Jacintha started her PhD in January 2009. In September 2009, due to a reorganisation at the NIOO, she moved to the Animal Ecology Department. In August 2010, her PhD project got funded by ALW NWO, entitled '*Host-pathogen interaction: the effects of avian influenza viruses on free-living mallards*'. This project was in collaboration with the Department of Viroscience of Erasmus MC. In January 2011, Jacintha moved with the NIOO to a new building in Wageningen.

Publications

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In progress

Verhagen, J.H.*, **J.G.B. van Dijk***, O. Vuong, T. Bestebroer, P. Lexmond, M. Klaassen and R.A.M. Fouchier. Migratory birds reinforce local circulation of avian influenza viruses. *Plos One* (in revision)
*contributed equally to this study

van Dijk, J.G.B., R.A.M. Fouchier, M. Klaassen and K.D. Matson. Minor differences in body condition and immune status between avian influenza virus infected and non-infected mallards: a sign of co-evolution? *Ecology and Evolution* (in revision).

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van Dijk, J.G.B.*, E. Kleyheeg*, M.B. Soons, B.A. Nolet, R.A.M. Fouchier and M. Klaassen. Weak negative association between avian influenza virus infection and movement behaviour in a key host species. Submitted manuscript.
*contributed equally to this study

National reviewed journals

van Dijk, J.G.B., R.A.M Fouchier and M. Klaassen. 2014. Vogelgriep in de natuur: wie of wat speelt een rol in de verspreiding. *Mens & Vogel* 52: 20-31.

van Dijk, J.G.B., E.W.M. Stienen, S. Gerritsen and F.A. Majoor. 2009. Reproductie van de Kokmeeuw in kust- en binnenlandkolonies. *Limosa* 82: 13-22.

Book chapter

van Dijk, J.G.B., R.A.M Fouchier and J.H. Verhagen. 2013. Hoofdstuk 5. Wetenschappelijk onderzoek in eendenkooien in Nederland: eenden en vogelgriepvirussen. *In* D. Karelse and F. Mandigers (editors). *Blauwgoed, helen en halven*. Werkgroep Ringwerk Eendenkooien Nederland (WREN).