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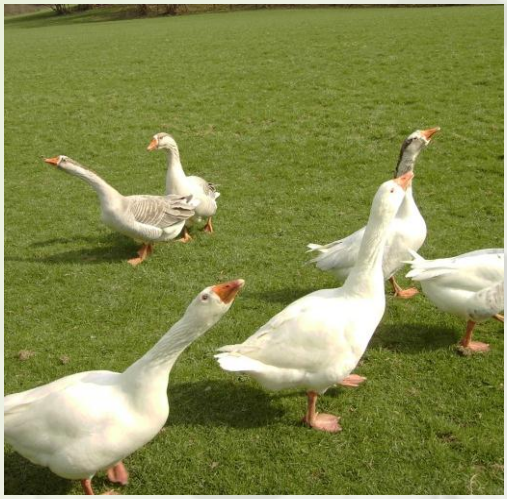
The transmission of H5 and H7 Low Pathogenic Avian Influenza viruses



Gerwin Claes

THE TRANSMISSION OF H5 AND H7 LOW PATHOGENIC AVIAN INFLUENZA VIRUSES

Gerwin Claes

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“The hardest thing of all is to find a black cat in a dark room, especially if there is no cat”

(Confucius)

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List of abbreviations

(NP-)ELISA	Enzyme-linked immunosorbent assay (directed towards the nucleoprotein)
(q)RRT-PCR	(quantitative) Reverse transcriptase real-time polymerase chain reaction
(v)RNA	(viral) Ribonucleic acid
AB	Antibiotics
AI(V)	Avian influenza virus
AIC	Akaike information criterion
BHI	Brain heart infusion broth
BSL	Biosafety level
C_1-chicken	First contact chicken
C_2-chicken	Second contact chicken
C-chicken	Contact chicken
CI	Confidence interval
CID_{50}	Fifty per cent chicken infectious dose
CL	Cloacal
dpe.	Days post-exposure
dpi.	Days post-inoculation
DPPA	Densely populated poultry area
EID_{50}	Fifty per cent egg infectious dose
FASFC	Federal agency for the safety of the food chain
EU	European Union
FS	Final size
GLM	Generalized linear model
HA / H	Hemagglutinin
HI	Hemagglutination inhibition
HPAI(V)	Highly pathogenic avian influenza (virus)
I-chicken	Inoculated chicken
LP(N)AI(V)	Low Pathogenic (Notifiable) Avian Influenza (virus)
NA / N	Neuraminidase
OIE	Office International des Epizooties
OP	Oropharyngeal
PCR	Polymerase chain reaction
R_0	Basic reproduction ratio
SIR	Susceptible - infectious – recovered
SPF	Specific pathogen free
WB-LPAIV	Low pathogenic avian influenza virus that was isolated from wild birds
β	Reproduction parameter / transmission rate

Chapter 1:

General introduction

Influenza A viruses are RNA-viruses that can infect a variety of vertebrate animals. The virus is diversified to such a degree that multiple strains are circulating among swine, horses, dogs and other domesticated animals. The virus can also be found in wildlife and is omnipresent in many wild bird species. In humans, influenza virus is known to have caused large outbreaks for centuries. Believing they were under some sort of celestial influence, 13^{th}-14^{th} century Italians used the term: "ex influentia colesti" to describe the disease that could affect virtually the entire population of a city (Cunha, 2004).

Influenza viruses that are isolated in birds (Avian influenza viruses, AIV) are of major importance for poultry species, since some of them have the ability to become extremely pathogenic and have caused outbreaks that led to massive mortality and considerable economic damage. Additionally, the zoonotic potential of avian influenza viruses and the close relation between human and avian influenza viruses have further increased worldwide interest for this virus. The thousands of strains that have been isolated provide an ever-growing insight in the mechanisms this virus has developed to maintain itself in host populations.

The genetic relationship between influenza strains from different species and the ability of the virus to cross the species-barrier make this virus a role model for the "one world, one health" concept.

1 The poultry industry

1.1 Introduction

Poultry is farmed mostly for meat & egg production but also for ornamental purposes, their feathers and oil. Poultry meat accounts for roughly 30% of meat production worldwide. Meat from chickens has accounted for 87% whereas turkey meat has accounted for 6,7% of the total poultry meat production in the year 2009. Commercial production and consumption of poultry meat products is continuously growing and is expected to continue to expand. The production of the second most important poultry product, eggs, is also rapidly growing and it is estimated that there are about 4,93 billion egg-laying hens in the world (Food and Agriculture Organization of the United Nations, 2010). Worldwide numbers of poultry stocks and other production animals for 2010 are presented in table 1.

The poultry sector has undergone a large modernization during the last century. Many technical innovations have changed the appearance of the sector. Traditional small-scale poultry farming with double-purpose breeds has made room for intensive commercial production systems with birds specially bred for meat or for egg production. Regional cultural differences determine the way in which poultry is raised in large areas throughout the world.
In Western Europe, poultry products are generally offered to the end-consumer as ready-to-consume or ready-to-cook end products. In these areas, poultry is mainly farmed in marketing systems that are referred to as "sector 1" (industrial & integrated systems with a high level of biosecurity and in which birds or bird products are usually marketed commercially (Food and Agriculture Organization of the United Nations, 2004)) or "sector 2" (commercial poultry production system with moderate to high biosecurity and birds/products usually marketed commercially (Food and Agriculture Organization of the United Nations, 2004)). Additionally, some poultry may be kept in village or backyard smallholder flocks with a low/minimal level of biosecurity from which poultry products are consumed locally (sector 4).

Table 1: Live animal stock in 2010 (adapted from FAOSTAT, 2013) (Food and Agriculture Organization of the United Nations).

Species	World (1000 animals)	Europe (1000 animals)	Belgium (animals)
Chickens	20.186.685,92	1.944.083,85	34.375.000
Ducks	1.254.365,28	84.000,10	255.000
Geese & guinea fowls	378.746,50	19.014,50	5.000
Turkeys	459.538,49	111.613,00	190.000
Cattle	1.465.154,68	124.453,27	2.593.000
Goats	970.224,13	17.112,11	22.000
Pigs	971.800,91	188.801,88	6.430.000
Sheep	1.127.046,76	129.957,24	120.000

In other parts of the world, most notably Southeast Asia, consumers prefer live-bird retailing and poultry is raised and sold at live bird markets (LBM) by small-scale producers (sector 3). The FAO defines this production sector as: "commercial poultry production systems with low

to minimal biosecurity and birds/products usually entering live bird markets" (Food and Agriculture Organization of the United Nations, 2004; Upton, 2007).
The types of poultry production systems have different characteristics regarding flock size, bird species and biosecurity measurements.

1.2 Professional poultry production

Sector 1 comprises highly industrialized enterprises where each step in the production chain is vertically integrated in one company. Since these companies work according to standard operating procedures and animals are housed indoor, the biosecurity is generally considered to be high and the probability of disease introduction is rather small. However, since often several 10.000s of animals are housed in a relatively small space, the impact of such an event may be very large. Sector 2 poultry holdings also comprise holdings with very large numbers of animals that are housed indoor, but the entire production chain is not integrated in one enterprise and relatively smaller enterprises cover only certain steps of the production chain. Therefore, despite high biosecurity levels are maintained in this sector as well, day-old chicks, broilers, laying hens or other resources are often bought from different enterprises, which implies that more complex contact structures may exist for these companies (Upton, 2007). In Belgium, these sector 1 and 2 poultry holdings are mainly situated in the northern part of the province of Antwerp and in West-Flanders. About 56% of professional poultry farms raise broiler chickens, 19% keep layers, 8% are multiplier farms, 10% are rearing farms, 1% keep ornamental birds and 5% of farms have multiple activities (Van Steenwinkel et al., 2011).

Breeder and broiler chickens are mostly housed on a floor that is covered with litter (wood shavings, straw, shredded paper, peanut hulls,…). This type of housing system requires little maintenance, but it may lead to higher concentrations of respirable dust particles and airborne micro-organisms. After every production cycle ideally, the manure is land-spread and the house is thoroughly cleaned to prevent carrying-on diseases to the next production cycle. The air quality, humidity, functioning of drinking and feeding systems, type of nutrition and bird health may affect litter moisture, leading to high incidences of hock burn or breast blisters. (Feddes et al., 2002; Proefbedrijf voor de veehouderij, 2011; Madelin and Wathes, 1989).

Layer chickens used to be often housed in battery cages housing 5-10 birds in cages at a density of +/- 400-700 cm²/bird. However, the limited space per bird and the barren floor impedes the birds to perform normal behavior and these ethical concerns have led to a ban of

cage housing in Europe. The use of conventional battery cages is forbidden in the EU since January 2013 and all existing stables for laying hens need to have adopted several measurements for improvement of animal welfare (Duncan, 2001; European Commission, 1999). Alternatively, enriched or furnished cages, in which more attention is given to the ethological needs of layer chickens, can be used. In these housing systems, group sizes vary from 5 birds per cage to 20 or more birds. European legislation imposes clear requirements on the sizes and design of these cages (the headroom for animals needs to be at least 45cm, there needs to be a perch in each cage and the area per hen needs to be at least 750cm², including 150cm² nest and litter area and the total cage area needs to be at least 2000cm²). Some European countries like Germany and Austria will also ban these cages however, hereby imposing floor-based housing systems on poultry farmers (Tauson, 2005).

Figure 1: Laying hens housed in enriched cages (left) and a multi-tier aviary system (photo courtesy of USA today and Potters equipment)

In floor-based or alternative housing systems for layer chickens, birds are no longer housed in small groups. The animals are kept on litter and are offered plenty of space to satisfy their ethological needs. Most alternative systems are single-tiered with a partly slatted floor. Housing systems, in which elevated slatted floors are mounted above the litter floor, i.e.: multi-tiered (aviary) systems, offer the possibility of increased stocking density. Daily inspection of animals is easier in these housing systems, but feed conversion ratios are higher and the average body weight of the hens is lower. Also, pecking or cannibalism may be increased, a poorer air quality may lead to health issues and problems with misplacing eggs or egg quality may occur (Proefbedrijf voor de veehouderij; Rodenburg et al., 2005; Tauson et al., 1999). In some poultry houses, the animals have access to a covered veranda

(wintergarden) in which the outdoor climate prevails, or the birds are offered the possibility of an outdoor environment. Alternatively, birds may be ranged outdoors in free-range and organic production systems.

1.3 Biosecurity in poultry holdings

The probability of introduction of a disease to a poultry holding is determined by many factors. These risk factors have been extensively described and analyzed in several studies, which all conclude that carefully applying biosecurity measurements leads to an important reduction of the risk of disease introduction (Welby et al., 2010). For most Belgian sector 1 and 2 poultry holdings, acceptable levels of biosecurity are generally maintained (Van Steenwinkel et al., 2011).

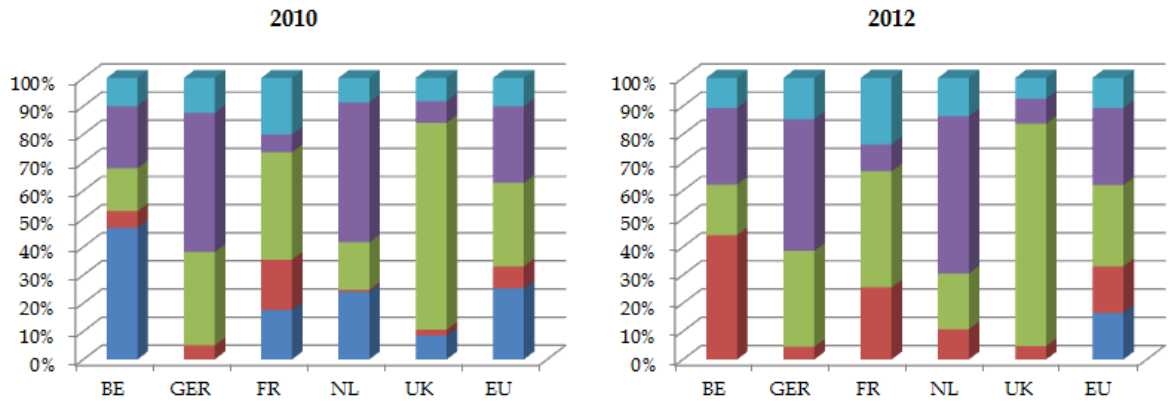

Figure 2: Proportion of laying hens housed in alternative housing systems (organic (light blue), aviary (purple), free-range (green)) and cage housing systems (enriched cages (red), conventional cages (dark blue)). Country codes: BE=Belgium, GER=Germany, FR=France, NL=the Netherlands, UK=United Kingdom, EU=European Union. Data obtained from: European Egg Processors Association, 2013.

The growing public awareness on welfare in animal husbandry and the prohibition on conventional cage housing (European Commission, 1999) have brought important changes to the poultry industry. In Belgium and other (mostly Western-) European countries, the share of laying hens that are kept in cages is decreasing and more alternative housing systems for laying hens are being installed (Figure 2) (Van Horne and Achterbosch, 2008; European Egg Processors Association; 2013). In these alternative housing systems however, the separation between animals and environment is less distinct. This implies that the animals are more exposed to potentially harmful bacteria, parasites and viruses (Berg, 2001). Particularly those

poultry holdings where the animals have outdoor access, contact with free-living animals may lead to the introduction of diseases (Koch and Elbers, 2006; Welby et al., 2010). Furthermore, if multiple species of domestic poultry are reared on the same holding, the possibility of disease introduction and dissemination is considered even higher (Koch and Elbers, 2006; Welby et al., 2010).

Contacts between poultry holdings (both professional and backyard) may facilitate the dissemination of pathogens to other farms as well. A survey carried out by Van Steenwinkel et al. (2011) has indicated that many professional farms and hatcheries trade live poultry and poultry products with local traders (46% and 16%) and/or with hobbyists (38% and 58%). Likewise, a substantial proportion of poultry holders says to visit bird shows on average once a year and there is much on- and off- movement of service providers, which increases the number of (indirect) contacts with other farms (Van Steenwinkel et al., 2011).

2 Avian Influenza

2.1 Introduction

Avian influenza viruses can be introduced to poultry holdings through contact with wild birds or other animals that are infected with the virus or that may act as mechanical vectors. Alternatively, the virus may be introduced to or transmitted between poultry holdings by human activities (Alexander, 1995).

Avian influenza virus is a subspecies of the Influenza A virus, comprising those viruses that are adapted to circulation in birds.

Influenza A is the only species in the genus influenzavirus A, which is further classified with the Influenzavirus B, Influenzavirus C, Isavirus, Quaranjavirus and Togothovirus genera to the family of the *Orthomyxoviridae* (International Committee on Taxonomy of Viruses, 2011).

Influenza A (in this thesis further referred to only as influenza virus) is a much diversified species of virus. It is therefore further classified according to the combination of two major glycoproteins that appear on the virus envelope; the Hemagglutinin (HA) and Neuraminidase (NA). To date, 18 different HA types and 11 different NA types have been discovered (Tong et al., 2013).

2.2 The influenza virion

2.2.1 The envelope & surface antigens

The influenza virion is a small (80-120nm diameter), pleomorphic particle which can have a spherical or a more filamentous shape. The outer envelope of the influenza virion is a lipid bilayer membrane. It has multiple characteristic rod-shaped and mushroom-like structures protruding from it: the hemagglutinin and neuraminidase glycoproteins.

The rod-shaped hemagglutinin glycoprotein (HA) is the most common and the largest surface antigen on the virus envelope. It is determinative for the virus' pathogenicity and is also the most important antigen against which protective antibodies are produced (Suarez and Schultz-Cherry, 2000). The HA is a trimer which consists of three HA0 proteins. Each of those HA0 proteins have several oligosaccharide chains that are attached to so-called glycosylation sites.

The NA is a tetramer composed of four identical glycosylated proteins that form a mushroom-like structure. It is four times less common on the viral membrane than the HA.

The third membrane protein, the matrix 2 (M2) protein, spans the virus envelope and the inner shell that lies underneath. The protein is much less represented on the virus membrane than the HA and NA glycoproteins (Lamb and Krug, 2001).

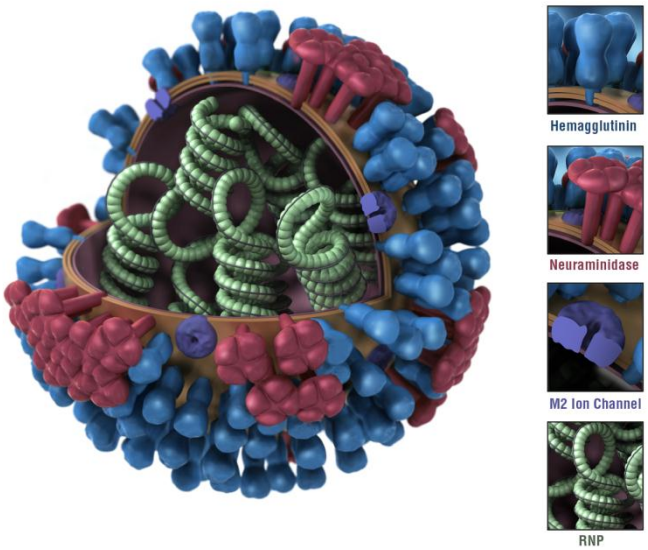

Figure 3: Three-dimensional representation of a generic influenza virion's ultrastructure. Photo courtesy of CDC/Douglas Jordan.

2.2.2 Other virus components

Directly underneath the envelope, the virion's inner shell offers rigidity. This shell is made of the matrix 1 (M1) protein, which is the most abundant protein in the influenza virion.

The genetic information of the influenza virion is encoded in eight negative-sense single stranded RNA segments ((-)ssRNA). These segments are bound to RNA polymerase complexes, and these complexes are bound to nucleoproteins (NP), which are the second most abundant protein in the influenza virion. These nucleoproteins bind firmly to each other and hereby form large, oligomeric complexes, called viral nucleoproteins (vRNPs), which lie at the center of the virion. The gene sequence of the NP protein is highly conserved among all influenza virus subtypes, which makes it a useful target for non-subtype specific testing (Portela and Digard, 2002).

The viral RNA polymerase complex is a trimer formed by the Polymerase A (PA), Polymerase B1 (PB1) and Polymerase B2 (PB2) subunits. This complex has an important role in the transcription of mRNA and the amplification of vRNA. The precise functioning of the subunits however is not yet fully understood (Nagata et al., 2008; Perales et al., 2000).

Another protein, the non-structural protein 1 (NS1), is a protein that can be found abundantly in infected cells, but appears not to be present in the influenza virion itself, which explains the origin of this protein's name. This protein plays a role in suppressing the host's immune response, more specifically by inhibiting the production of interferon. Several studies suggest that the NS1-molecule is also associated with the virulence of the virus (Li et al., 2006). The nuclear export protein (NEP) was originally thought to be a non-structural protein as well and was therefore initially named as such (non-structural protein 2 (NS2)) (Yasuda et al., 1993). However, it has been proven that this protein is present in the influenza virion.

2.3 Avian influenza virus

Of the currently known 18 HA and 11 NA subtypes, all except for the recently discovered H17 and H18 subtypes (which have only been observed in bats (Tong et al., 2012, 2013)) have been observed in birds. These HA and NA subtypes apparently can occur in any combination (Fouchier et al., 2005). Based on the symptoms the virus causes in chickens, AIVs are additionally classified as either low pathogenic avian influenza virus (LPAIV) or highly pathogenic avian influenza virus (HPAIV).

2.3.1 The life cycle of avian influenza virus

In order to be infectious, the HA0 proteins need to be cleaved extracellularly in HA1 and HA2 proteins. LPAIVs are cleaved only extracellularly by trypsin-like proteases (Guo et al., 2008). Since these proteases are secreted only by epithelial cells lining the respiratory and intestinal tracts, LPAIVs can only replicate in these organs (Klenk et al., 1975). The HA0 proteins of HPAIVs however, can additionally be cleaved intracellularly by ubiquitous proteases, of which furin is one of the most important ones since it can be found in cells throughout the body (Guo et al., 2008; Stieneke-Grober et al., 1992). Consequently, HPAIVs can infect a wide range of cell types and infected animals go through a systemic infection characterized by viremia, massive virus replication and cellular damage in multiple cycles (Swayne, 1997).

2.3.2 Low Pathogenic avian influenza (LPAI)

LPAIVs have been observed among HA subtypes 1-16 (Fouchier et al., 2005). Outbreaks with LPAIVs generally do not cause severe symptoms in affected poultry and may sometimes even be subclinical, depending on the poultry species and the virus strain. Symptoms are caused by localized virus replication and may include sneezing, coughing, lethargy or diarrhea. Some LPAI outbreaks (most notably H1, H3, H5, H6, H7 and H9 outbreaks) may lead to more severe symptoms like dyspnea, pulmonary congestion, swelling of the sinuses and discharge coming from nares and eyes, especially if opportunistic pathogens are involved (Ficken et al., 1989; Nili and Asasi, 2002; Tang et al., 2005; Webby et al., 2002). Gallinaceous poultry species are often found to suffer more from LPAIV infections than domesticated waterfowl do (Tumpey et al., 2004; Morales et al., 2009; Jackwood et al., 2010; Mundt et al., 2009). Because of the variety in symptoms, secondary infections and because LPAI infections may sometimes be subclinical, many LPAIV outbreaks may remain unnoticed (Swayne and Pantin-Jackwood, 2006).

Mutations that lead to the insertion of multiple basic amino acids at the cleavage site of the HA (vide infra) may lead to a transition of the virus into a highly pathogenic variant, which causes much more severe symptoms. Such transitions have been observed for LPAIVs belonging to the H5 and H7 subtypes (Garcia et al., 1996; Spackman et al., 2003). At least four mechanisms leading to the formation of a polybasic cleavage site have been described; simple site mutations, accumulated nucleotide insertion(s), tandem duplications and recombinations (Perdue, 2008).

2.3.3 Highly Pathogenic Avian Influenza (HPAI)

Highly pathogenic Avian Influenza, which was referred to as fowl plague until 1981, causes very severe symptoms in poultry. In modern-day large poultry houses, a sudden increase in mortality in just a matter of days is usually the first alarming symptom (Stegeman et al., 2004). Chickens affected with HPAI exhibit severe respiratory illness and intestinal disorders. Sick birds are often seen with ruffled feathers, sinusitis, subcutaneous hemorrhages, swelling of the head and focal necrosis of the comb and wattles. Nervous disorders such as torticollis can be observed as well. Death usually follows within 2-7 days after the onset of the first clinical signs. Post-mortem examination may reveal peritonitis, tracheitis and a swollen liver and spleen. Histological examination mostly reveals lesions as a result of virus replication in many different organs (Elbers et al., 2004; Mutinelli et al., 2003; Swayne, 1997).

The symptoms caused by HPAIV are usually more pronounced in chickens and turkeys. In other poultry species like pheasants, quails and ostriches, the infection does not spread as fast, symptoms are less severe and mortality rates are usually lower (Mutinelli et al., 2003; Perkins and Swayne, 2001). Infection of domestic ducks and geese or wild birds with HPAIV usually does not cause severe disease or causes no disease at all (Alexander et al., 1986; Koch and Elbers, 2006).

Evolution from LPAI to HPAI has only been observed for LPAIVs belonging to the H5 and H7 subtypes may evolve towards a HPAIV, these two LPAI subtypes (hereafter referred to as LPNAIVs) are also notifiable diseases and also targeted in surveillance programs (European Commission, 2005).

2.4 Stability outside the host

The sensitivity of influenza virions to chemicals is determined by the presence of the viral envelope. It has been shown that the presence of a lipid viral envelope makes the virus highly susceptible to all disinfectants (De Benedictis et al., 2007). Most importantly, organic debris offers a protective environment for the virus. Therefore, removal of organic material is the cornerstone of every intervention intending to prevent further spread. Soaps and detergents have a surfactant effect on the viral envelope and are therefore not only efficacious in removing dirt and organic components, but are also suitable for inactivating all enveloped viruses (Birnbaum and O'Brien, 2008; De Benedictis et al., 2007; Greatorex et al., 2010).

Chemical inactivation of the virion often involves the use of oxidants which oxidize peptide links and lipids. When used in higher concentrations, they may also cause damage to the vRNA. Because it is inexpensive and easily available, the most widely used oxidant for AI

disinfection is chlorine. Gaseous formaldehyde is frequently used for disinfection of materials that must be kept dry. Alcohols are mainly used for decontamination of hands and forearms of laboratory personnel or materials (De Benedictis et al., 2007).

Heat treatment accelerates the natural inactivation of the virus and can therefore rapidly decrease the virus load. Several methods for heat treatment have been evaluated including microwaves, pasteurization, autoclaving and composting of poultry litter (De Benedictis et al., 2007). Other physical inactivation methods are ultraviolet light or ionizing radiation. These cause damage to the viral RNA, resulting in inactivation of the virus. A major advantage of ionizing radiation is its ability to penetrate into or through most biological materials (Lowy et al., 2001).

The impact of meteorological conditions on influenza survival has been extensively investigated. Whereas survival of influenza viruses is increased at low temperatures, results on the impact of air humidity are sometimes contradictory. Influenza survival in water bodies is found to be prolonged at low temperatures and low salinity (Brown et al., 2007a; Stallknecht et al., 1990b; Weber and Stilianakis, 2008). In freshwater bodies, it is estimated that influenza viruses may persist for two to three months at a temperature of 10°C and for over six months at a temperature of 0°C (Nazir et al., 2010). Additionally, influenza viruses have been proven to survive well in feces and lake sediments (Chumpolbanchorn et al., 2006; Lang et al., 2008; Nazir et al., 2011). After inactivation of the virion, the vRNA can often remain present for several days (Guan et al., 2009).

3 Epidemiology of Avian Influenza

3.1 The natural reservoir of AIV

AIVs are endemic in many wild bird populations, which are considered the natural reservoir of AIVs. In the natural reservoir, the virus is assumed to be in evolutionary stasis, which is characterized by a low rate of genetic drift (Webster, 1999). Aquatic wild birds, especially species of the order *Anseriformes* harbor a wide variety of antigenic subtypes and are the most important species for the perpetuation of AIVs. Since all known AIVs in aquatic wild birds are of low pathogenicity (with the exception of the Asian H5N1 HPAIV (vide infra)) and also cause no or minimal disease, the relationship between the host and the virus is practically commensal (Fouchier and Munster, 2009). Also, infected aquatic wild birds generally shed

large amounts of virus whilst the humoral immune response in these species is generally weak and transient (Alexander et al., 1986; Kida et al., 1980; Webster et al., 1978).

Of all species in the order *Anseriformes,* wild duck species are most frequently found to be infected with LPAIVs. Average prevalence rates of 10,1% are estimated for dabbling duck spp and 1,6% for diving duck spp. (Olsen et al., 2006). However, these rates vary throughout the year. In early fall, just before migration, prevalence rates peak. In this period, juvenile birds are most frequently affected; with some reports even mentioning prevalence rates of over 60% (Hinshaw et al., 1980). By early spring, the prevalence rate has dropped to only a few percentages and by the time the animals return to their breeding grounds, prevalence can be <1%. Geese and swans are less frequently infected than duck species (1,0% for goose spp and 1,9% for swan spp; (Olsen et al., 2006)). However, these birds tend to congregate in large groups on agricultural fields and may thus transfer the virus to domesticated bird species. Also, since domesticated geese and swans are often kept alongside chickens in many backyard smallholder flocks, these animals may attract wild related species (Alexander, 2000).

The migratory behavior of these species leads to an important dissemination of the virus along the migratory flyways they use. Animals tend to stop several times during migration and hereby often choose the same stopover sites. Thus, large densities of aquatic birds of different populations can be present at these favorable stopover sites leading to transmission of LPAIVs between them (Hoye et al., 2011; Krauss et al., 2007).

Species that belong to the order of the *Charadriiformes* may harbor LPAIVs as well, although the prevalence in these populations is generally smaller than in species of the *Anseriformes* order (1,4% for gull spp and 0,9% for tern spp)(Olsen et al., 2006). Also, differences in the prevailing LPAIV subtypes are observed between these orders, suggesting that genetically different lineages circulate in these species and that transmission of viruses between animals from these two orders is rather rare (Munster et al., 2007; Olsen et al., 2006).

Transmission of LPAIVs occurs mainly through the fecal-oral route (Fouchier and Munster, 2009; Webster RG et al., 1992). Indeed, several experimental infection studies have mentioned that AIV replication in wild aquatic birds occurs preferentially in the intestinal tract (Kida et al., 1980; Pillai et al., 2010; Slemons and Easterday, 1977; Vandalen et al., 2010; Webster et al., 1978). As a result, viruses are shed in feces and are thus deposited on shores, in nests and in water reservoirs. Since LPAIVs can remain infectious in aqueous environments for a long time (Brown et al., 2007a; Stallknecht et al., 1990a), the virus can be transmitted to other aquatic wild birds by drinking or soaking the head. This route is widely

recognized as the primary route of LPAIV transmission in wild aquatic birds (Alexander, 1995).

3.2 Avian Influenza in poultry

Poultry species (fowl, turkeys, guinea fowl, ducks, geese, quails, pigeons, pheasants, partridges and ratites reared or kept in captivity for breeding, the production of meat or eggs for consumption, or for re-stocking supplies of game (European Commission, 2009)) are not a natural reservoir for AIVs. It is believed that outbreaks of AIV in poultry are introduced by contact with wild aquatic birds, and that the current lineages of AIVs that are circulating in poultry species have once been introduced in the same way (Jackwood et al., 2010; Krauss et al., 2004; Munster et al., 2005; Swayne, 2008; Webby et al., 2002). Transmission of LPAIVs from the wild bird reservoir to poultry may occur as a result of direct or indirect contact. Since many LPAIV outbreaks have been linked to direct contact between wild birds and poultry, this route is generally considered to be the most important route (Alexander, 2007a; Halvorson, 2002). Moreover, outbreaks of LPAIVs in poultry are mostly located in regions where many wild birds are found or in months when wild birds are present in the region. Likewise, the housing of domestic waterfowl also appears to attract wild birds, which increases the possibility of LPAIV introduction (Koch and Elbers, 2006).

Since HPAIVs do not circulate among wild birds (except for Asian H5N1 HPAIV (vide infra)), the introduction of AIVs from wild aquatic birds to poultry generally involves strains of low pathogenicity. Additionally, the series of mutations leading towards the insertion of multiple basic amino acids at the cleavage site of LPNAIVs is thought to occur only in poultry species. It is thus believed that every HPAI outbreak can be linked to the introduction of a LPNAIV from the wild bird reservoir to poultry (Garcia et al., 1996; Koch and Elbers, 2006; Spackman et al., 2003).

Poultry and other land-based birds live in very different environments as compared to aquatic wild birds; water bodies are not as dominant in their environments. Therefore, it is suggested that LPAIV transmission occurs via different routes in poultry (Fouchier and Munster, 2009). However, very little is known on the dynamics of LPAI transmission in poultry and research is needed to create a better understanding on this matter. The gathered knowledge is of high importance for designing LPNAI surveillance programs.

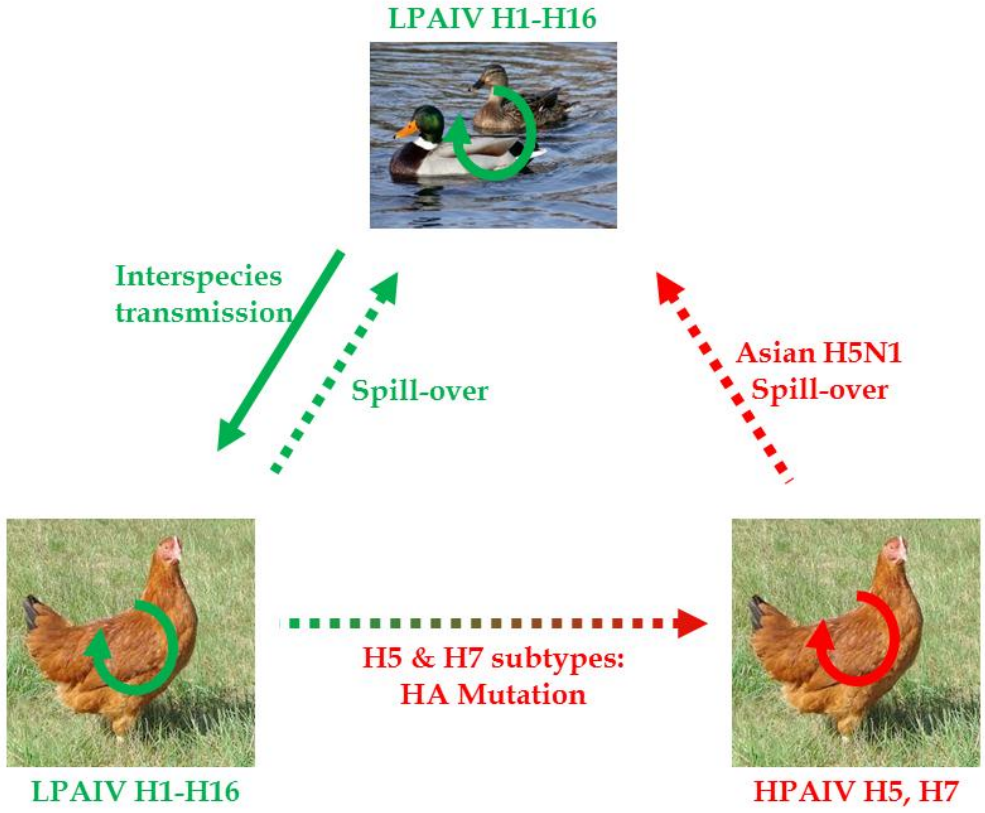

Figure 4: The epidemiology of LPAIVs and HPAIVs between aquatic wild birds and poultry (adapted from: Swayne, 2008).

3.3 LPNAI outbreaks in poultry

Since the course of a LPAI infection in poultry is generally not severe, many LPAI outbreaks in poultry have limited economic impact or remain unnoticed. Moreover, before the transition of a LPNAIV into a highly pathogenic variant was witnessed in the 1980s, very little attention was paid to LP(N)AIVs and very few data on these viruses is available in wild birds before this time.

LPAI outbreaks occur rather frequently in poultry. A paper by Alexander (2007) lists sixty LPAIV strains isolated from poultry and other captive birds between 2002 and 2006, in Europe, Asia, Africa and Australia. The actual number of LPAI outbreaks in poultry may be even higher, since many outbreaks may remain unnoticed (Alexander, 2007b). An overview of the LPNAI outbreaks in Europe since 2006 is presented in table 2.

Some LPNAIVs may circulate among poultry holdings for some time before they are finally detected. Several LPNAI outbreaks have thus led to the formation of a stable lineage of the

virus in poultry and have been able to spread over large regions. Italy for instance, is coping with several LPNAIVs circulating in densely populated poultry areas (DPPAs) since the second half of the 1990s. In 1999-2000, a H7N1 LPAIV that had become endemic in poultry in the Veneto and Lombardia regions, affecting at least 199 farms led to the emergence of a H7N1 HPAIV (Capua et al., 2003). Whilst authorities were able to eradicate this HPAIV, viruses related to the ancestor H7 LPAIV are still regularly encountered in Italy and, less commonly, other countries throughout Europe (Brown, 2010a; Capua et al., 2000; Cecchinato et al., 2010). Likewise, a H5N2 LPAIV that has caused the emergence of a HPAIV in Mexico during 1994-1995 is still circulating throughout Central America. In the northeastern part of the United States of America, a H7N2 LPAIV that had become endemic in poultry has circulated among live bird markets and backyard smallholder flocks for 13 years. In April 2006, the virus was finally eliminated, fortunately without having evolved into a HPAIV (Trock and Huntley, 2010). Recently, several subtypes of LPNAIVs have been introduced in poultry farms across Germany, the Netherlands and Denmark, including one farm positive by serology in Belgium (Steensels, 2013). Oftentimes, only one or a few farms are involved. Therefore, direct introduction from the wild bird reservoir, without the virus being endemic, is assumed for many of these outbreaks (Koch, 2013). However, the molecular similarity between H7N7 LPAIVs isolated at several occasions in Germany and the Netherlands since 2009 might suggest that an endemic situation has been reached for this virus (Probst et al., 2012).

Although similar situations with non-H5/H7 LPAIVs may seem less worrying, these viruses should not be treated as unimportant. They can cause severe illness and can be particularly harmful for the poultry industry. Therefore, eradication (depopulation) or control measures (vaccination) are sometimes applied to non-H5/H7 LPAIVs outbreaks (Senne, 2007). Examples are: USA (H6N2), Hong Kong (H6N1), Italy (H3N2), China (H3N2, H3N6) and Asia & the Middle-east (H9N2). Among these, the H9N2 subtype has become very widespread and, more recently, has become established in poultry throughout large parts of Asia. In many case reports on these H9N2 LPAIVs, very high mortality rates, sometimes even similar to those observed in HPAI outbreaks, have been observed in poultry flocks. In these cases, secondary infections (frequently with Infectious bronchitis virus and *Mycoplasma gallisepticum*) are always involved whilst the LPAIVs themselves do not produce severe symptoms in affected poultry under experimental conditions (Alexander, 2007a; Cecchinato et al., 2010; Liu et al., 2003; Nili and Asasi, 2002). However, the presence of H9N2 LPAIVs in these outbreaks and the role this virus may play in aggravating symptoms is worrisome. It

is therefore believed that this subtype may cause more and more problems in the future (Alexander, 2010).

Table 2: Overview of LPNAI outbreaks in Europe since 2006 (adapted from: Brown, 2007b, 2008, 2009, 2010a, 2010b and 2011; World animal health information database, 2013; ProMED-mail, 2013).

Year	LPAI subtype	Description	Country(ies) involved	Additional reference
2006-present	Multiple LPNAIVs including: H5N2, H7N3, H7N7	Multiple outbreaks in rural and commercial poultry holdings	Italy	Cecchinato et al., 2010
2006	H5N2	1 mallard holding	Denmark	
2006	H5N3	2 gamebird holdings	Denmark	
2006	H5N3	1 ostrich holding	Germany	
2006	H7N3	1 commercial chicken farm	United Kingdom	
2006	H7N7	Chickens	The Netherlands	
2006 - 2007	H5N1, H5N2, H5N3	Commercial duck holdings	France	Briand, 2010
2007	H5N2	Free-range duck farm	Portugal	
2007	H7N2	2 backyard holdings	United Kingdom	
2008	H5N?	Goose breeder farm with outdoor access	Belgium	Marche et al., 2013
2008	H5N1	Rural farm	Italy	Cecchinato et al., 2010
2008	H5N2	Ornamental birds at one farm	Belgium	Marche et al., 2013
2008	H5N3	2 red-legged partridge holdings and one broiler holding	Portugal	
2008	H7N?	Backyard holding with chickens and geese	Norway	
2008	H7N1	2 outbreaks in rural farms	Italy	Cecchinato, 2010
2008	H7N1	1 holding with ducks and geese	Denmark	

2008-2009	H5N3	Zoo waterfowl, mixed backyard holding, 33 commercial holdings in Germany and possibly one in Poland	Germany, Poland	
2009	H5N1, H5N3, H5N?	3 outbreaks involving several duck farms	France	
2009	H5N3	Mallard holding	Czech republic	
2009	H5N3	Duck holding	Spain	
2009	H5N7	4 meat turkey farms	Italy	Terregino, 2010
2009	H7N9	Breeding geese farm	Czech Republic	
2009-2010	H5N2	2 outbreaks in free-range mixed poultry holdings	Germany	
2009 - present	H7N7	Several outbreaks in commercial turkey and chicken farms, mixed holdings and 1 zoo bird. Some outbreaks possibly connected through unperceived virus circulation in domestic poultry/zoo birds.	Germany, the Netherlands, Denmark	Probst et al, 2012
2010	H7N1	2 duck holdings	Denmark	
2010	H7N4	Free range layer hens	The Netherlands	
2011	H7N?	Swans in a commercial holding	The Netherlands	
2011	H7N1	Free range layer hens	The Netherlands	
2012	H5N2	Pheasants reared in open pens	Ireland	
2012	H5N2	Turkey holding	The Netherlands	
2012-2013	H5N1	Seven holdings, species unspecified	Germany	
2013	H7N1	Breeder chickens	Spain	
2013	H7N1	Free range layer hens	The Netherlands	
2013 - present	H7N1	not specified	The Netherlands	

3.4 HPAI outbreaks in poultry

Much more information is available on HPAI than on LPAI outbreaks. Indeed, HPAI outbreaks always have severe consequences, and animal plagues have been documented since the Greco-Roman period. Although the causative agent of many of these historical outbreaks is unclear, several descriptions of disease outbreaks are analogous to what is observed in HPAIV outbreaks at present (Fleming, 1871; Heusinger, 1847). The first reliable scientific report on an HPAI outbreak is an article by Italian scientist Perroncito, published in the late 1870s. Perroncito describes an illness that affected poultry in the surroundings of Torino during the fall of 1877 and the winter of 1877-1878. While affected poultry was at first not seriously ill, the disease suddenly started causing high mortality and spread quickly throughout a larger area (Perroncito, 1878; Ruiz and Vallès, 2010). It was later revealed that this outbreak likely involved the transition of a LPAIV into a highly pathogenic variant (Alexander and Brown, 2009). Since then, several other HPAI outbreaks have been described.
Between 1901 and the 1930s, major outbreaks of fowl plague occurred all over the world, the spread of the virus often aided by a late detection of the outbreaks and wrongful or panicky settlement. However, several outbreaks seemed to have been controlled by restricting the shipping of live poultry, depopulation, quarantine and disinfection (Köhler and Köhler, 2001; Lupiani and Reddy, 2009; Petek, 2003).
The first outbreak which was with certainty caused by a HPAIV occurred in Scotland, in 1959. A list of HPAI outbreaks from then on is presented in table 3.

Unlike LPAIVs, HPAIVs do not tend to form stable lineages in poultry. Indeed, HPAI outbreaks result in a ruthless spread of the virus and the killing off of a large proportion (if not all) of the available hosts. Therefore, the emergence of a HPAIV can be considered as an erratic evolution of a LPNAIV. The transition of a LPNAIV into a HPAIV has been witnessed in some past outbreaks, namely those in the United States of America (1983), Mexico (1994-1995), northern Italy (1999-2000), Chile (2002) and Canada (2004) (Bean W.J. et al., 1985; Capua et al., 2000; Horimoto et al., 1995; Pasick et al., 2006; Suarez et al., 2004). This transition is also assumed to have occurred in the 2003 Dutch-Belgian-German H7N7 HPAI outbreak, although the progenitor LPAIV had not been isolated (Elbers et al., 2004; Stegeman et al., 2004).
The most worrisome HPAI-outbreak is the still ongoing Asian outbreak of H5N1 HPAIV that came into existence 17 years ago. This can be considered the most severe HPAI outbreak of

modern times. Unlike other HPAIV outbreaks, this virus has managed to spill-over from domesticated bird species to the wild bird reservoir and, despite its ability to cause sickness and mortality in these wild birds, to become established in them (Koch and Elbers, 2006; Shortridge, 1999; Sims et al., 2003; Sims et al., 2006; Xu et al., 1999). Whilst the virus originated in Hong Kong and other parts of southern China, by the beginning of 2004, it appeared to have become endemic in poultry and wild birds throughout south-east Asia (World animal health organisation (OIE)) and was being spread among migratory flyways across the Himalayas to the south and westward to Europe and Africa (Chen et al., 2005; Ellis et al., 2004). At its peak, in 2006, 63 countries across Asia, Europe and Africa had reported H5N1 HPAIV. Despite the number of countries reporting the disease has decreased by now, there is still evidence that the virus continues to spread. At the time of this writing, Asian H5N1 HPAIV remains endemic in some Asian countries and Egypt. In Europe, the last events of H5N1 HPAI were detected in Germany in 2008 and Romania, Bulgaria and Russia in 2010 (World animal health information database, 2013).

Table 3: Summary of HPAI outbreaks since 1959. Table adapted from: (Alexander and Brown, 2009; Koch and Elbers, 2006; Lupiani and Reddy, 2009; Swayne, 2012; World animal health organisation (OIE)).

Year	Subtype	Description	Area
1959	H5N1	1 Chicken farm	UK (SCT)
1961	H5N3	+/- 1.300 Common Terns	South Africa
1963	H7N3	29.000 Turkeys	UK (ENG)
1966	H5N9	8.000 Turkeys	Canda (ON)
1966	H7N7	not specified	Australia
1976	H7N7	58.000 Chickens & Ducks	Australia (VIC)
1979	H7N7	1 Chicken farm & 1 Goose farm	Germany
1979	H7N7	9.000 Turkeys	UK (ENG)
1983-84	H5N2	17.000.000 Chickens & Turkeys	USA (PA)
1983	H5N8	307.000 Turkeys, Chickens and Ducks	Ireland
1985	H7N7	240.000 Chickens	Australia (VIC)
1991	H5N1	8.000 Turkeys	UK (ENG)
1992	H7N3	18.000 Chickens & ducks	Australia (VIC)
1994	H7N3	22.000 Chickens	Australia (QLD)
1994-95	H5N2	Chickens (number unknown)	Mexico
1994, 2004	H7N3	>6.000.000 Chickens	Pakistan
1997	H7N4	160.000-310.000 Chickens & Emus	Australia (NSW)
1996 (ongoing)	H5N1	Wild Birds, 100s of millions of chickens and other domestic species	Eurasia, Africa
1997	H5N2	6.000-8.000 Chickens, Turkeys, Guinea-fowl, Quail, Ducks, Pheasants, Pigeons, Geese	Italy

1999-2000	H7N1	14.000.000 Chickens, Turkeys, Guinea-fowl, Quail, Ducks, Pheasants, Ostriches	Italy
2002	H7N3	600.000 Chickens and Turkeys	Chile
2003	H7N7	>30.000.000 Chickens	The Netherlands, Belgium, Germany
2004	H5N2	6.600 Chickens	Texas
2004	H7N3	>1.700.0000 poultry	Canada (BC)
2004-2006	H5N2	>30.000 Ostriches	South Africa, Zimbabwe
2005	H7N7	219.000 Chickens	North Korea
2007	H5N1	160.000 Turkeys	UK (ENG)
2007	H7N3	50.000 chickens in 1 farm	Canada (SK)
2008	H7N7	6.528 Turkeys	UK (ENG)
2009	H7N7	300.000 layer chickens in 1 farm	Spain
2011-2013	H5N2	50.000 Ostriches in 50 farms	South Africa
2012	H7N1	not specified	South Africa
2012	H5N2	831 chickens	Taipei, China
2012-2013 (ongoing)	H7N3	>6.000.000 layers and breeders	Mexico
2012	H7N7	45.000 free range layer chickens on 1 farm	Australia (NSW)
2013	H7N7	6 layer farms	Italy
2013 (ongoing)	H7N2	At least 2 layer farms (October, 2013)	Australia (NSW)

3.5 Zoonotic aspect of avian influenza

Transmission of HPAIVs to humans has been observed in several HPAI outbreaks. Typical symptoms for HPAIV infection in humans are conjunctivitis and/or influenza-like symptoms. Some infections, however, can be particularly serious and have a high case fatality rate (World health organisation, 2013). The Asian H5N1 HPAIV is especially virulent to humans: By March 12, 2013, infection with this virus has had a fatal outcome in 371 of 622 reported human cases. Fortunately, HPAIV transmission between humans has not yet been clearly observed, and humans can be considered as dead-end host (Trampuz et al., 2004; Tweed et al., 2004; Van Reeth, 2007). However, if a recombination between Asian H5N1 HPAIV and a human influenza strain occurs, a descendant possessing the pathogenicity of the avian and the transmissibility of the human strain might arise, possibly leading to a new worldwide pandemic (Alexander and Brown, 2009; Alexander, 2011).

Occasionally, LPAIVs can be transmitted from birds to humans as well. These infections are generally not severe and mostly cause conjunctivitis or an influenza-like disease (Van Reeth, 2007). However, recently (April, 2013), a LP H7N9 AIV which causes no symptoms in infected poultry has been reported to cause serious illness in humans in China (World health organisation, 2013), indicating that LPAIVs can be more threatening than generally believed.

4 Surveillance and Control of LPNAI and HPAI

Because of the economic impact of HPAI outbreaks and the zoonotic potential of these viruses, all HPAIVs are to be reported to the World Animal Health Organization (OIE). For their ability of becoming highly pathogenic through mutations, LPAIVs belonging to the H5 and H7 subtypes have become increasingly important and from 2005 onwards, these H5/H7 LPAIVs are also included in the OIE's list of notifiable diseases (World animal health organisation (OIE), 2013).

To anticipate a timely reaction in case of introduction of LPNAIVs and/or emergence of HPAIVs, early warning systems have been installed. These systems have been made possible by installing efficient disease reporting protocols (syndromic surveillance), conducting active surveillance programs (swabbing, serology) and epidemiological analyses and by making information on outbreaks readily available worldwide (Martin et al., 2007). The control and surveillance of LPNAIVs and HPAIVs in European member states is directed towards both poultry and wild birds. European member states are obliged to conduct active and passive

surveillance to detect the prevalence of LPNAI and HPAI infections in poultry. Regarding wild birds, member states have to contribute to the knowledge on the threats posed by wild birds regarding LPNAIVs and HPAIVs, also by means of active and passive surveillance programs.

The methods to be used for the diagnosis of avian influenza are defined in the "Diagnostic Manual for avian influenza" (European Commission, 2006). The measurements to be taken in case of an outbreak are set out in council directive 2005/94/EC of 20 December 2005 on community measures for the control of avian influenza (European Commission, 2005). In this chapter, the surveillance strategies, diagnostic procedures and outbreak control measures for LPNAIV and HPAIV are described.

4.1 Passive surveillance

4.1.1 Wild birds

In wild birds, passive surveillance involves the examination of wild birds that are suspected of being infected with AIV. The major aim is to detect the emerging Asian H5N1 HPAIVs in wild bird populations as soon as possible. The surveillance is directed towards high risk species, i.e.: *Anseriformes* and *Charadriiformes*.

Carcasses from groups of birds that are found dead must be sent to the national reference laboratory. Also, wild birds that are found sick, suffering influenza-like symptoms must be sampled for virological diagnosis. The obtained samples (swabs or organ samples) are to be examined for AIV with molecular diagnostic tests (European Commission, 2007).

4.1.2 Poultry

Passive surveillance in the poultry industry is based on the obligation of reporting AI-suspicions to the competent authority. Concretely, if an infection with AI is suspected in a poultry holding (European commission, 2006), the owner has to notify an official veterinarian or the competent authority (Federal agency for the safety of the food chain (FASFC)). Within 12 hours, the official veterinarian has to visit the holding for examination of the animals, collection of samples for virological and serological analyses and has to send these samples to the national reference laboratory. The official veterinarian also has to inform the competent authority of his findings and actions.

Samples for virological analysis should include at least five sick/dead birds (if present) and/or at least 20 tracheal/oropharyngeal and 20 cloacal swabs, focusing on sick birds. These samples must be analyzed using either classical (virus isolation in embryonated fowl eggs) or

molecular (conventional RT-PCR or 'real-time' single step RT-PCR) virological diagnostic techniques. Samples for serological analysis should include 20 blood samples. Those samples must be tested for presence of anti-AIV antibodies using the haemagglutination-inhibition (HI) test. Alternatively, the agar gel precipitin test may be used (European commission, 2006). If the national reference laboratory detects live virus, viral RNA or anti-AIV antibodies, the AIV outbreak is confirmed. The genotype and pathotype of the virus must then be determined and the outbreak must be declared to the Community reference laboratory (Federal public service public health, food chain safety and environment, 2008).

4.2 Active surveillance

4.2.1 Wild birds

Active surveillance of wild birds involves the sampling of (apparently) healthy wild birds for AI diagnosis. The central aim is to enable assessment of the prevalence of LPNAIVs in wild birds and to estimate the possibility of transmission by contact between infected wild birds and poultry leading to the introduction of the virus to poultry holdings (Federal public service public health, food chain safety and environment, 2008). In addition, these surveillance programs aim at ensuring an early detection of H5N1 HPAI in each country in wild birds in which they do not always induce clear clinical symptoms (Veterinary Laboratory Agency, 2010).

Like passive surveillance, the active AI surveillance in wild birds also focuses on certain target species, mostly belonging to the orders of the *Anseriformes* and *Charadriiformes*. Sampling consists of taking oropharyngeal and cloacal swabs (or alternatively, fresh fecal samples) of captured or hunted healthy wild birds. Then, these samples are to be analyzed with molecular assays (real time RT-PCR) (European Commission, 2007). Active AI surveillance in wild birds was compulsory in member states of the European Union since 2005 (European Commission, 2005). However, despite results from these active surveillance programs were valuable from a scientific point of view, the total number of samples that yielded LPNAIVs or an HPAIV was low and rarely above 2% (Veterinary Laboratory Agency, 2010). Since many resources were thus lost on negative samples, these active surveillance programs are no longer compulsory from 2011 onwards (European Commission, 2005). However, voluntary active AI surveillance is still being carried out by some EU member states (including Belgium).

Serological surveillance of healthy wild birds is not covered in the surveillance programs set out by the European commission (European Commission, 2007). However, serological data may provide important information on the AIV prevalence in wild bird populations. Indeed, virological methods only give positive results if the animal was sampled during an active AIV-infection. Serology however, includes past exposure to AIV and may enable better assessment of the presence of AIVs in wild bird populations over a longer time span (Charlton et al., 2009). Unfortunately, the analysis of wild bird sera for AIV surveillance is not yet optimized. The HI assay, which is the reference test for AIV, and the AGID test do not perform well for sera from many wild bird species and are costly and time-consuming (Higgins, 1989; Spackman et al., 2008). Therefore, reliable diagnostics for the analysis of wild bird sera are required.

4.2.2 Poultry

In HPAI outbreaks, the time span between the onset of clinical signs and diagnosis is often a week or more (Elbers et al., 2004). From the 1999-2000 Italian H7N1 and the 2003 Dutch-Belgian-German H7N7 HPAI outbreaks, it was learned that these delays may allow the virus to spread quickly to multiple farms, by which the outbreak becomes more difficult to resolve (Capua and Marangon, 2000; Elbers et al., 2004). To prevent the future development of such outbreaks, it has become clear that permanent surveillance programs need to be installed, next to the aforementioned passive AI surveillance.

European member states are now obliged to develop and apply surveillance programs that allow early detection and prevention of the spread of LPNAIVs in poultry. The objective of these active surveillance programs is to detect the virus before it has the chance of becoming widespread and/or highly pathogenic. In a nutshell, blood samples from different species of poultry are to be collected on a yearly basis. If anti-LPNAIV antibodies are detected, the holding is visited again for collection of swabs. The outbreak is confirmed if virus or viral RNA is detected in these swabs.

Guidelines for designing the active surveillance program are specified in commission decision 2010/367/EU (European Commission, 2010). A member state's surveillance program must be able to detect a LPNAI circulation with 95% certainty, in a theoretical design prevalence of 5%. For turkey, goose and duck holdings, the aforementioned level of certainty must be 99% (European Commission, 2007; 2010). The collected samples are to be tested using an HI assay, which is considered as the reference test. Alternatively, initial screening can be carried

out with another test such as ELISA. Positive results still need to be confirmed with HI test, however.

In the Belgian surveillance program, sampling is anticipated in all commercial duck, goose, turkey, chicken breeder and laying hen holdings with at least 200 animals. Game bird holdings and holdings with at least 15 ratites have been occasionally included in the surveillance program between 2005 and 2012. Backyard flocks and broiler holdings are normally not sampled (Federal agency for the safety of the food chain Federal agency for the safety of the food chain , 2005; 2006; 2007; 2009; 2013b). For the 2012 surveillance program, Belgium reported samples from 25 duck holdings, 2 goose holdings, 49 turkey holdings, 145 chicken breeder holdings, 400 laying hen holdings, 18 game bird holdings, 4 broiler chicken holdings and 1 "other" (Animal health and veterinary laboratories agency, 2010).

The selection of holdings to be sampled must be done using either a representative or a risk-based sampling approach. In a representative approach, a stratified sampling is conducted, ensuring that samples are representative for the entire member state and that each category of poultry holdings is included. In a risk-based sampling approach, knowledge on which sectors of the poultry industry are more likely to be infected by LPNAIVs is applied for the selection of holdings (Animal health and veterinary laboratories agency, 2012).

Ideally, the selection of holdings is done based on a risk assessment. In this kind of sampling approach, poultry holdings that are more at risk for being infected with LPNAIV are given extra attention when designing the sampling round. The development of a risk-based sampling approach is urged upon by the European Union and the community reference laboratory (Veterinary laboratory agency, 2008; 2009; 2012; Commision, 2007; 2010). However, risk-based surveillance must be applied with care and has to be based on thorough analysis that identifies the risk factors for LPNAIV infection in a specific member state. At the beginning of this thesis, none of the EU member states had fully developed and applied such a risk-based surveillance strategy. However, for the Belgian surveillance program, more focus is being laid on certain sectors of the poultry industry since 2007; turkey, duck and goose holdings are sampled twice/year instead of just once/year, and holdings that are situated in high risk areas, as defined by the FASFC (Federal agency for the safety of the food chain, 2013a), are to be sampled twice/year as well (Federal agency for the safety of the food chain, 2007). In the 2012 surveillance program, a risk-based sampling design was carried out by additionally considering outdoor access of animals and the situation of a holding in a densely populated poultry area. According to the most recent report by the EU Reference Laboratory

for avian influenza, nine other EU member states have used a risk-based sampling approach in 2012 (Animal health and veterinary laboratories agency, 2012).

4.3 Outbreak control measurements

4.3.1 HPAI outbreak measurements

In case of an HPAI outbreak, measurements must be applied to prevent further spread of the disease and the large-scale crippling effects it has on the poultry sector (European Commission, 1992). More specifically, in poultry holdings where the presence of an HPAIV has been detected, all on- and off-movements of people, vehicles, animals or animal products are prohibited. Additionally, all birds are killed and carcasses, poultry- and waste products must be destroyed. Housing facilities are thoroughly cleaned and disinfected and no poultry may be reintroduced for a period of 21 days. Additionally, the competent authority must establish protection and surveillance zones around the affected holdings and must immediately set in motion an epidemiological inquiry to identify contact holdings and to prevent further spread of the virus (van den Berg and Houdart, 2008).

4.3.2 LPNAI outbreak measurements

Since LPNAI outbreaks are not as dramatic as HPAIV outbreaks, these outbreaks do not always need to be dealt with as rigorously. Belgian legislation provides that holdings where an outbreak of LPNAIV is confirmed must be depopulated, but animals may be transported (under strict conditions) to the slaughter house.

If no virus or viral RNA can be detected however, it is likely that the virus is no longer circulating. Therefore, these holdings are no longer considered “infected” and depopulation is not necessarily imposed. Instead, it can be decided to do a follow-up, ensuring that a re-emergence of the virus can be detected in time (Veterinary laboratory agency, 2009).

4.3.3 Vaccination

Vaccination against notifiable avian influenza is prohibited in all of Europe since it is feared that the use of ineffective vaccines or a poor vaccination method will lead to virus replication and transmission without the animals exposing disease symptoms, thus giving the virus the opportunity to spread and to become endemic without being detected. Additionally, vaccination can increase the evolutionary pressure on AIVs and can thus lead to an increased rate of antigenic drift (Webster and Hulse, 2004; Webster et al., 2006).

However, if vaccination campaigns against notifiable AIVs are properly managed, they can prove very useful and eventually lead to the eradication of the virus, while limiting the number of healthy poultry to be destroyed. Therefore, emergency and preventive vaccination may be applied in some cases. This request for emergency vaccination must be thoroughly motivated and presented for approval to the European commission. Emergency and prophylactic vaccination campaigns have been carried out in Italy between 2000 and 2006. Since 1999, this country has faced five H7 and one H5 LPAI outbreaks. Emergency vaccination, combined with the depopulation of infected farms and contact farms, has proven to aid in controlling the outbreaks. Furthermore, since multiple LPNAIV outbreaks of genetically unrelated viruses emerged in the same area thereafter, it was decided to start a prophylactic vaccination campaign with a bivalent vaccine (Capua and Marangon, 2007).

5 Studying the transmission of LPNAIV

Compared to a representative sampling approach, risk-based sampling can increase the confidence in detecting LPNAIV in poultry holdings, hereby increasing the efficacy of the active surveillance. (Welby et al., 2010a; European Commission, 2010). Studying the transmission of LPNAI isolates allows estimating the risk of the isolate becoming established in poultry holdings. Also, transmission experiments can be used to investigate the importance of risk factors for the transmissibility of LPNAIVs or to identify new risk factors. By exposing susceptible animals to LPNAI–inoculated animals, transmission of the virus can be studied closely.

5.1 Quantifying LPNAIV transmission

Since the outcome of transmission experiments can be described by multiple parameters (such as the number of susceptibles that have become infected, the time to infection and the infectious period (Yee et al., 2009)) it is sometimes difficult to compare one experiment to another. Therefore, an analysis comprising those parameters into one parameter allows the comparison of transmission experiments and is very helpful in demonstrating effects from intervention strategies (like vaccination (de Jong and Kimman, 1994; van der Goot et al., 2005; van der Goot et al., 2007)) and climatological differences (Lowen et al., 2007).

Transmission experiments are often analyzed according to the stochastic susceptible-infectious-recovered (*SIR*) model. In this model, each individual is considered susceptible, infectious, or recovered. It describes transmission by two parameters, being the transmission

rate (β) (alternatively: transmission parameter) and the recovery rate (α). The overall transmissibility of the virus can additionally be quantified by a single parameter, the basic reproduction ratio (R_0). In words, the basic reproduction ratio is essentially the number of secondary cases caused by one typically infectious animal throughout its entire infectious period when housed in a fully susceptible population. If $R_0 < 1$, infection with the virus will die out spontaneously and it is suggested that only minor outbreaks can occur. Alternatively, if $R_0 > 1$, the virus is able to spread and both minor and major outbreaks can occur. High R_0 values indicate the possibility of a major epidemic (de Jong, 1995).

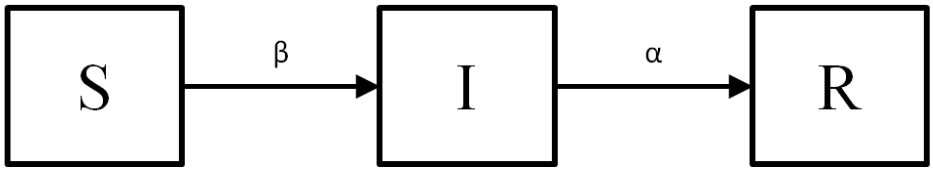

Figure 5: In the stochastic Susceptible-Infectious-Recovered model, pathogen transmission is described by the rate by which susceptibles (S) become infectious, or the transmission rate (β), and the rate by which infectious animals (I) recover from infection (R), or the recovery rate (α).

When using the SIR model, some assumptions are made. Firstly, it is assumed that the contact rate γ of each individual with other individuals is constant. Also, each individual is estimated to make a fixed number of contacts per unit of time, which implies that the transmission is assumed to be frequency-dependent (Bouma et al., 2009). Secondly, the probability p that transmission takes place given a contact is also considered a constant.

Choosing the most suitable method for estimating the parameters β, α and R_0 depends on the type of available data and the experimental design. In this thesis, two methods are used to estimate R_0; the Final Size (FS) method and the Generalized Linear model (GLM) as described in Velthuis et al (2007) (Velthuis et al., 2007).

As suggested by its name, the FS method only considers the final state of the experiment for estimating R_0. Hence, the route by which the end state is reached is ignored in this method and thus, R_0 can be estimated directly without the need of estimating β and α, according to the method of De Jong and Kimman (de Jong and Kimman, 1994). Contrarily, the more complicated GLM uses data from every time interval separately to make an estimation of β.

By calculating α by means of the infectious period of infected individuals, R_0 can be estimated as the quotient of β and α (Velthuis et al., 2007).

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Chapter 2:

Aims of the thesis

Since LPAIVs do not cause severe or pathognomonic symptoms in affected poultry, LPAI infections can easily be missed or misdiagnosed. This may allow the virus to circulate in the affected poultry holding, to spread to other holdings and thus to become established in a large part of the poultry industry. If this involves LPAIVs of the H5 or H7 subtypes, a highly pathogenic variant can emerge as the result of mutations. Since these highly pathogenic progenitors can spread rapidly and cause severe sickness in poultry, such outbreaks can inflict serious economic problems.

In the European Union, the emergence of HPAIVs is fought by aiming at detecting LPNAIVs before they have the opportunity of becoming established in a large number of poultry holdings. Hereto, since 2006, EU member states have installed active LPNAIV surveillance programs that are based on a serological screening of poultry holdings. To increase the confidence by which circulating LPNAIVs can be detected in poultry holdings, these active surveillance programs need to be as efficient as possible. Therefore, identifying the risk factors involved in LPNAIV outbreaks enables focusing the active surveillance programs on those holdings that are more at risk and will contribute to an earlier detection of LPNAIVs.
In the context of the active surveillance programs in poultry holdings, our main objectives were:

- Designing experimental models that can be used to investigate the transmission potential of LPNAIVs and to define possible risk or protection factors in LPNAI transmission, such as:
    - Housing conditions: chickens in a cage-based or a floor-based housing system.
    - Access of wild birds to poultry premises and, more specifically, the possibility of contamination of drinking water or surface contamination.
    - Mixed housing of domesticated aquatic birds and land-based poultry.
- Combining results from these models to provide insights on the infectivity and transmissibility of LPNAIVs and to determine virus characteristics that could be used as marker for estimating the potential of spreading of LPNAI isolates found in wild birds and in poultry and the subsequent risk of epidemics.

In addition, to estimate the risk of introduction of LPNAIVs from wild birds to poultry, active surveillance programs based on a serologic screening can also be envisaged in wild birds. In the context of the active LPNAI surveillance in wild birds, we additionally aimed to:

- Investigate the reliability of results obtained by multispecies ELISA kits used for the analysis of wild bird sera.

Chapter 3:

The impact of viral tropism and housing conditions on the transmission of three H5/H7 low pathogenic avian influenza viruses in chickens

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1 Summary

In this study, shedding and transmission of three H5/H7 low pathogenic avian influenza viruses (LPAIVs) in poultry was characterized and the impact of floor system on transmission was assessed. Transmission experiments were simultaneously conducted with two groups of animals housed on either a grid or a floor covered with litter. Transmission was observed for H5N2
A/Ch/Belgium/150VB/99 LPAIV. This virus was shed almost exclusively via the oropharynx and no impact of floor system was seen. Transmission was also seen for H7N1 A/Ch/Italy/1067/v99
LPAIV, which was shed via both the oropharynx and cloaca. A slight increase in transmission was seen for animals housed on litter. H5N3 A/Anas Platyrhynchos/Belgium/09-884/2008 LPAIV did not spread to susceptible animals, regardless of the floor system. This study shows that environmental factors such as floor systems used in poultry barns may act upon the transmission of LPAIVs. However, the level of influence depends on the virus under consideration and, more specifically, its principal replication sites.

2 Introduction

Avian Influenza is a disease of major importance for poultry. It is caused by type A Influenza Viruses which can infect a wide variety of animal species including many wild bird and poultry species, swine and humans (Alexander et al., 2000). It is a very diverse virus and 16 Hemagglutinin (HA) and 9 Neuraminidase (NA) subtypes have been discovered in birds up to now (Fouchier et al., 2005). Most HA and NA subtypes can be found in many possible combinations in wild water fowl, which are the virus' natural reservoir (Olsen et al., 2006).
Avian influenza viruses are typically classified in two pathotypes; highly pathogenic avian influenza (HPAI) and low pathogenicity avian influenza (LPAI), based on the symptoms developed in chickens. Highly pathogenic avian influenza viruses (HPAIVs) (formerly: bird flu or fowl plague) cause severe illness and high mortality in chickens. These virulent phenotypes have only been observed among the H5 and H7 serotypes. Infection of chickens with Low Pathogenic Avian Influenza Viruses (LPAIVs) can be asymptomatic or can cause

mild to severe respiratory sickness and/or general illness which may or may not be associated with secondary infections. Low Pathogenic Avian Influenza is found among HA serotypes 1 through 16 (including H5 and H7) (Swayne et al., 2006). The circulation of H5 and H7 LPAIVs in poultry populations can lead to mutations resulting in the insertion of basic amino acids at the HA0 cleavage site. Since this causes the virus to become highly pathogenic, these two subtypes are classified as notifiable viruses (Commission of the European Communities, 2006).

Direct or indirect contact between poultry and wild birds can lead to the introduction of AIV in the poultry sector. Therefore, location in the vicinity of wild bird breeding grounds or major flyways, the possibility of close direct contact between wild birds and domestic birds or breaches in biosecurity measures may increase the risk of introducing H5/H7 AIV into poultry holdings (Koch et al., 2006; Welby et al., 2010). Such events in which an H5/H7 LPAIV was introduced in the poultry sector and subsequently transformed into a highly pathogenic form of the virus has been observed during the 1983-1984 outbreak in Pennsylvania, the 1993-1994 outbreak in Mexico and the 1999 outbreak in Italy (Capua et al., 2010; Bean et al., 1985; Horimoto et al., 1995).

Precise estimation if an H5/H7 LPAI outbreak poses a risk of giving rise to an outbreak of HPAIV is impossible. However, it is generally assumed that the wider the circulation of H5/H7 LPAIV in a population, the higher is the probability of an HPAIV to emerge (Alexander, 1995). Therefore, studying the transmissibility of LPAIV isolates that have been isolated in poultry and wild birds can provide more insight in this process. A better understanding of the transmission of H5 and H7 LPAIVs is needed for controlling the circulation of these viruses and hereby reducing the risk of them to become highly pathogenic.

Quantification of disease transmission can be done by using the basic reproduction ratio (R_0), which is essentially the average number of susceptible individuals that are infected by one typical infectious individual during its entire infectious period in a fully susceptible population (Diekmann et al., 1990). This definition implies that an infection may spread throughout a susceptible population if $R_0>1$, and otherwise may die out if $R_0<1$ (Velthuis et al., 2007).

The survival of AIV in water, poultry litter and on surfaces has been demonstrated, indicating that fomites may contribute in AIV-transmission (Reis et al., 2012; Rohani et al., 2009; Tiwari et al., 2006). Moreover, poultry housing systems have been suggested to affect transmission of AIV in the initial stage of an outbreak (Tsukamoto et al., 2007) and Chen et al. have suggested the spread of an H5N1 HPAIV between ducks to be compromised by housing of the animals on a grid (Chen et al., 2004). Considering the role of these factors in the circulation of AIV in poultry can highly increase our understanding of the transmission of the disease.

In this study, the transmissibility of two poultry-origin LPAIVs and one duck-origin LPAIV was assessed in a series of transmission experiments involving direct contact between chickens. To provide more insight into the process of LPAIV transmission, the viruses that were selected for the present study have different infective properties for poultry, more specifically regarding their tissue tropism (oropharyngeal vs. cloacal replication). In addition, experiments were conducted pairwise on different floor systems to assess the impact of virus properties and floor system on virus transmission.

3 Methods

3.1 Viruses

Three LPAIVs were used in the present study. Low Pathogenic Avian Influenza Virus *H5N2 A/Ch/Belgium/150VB/99* was isolated by the Veterinary and Agrochemical Research Institute (VAR) in 1999. This isolate was obtained from chickens in a smallholder flock where approximately 100 chickens and 20 ducks were held. The chickens experienced 10% mortality associated with clinical signs such as depression, diarrhoea and respiratory distress. This virus was probably introduced by purchasing 10 chickens from a local market 10 days earlier (Meulemans et al., 2000). A second egg-passage of this virus was used for inoculation of the animals.

A second LPAIV, *H7N1 A/Ch/Italy/1067/v99*, was isolated from chickens by the Istitutio Zooprofilattico Sperimentale (IZS) during the 1999 LPAI epidemic in northeastern Italy (Capua et al., 2010). A fourth egg-passage of this virus was used for inoculation of the animals.

The third LPAIV used in this study, *H5N3 A/Anas platyrhynchos/Belgium/09-884/2008*, was isolated in Belgium from the cloacal swab of a wild mallard duck that was sampled as part of a long-term wild bird monitoring programme that ran from August 2008 to April 2009 (Van Borm et al., 2011). A second egg-passage of this virus was used for inoculation of the animals.

3.2 Animals

All animal experiments were conducted on SPF chickens, delivered by Lohmann-Valo (Cuxhaven, Germany). The animals were housed in biosafety level 3 isolators (type: HM1500, Montair Process Technology B.V., Kronenberg, the Netherlands) from the day of hatching until the end of the experiment. The isolators have a floor surface of 1,2 m² and the internal volume measures 0,9 m³. A negative air pressure of 45 ± 5 m^3/hour was maintained during the entire course of the experiment. Each animal experiment was conducted under the authorization and supervision of the Biosafety and Bioethics Committee at the VAR, following national and European regulations.

3.3 Experimental design

Three transmission experiments were conducted, each one using one of the three viruses described above. Each transmission experiment comprised two (for the H5N2 and H7N1 viruses) or one (for the H5N3 virus) trial(s) in which virus transmission was studied in two separate groups of SPF chickens, hereafter referred to as subtrials or housing groups (Figure 1). Animals from these two subtrials were housed in different isolators, with a different floor system. In one subtrial, animals were housed on grid flooring, which allowed droppings to pass through. In the other subtrial, the floor of the isolator was covered with plastic on which approximately 1,5kg of litter (wood shavings, Agrospan Houtkrullen, Vividerm, Bekkevoort, Belgium) was spread. To reduce other variation between the two floor systems as much as possible, both subtrials were conducted at the same time, with the same lot of SPF chickens and the same inoculum. The second trial of a transmission experiment was a repetition of the first trial.

In each subtrial, six SPF chickens were oculo-nasally inoculated at 4-6 weeks of age with a 10^6 EID_{50}/dose virus solution. These animals are hereafter referred to as seeders. One day after inoculation, six naïve SPF chickens, hereafter referred to as contacts, were introduced in the isolator. This is the reference time point used in this article and will be referred to as 0

days post exposure (dpe). Oropharyngeal (OP) and cloacal (CL) swabs were taken from the seeders from 0dpe until 9dpe. For the contacts, OP and CL swabs were taken from 1dpe until 10dpe. At 14 and 21dpe, blood samples were taken from all animals.

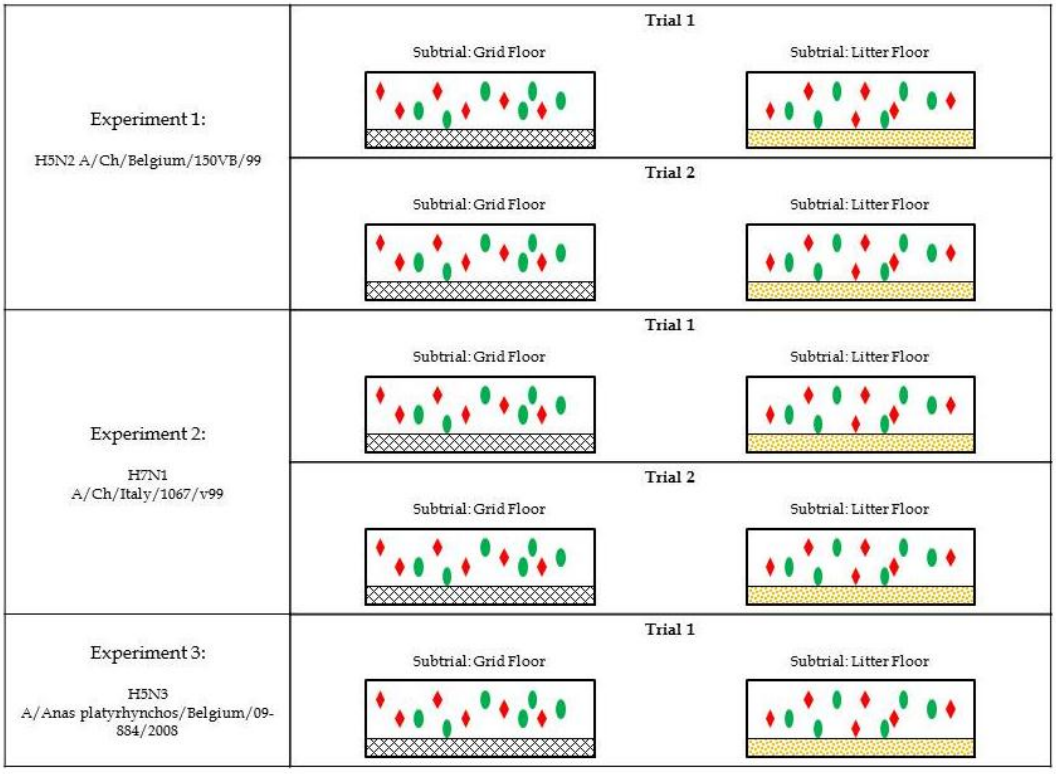

Figure 1: Schematic illustration of the design of transmission experiments conducted in this study. Experiments with H5N2 A/Ch/Belgium/150VB/99 and H7N1 A/Ch/Italy/1067/v99 comprised two separately conducted trials. The experiment with H5N3 A/Anas platyrhynchos/Belgium/09-884/2008 comprised only one trial. Each trial included two subtrials which were simultaneously conducted in different isolators; in one isolator, animals were housed on a grid floor; in the other isolator the floor was covered with litter. In each isolator, six seeders (red diamonds) and six contacts (green ovals) were housed.

3.4 Sample handling

After sampling, the OP and CL swabs were immediately submerged in a falcon tube filled with 1,5ml storage medium containing brain-heart infusion broth enriched with antibiotics (BHI+AB) (10^6 U/l penicillin G, 2 g/l streptomycine, 1g/l gentamycine sulfate and 66ml/l kanamycine sulfate 100x). Drinking water was sampled by collecting 1,5ml of drinking water

and pouring it in 1,5ml double concentrated BHI+AB medium, to yield the same concentration of medium and antibiotics in drinking water samples as the one present in swab samples. Swabs and water samples were subsequently stored at -80°C, awaiting further analysis. Sera from blood samples were stored at -20°C.

3.5 Detection and quantification of viral RNA in samples using one-step real-time RT-PCR

Viral RNA (vRNA) was semi-automatically extracted from 50µl thawed sample material using a KingFisher magnetic particle processor and the MagMaxTM AI/ND-96 Viral RNA Kit (Ambion Inc., Austin, Texas), according to the manufacturer's protocol. The Quantitect Probe RT-PCR kit (QiagenGmBH, Hilden, Germany) was used to prepare a total of 25µl reaction volume (containing 2µl of purified RNA) for amplification of matrix gene in a Biosystems 7500 real time PCR cycler (Applied biosystems, Lennik, Belgium) (Van Borm et al., 2007). A series of 1:10 dilutions of synthetic matrix RNA was run simultaneously in each RRT-PCR run to calculate the number of vRNA copies in each sample. Then, a series of 1:10 dilutions of the stock solution of each virus was analyzed to create a standard curve from which EID_{50} equivalents per ml sample medium (EID_{50}eq/ml) of each sample could be calculated. Results were finally expressed as EID_{50}eq/ml storage medium or drinking water. Samples with an RNA concentration smaller than $10^{0,0}$ EID_{50}eq/ml were considered negative. The selection of samples to be analyzed was done based on data needed for assessment of transmission parameters.

3.6 Serology

Serum samples were tested for antibodies directed towards the viral nucleoprotein with IDScreen influenza A antibody competition ELISA kit (IDvet, Montpellier, France). All tests were conducted according to the manufacturer's instructions. In the data analysis, serum samples with a doubtful result were considered positive.

3.7 Statistical analysis

Reproduction ratios were estimated using the Susceptible-Infectious-Recovered (SIR)-model, as described in Velthuis et al, 2007 (Velthuis et al., 2007). In a SIR-model, fully susceptible individuals that are in contact with infectious individuals can either stay susceptible or become infectious and finally recover from infection. The number of Susceptible, Infectious

and Recovered animals for each time period as well as the number of new cases (animal passing from exposed to infectious state) observed in the same time period were recorded. Animals were considered infected when anti-AIV antibodies were present at either 14dpe, 21dpe or both.

Basic reproduction ratios (R_0) were estimated using 2 different methods. In the first method, R_0 was estimated using following formula:

$$R_0 = \frac{\beta}{\alpha}$$

In this formula, transmission rate (β) was obtained following a generalized linear model (GLM) developed in SAS 9.2 and described in Velthuis et al (2007) (Velthuis et al., 2007; Gonzales et al., 2011; Gonzales et al., 2012). The parameter log β was estimated by modeling the number of new infections upon contact, using the offset function ln(IΔt/N), and complementary log-log function. A back transformation of log β was required to obtain β. The recovery rate (α) was calculated as the arithmetic mean of the individual infectious periods (I.P.) of all infected contact animals. The 95% confidence intervals (95%C.I.) and p-values used to determine whether the results were significant or not were the ones obtained for the parameter log β. The goodness of fit of the GLM model was assessed with the Akaike Information Criterion. For the calculation of transmission parameters with this method, contact animals were considered infected when seroconversion was demonstrated at 14 and/or 21dpe and when virus shedding above $10^{1,3}$ EID_{50}eq/ml was observed at least once. The same cut-off value was maintained for determining the infectious periods.

In the second method, values for R_0 were estimated according to the Final Size (FS) model, using the Maximum Likelihood Estimator (Gonzales et al., 2011; Dewulf et al., 2001 and 2002; Velthuis et al., 2002; van der Goot et al., 2003):

$$R_0 = max \prod_{i=1}^{n} Prob(x_i \,.\, R_0 | N.S_0.I_0)$$

Confidence intervals were constructed symmetrically around the estimate of R_0, in accordance to the method described by Bouma et al. (2000) (Bouma et al., 2000). For this method, animals were considered infected if seroconversion was demonstrated at either 14 or 21dpe.

4 Results

4.1 Transmission of H5N2 A/Ch/Belgium/150VB/99

In trial 1, the seeders shed virus from the day after inoculation (0dpe) until at least 4dpe, except for one animal in the grid subtrial, which commenced virus shedding at 2dpe (Table 1A). Virus shedding was nearly always via the oropharynx. Cloacal virus shedding was demonstrated in only one inoculated animal, in the litter subtrial, at 5dpe (Table 1B). In both subtrials, virus shedding was observed in all contact animals. In the grid subtrial, one contact animal was found positive by RRT-PCR on two separate days and did not seroconvert. All other contact animals in this subtrial shed virus for several consecutive days and seroconverted (Table 1A). In the litter subtrial, one contact animal was found positive by RRT-PCR for only two days as well, however, in this case these were consecutive days, larger EID_{50}eq/ml were demonstrated in these swabs and the considered animal seroconverted. All other contact animals in the litter subtrial demonstrated virus shedding for several consecutive days and seroconverted (Table 1B). Viral RNA was found in drinking water until 8dpe for the grid subtrial and 5dpe for the litter subtrial.

In trial 2, all seeders from both subtrials shed virus from 0dpe until at least 3dpe. Virus shedding was practically always via the oropharynx and cloacal virus shedding was only rarely observed. In the grid subtrial, virus shedding was observed in two contact animals, both of which were positive from 1 or 2dpe until 7 or 8dpe. Both these animals were the only ones from this group to seroconvert (Table 1C). In the litter subtrial, two contact animals showed a similar pattern of virus shedding and were the only ones to seroconvert as well. In contrary to the group housed on grid, two more contact animals from the litter housing group were found positive by RRT-PCR for one day, however without seroconverting (Table 1D). Drinking water contained vRNA from 0 or 1dpe until 9dpe.

Table 1 (next two pages): Results for transmission experiments with H5N2 A/Ch/Belgium/150VB/99 LPAIV. Virus shedding is expressed as $\log_{10}$ EID_{50}eq/ml storage medium for oropharyngeal and cloacal swabs. Light grey squares = OP swab with virus concentration above $10^{1,3}$ EID_{50}eq/ml and CL swab below or equal to $10^{1,3}$ EID_{50}eq/ml or not tested, dark grey squares = CL swab with virus concentration above $10^{1,3}$ EID_{50}eq/ml and OP swab below or equal to $10^{1,3}$ EID_{50}eq/ml or not tested, black squares = OP and CL swabs both containing a virus concentration above $10^{1,3}$ EID_{50}eq/ml. Virus in drinking water is expressed as $\log_{10}$ EID_{50}eq/ml. Immune response at 14 and 21days post exposure as determined by ELISA test. +=positive, ±=doubtful; -=negative; n.t.=not tested; n.s.=no sample; Exp.=Exposure. A=Trial 1-Subtrial 1; B=Trial 1-Subtrial 2; C=Trial 2-Subtrial 1; D=Trial 2-Subtrial 2.

H5N2: TRIAL 1

Subtrial: GRID

A.

	Days Post-exposure											Serology	
	0	1	2	3	4	5	6	7	8	9	10	14	21
	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL		
Seeder 1	3,1|-	5,3|-	3,9|-	5,2|-	4,3|n.t.	3,1|-	-	-	n.t.|-	-	n.s.	+	+
Seeder 2	-	-	1,4|-	5,5|-	4,9|n.t.	5,3|-	5,0|-	4,2|-	2,1|-	-	n.s.	+	+
Seeder 3	4,1|-	3,6|-	4,4|-	4,5|-	5,0|n.t.	2,8|-	3,2|-	-	-	-	n.s.	+	+
Seeder 4	3,2|-	4,2|-	3,1|-	5,1|-	4,0|n.t.	-	3,6|-	-	-	-	n.s.	+	+
Seeder 5	2,5|-	5,6|-	4,6|-	n.t.|-	n.t.	3,7|-	3,4|-	4,1|-	3,8|-	-	n.s.	+	+
Seeder 6	5,0|-	4,3|-	5,2|-	n.t.|-	n.t.	3,6|-	4,1|-	4,5|-	2,4|-	-|1,0	n.s.	+	+
Contact 1	Exp.	1,2|-	-	2,3|-	-	-	-	n.t.	n.t.	n.t.	-	-	-
Contact 2	Exp.	2,1|-	-	3,2|-	4,6|n.t.	4,8|n.t.	4,7|-	4,0|-	3,2|-	2,1|-	-	+	+
Contact 3	Exp.	1,3|-	3,9|-	3,8|-	n.t.	n.t.	3,9|-	2,6|-	-	-	-	+	+
Contact 4	Exp.	-	-	4,3|-	n.t.	n.t.	4,4|-	4,2|-	3,0|-	2,9|-	-	+	+
Contact 5	Exp.	-	-	3,0|-	4,3|n.t.	3,9|n.t.	2,4|-	3,2|-	-	-	-	+	+
Contact 6	Exp.	1,8|-	3,9|-	3,8|-	4,0|n.t.	4,4|n.t.	2,7|-	-	-	-	0,2|-	+	+
Drinking Water	-	2,9	2,2	4,2	2,8	2,9	2,4	0,9	1,2	0,7	-		

Subtrial: LITTER

B.

	Days Post-exposure											Serology	
	0	1	2	3	4	5	6	7	8	9	10	14	21
	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL		
Seeder 1	3,4|-	5,0|-	4,5|-	n.t.	5,2|n.t.	1,9|3,1	n.t.|-	-	n.t.	0,5|-	n.s.	+	+
Seeder 2	5,1|-	4,3|-	5,7|-	n.t.	4,2|n.t.	3,0|-	3,5|n.t.	-	1,3|-	-	n.s.	+	+
Seeder 3	3,0|-	5,8|-	4,5|-	n.t.	5,2|n.t.	3,0|-	2,7|n.t.	-	n.t.	-	n.s.	+	+
Seeder 4	4,0|-	5,0|-	5,0|-	n.t.	5,5|n.t.	2,8|-	-|n.t.	0,7|-	n.t.	-	n.s.	+	+
Seeder 5	4,1|-	5,4|-	5,2|-	n.t.	4,8|n.t.	-	-|n.t.	n.t.	n.t.	-	n.s.	+	+
Seeder 6	3,3|-	6,0|-	5,5|-	n.t.	4,5|n.t.	2,3|-	2,8|n.t.	-	n.t.	-	n.s.	+	+
Contact 1	Exp.	-	-	2,3|-	4,5|n.t.	3,3|n.t.	2,5|-	2,3|-	-	1,8|-	1,0|-	+	+
Contact 2	Exp.	-	-	4,5|-	2,1|-	-	-	n.t.	n.t.	n.t.	-	+	+
Contact 3	Exp.	3,5|-	4,5|-	4,2|-	4,6|-	2,7|-	-	-	n.t.	n.t.	-	+	+
Contact 4	Exp.	-	3,4|-	4,6|-	n.t.	n.t.	4,2|-	-	1,6|-	-	-	+	+
Contact 5	Exp.	1,1|-	3,0|-	4,7|-	n.t.	n.t.	4,4|-	2,3|-	-	-	-	+	+
Contact 6	Exp.	2,1|-	2,8|-	4,3|-	n.t.	n.t.	4,1|-	-	-	-	-	+	+
Drinking Water	-	3	2,5	1,4	1,5	1,6	-	-	-	-	-		

H5N2: TRIAL 2

Subtrial: GRID

C.

	Days Post-exposure											Serology	
	0	1	2	3	4	5	6	7	8	9	10	14	21
	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL		
Seeder 1	2,2|-	3,9|-	n.t.	n.t.	n.t.	3,7|n.t.	0,4|-	-	n.t.	n.t.	n.s.	+	+
Seeder 2	2,5|-	3,1|-	n.t.	n.t.	n.t.	3,3|n.t.	-|6,0	-	n.t.	n.t.	n.s.	+	+
Seeder 3	4,8|-	3,8|-	n.t.	n.t.	n.t.	3,8|n.t.	3,0|-	-	-	n.t.	n.s.	+	+
Seeder 4	2,5|-	3,4|-	n.t.	n.t.|-	n.t.	3,2|n.t.	3,4|n.t.	1,4|-	-|0,8	n.t.	n.s.	+	+
Seeder 5	4,1|-	3,8|-	3,8|-	2,1|-	-	-|n.t.	-|n.t.	n.t.|-	n.t.	n.t.	n.s.	+	+
Seeder 6	4,1|-	3,8|-	n.t.	n.t.|-	n.t.	3,2|n.t.	1,3|n.t.	-	n.t.	n.t.	n.s.	+	+
Contact 1	Exp.	-|n.t.	-|n.t.	-	-|n.t.	-|n.t.	-	n.t.	n.t.	n.t.	n.t.	-	-
Contact 2	Exp.	-|n.t.	-|n.t.	-	-|n.t.	-|n.t.	-	n.t.	n.t.	n.t.	n.t.	-	-
Contact 3	Exp.	-|n.t.	3,0|n.t.	4,2|-	n.t.	3,5|n.t.	4,2|-	3,6|-	-	-	n.t.	+	+
Contact 4	Exp.	1,1|n.t.	-|n.t.	2,3|-	2,3|-	-	4,2|-	4,2|-	3,5|-	-	-	+	+
Contact 5	Exp.	-|n.t.	-|n.t.	-	-|n.t.	-|n.t.	-	n.t.	n.t.	n.t.	n.t.	-	-
Contact 6	Exp.	-|n.t.	-|n.t.	-	-|n.t.	-|n.t.	-	n.t.	n.t.	n.t.	n.t.	-	-
Drinking Water	2,7	2,1	4,4	3,6	5	4,9	3,8	3,8	3,5	3,3	-		

Subtrial: LITTER

D.

	Days Post-exposure											Serology	
	0	1	2	3	4	5	6	7	8	9	10	14	21
	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL		
Seeder 1	3,7|-	3,8|-	n.t.	n.t.	n.t.	3,3|n.t.	-	-|n.t.	n.t.	n.t.	n.s.	+	+
Seeder 2	3,5|-	3,3|-	n.t.	4,9|n.t.	2,5|-	1,5|-	-	-|n.t.	n.t.	n.t.	n.s.	+	+
Seeder 3	4,2|-	3,8|-	n.t.	n.t.	n.t.	3,1|n.t.	-	-|n.t.	n.t.	n.t.	n.s.	+	+
Seeder 4	4,9|-	4,5|-	n.t.	5,6|n.t.	1,7|-	-	n.t.	n.t.	n.t.	n.t.	n.s.	+	+
Seeder 5	4,6|-	3,7|-	n.t.	n.t.	4,0|-	1,5|1,1	-	-|n.t.	n.t.	n.t.	n.s.	+	+
Seeder 6	5,2|-	5,1|-	n.t.	n.t.	n.t.	4,5|n.t.	-|1,0	-	n.t.	n.t.	n.s.	+	+
Contact 1	Exp.	1,8|n.t.	2,9|n.t.	-	3,4|n.t.	3,5|-	3,1|-	-	-	n.t.	n.t.	+	+
Contact 2	Exp.	n.t.	n.t.	-	n.t.	n.t.	-	n.t.	n.t.	n.t.	n.t.	-	-
Contact 3	Exp.	-|n.t.	5,3|n.t.	5,8|-	5,9|n.t.	4,0|n.t.	2,6|-	1,3|-	-	-	n.t.	+	+
Contact 4	Exp.	-|n.t.	-|n.t.	3,6|-	-|n.t.	-	-	n.t.	n.t.	n.t.	n.t.	-	-
Contact 5	Exp.	-|n.t.	-|n.t.	-	n.t.	n.t.	-	n.t.	n.t.	n.t.	n.t.	-	-
Contact 6	Exp.	-|n.t.	-|n.t.	-	n.t.	-|n.t.	1,6|-	-	n.t.	n.t.	n.t.	-	-
Drinking Water	-	3,3	4,7	3,8	4	2,9	-	2,5	1,9	3,2	-		

In general, transmission of H5N2 A/Ch/Belgium/150VB/99 was different in both trials. Despite virus shedding in seeders was similar in all groups for both trials, less contact animals had become infected in trial 2 than in trial 1. Virus transmission leading to seroconversion always occurred during the 3 days following introduction of the contact animals and no cloacal virus shedding was observed during this time period.

4.2 Transmission of H7N1 A/Ch/Italy/1067/v99

In trial 1, the seeders shed virus from the day after inoculation until at least 5dpe. Oropharyngeal virus shedding preceded combined virus shedding via the oropharynx and cloaca in three animals for the grid subtrial (Table 2A) and five animals for the litter subtrial (Table 2B). In the grid subtrial, only one contact animal seroconverted. This animal shed virus for 7 consecutive days. Three other contact animals were found positive by RRT-PCR on several occasions but did not seroconvert (Table 2A). In the group housed on litter, all animals were found positive by RRT-PCR throughout most of the experiment. Cloacal virus shedding was observed in two contact animals and five contact animals seroconverted (Table 2B). Drinking water contained vRNA throughout most of the experiment

Table 2 (next two pages): Results for transmission experiments with H7N1 A/Ch/Italy/1067/v99 LPAIV. Virus shedding is expressed as $\log_{10}$ EID_{50}eq/ml storage medium for oropharyngeal and cloacal swabs. Light grey squares = OP swab with virus concentration above $10^{1,3}$ EID_{50}eq/ml and CL swab below or equal to $10^{1,3}$ EID_{50}eq/ml or not tested, dark grey squares = CL swab with virus concentration above $10^{1,3}$ EID_{50}eq/ml and OP swab below or equal to $10^{1,3}$ EID_{50}eq/ml or not tested, black squares = OP and CL swabs both containing a virus concentration above $10^{1,3}$ EID_{50}eq/ml. Virus in drinking water is expressed as $\log_{10}$ EID_{50}eq/ml. Immune response at 14 and 21days post exposure as determined by ELISA test. +=positive, ±=doubtful; -=negative; n.t.=not tested; n.s.=no sample; Exp.=Exposure. A=Trial 1-Subtrial 1; B=Trial 1-Subtrial 2; C=Trial 2-Subtrial 1; D=Trial 2-Subtrial 2.

H7N1: TRIAL 1

Subtrial: GRID

A.

	Days Post-exposure											Serology	
	0	1	2	3	4	5	6	7	8	9	10	14	21
	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL		
Seeder 1	3,1|-	6,0|-	2,5|-	4,8|-	4,5|n.t.	3,7|-	3,1|-	2,2|-	-	-	n.s.	+	+
Seeder 2	4,1|-	3,1|-	2,6|0,8	3,7|-	n.t.	3,8|-	-	-	-	-	n.s.	+	+
Seeder 3	4,3|-	4,3|-	3,2|-	4,2|-	n.t.	4,0|-	0,9|-	0,6|-	-	-	n.s.	+	+
Seeder 4	5,6|-	5,4|-	5,7|-	5,8|n.t.	5,0|n.t.	3,6|-	2,7|-	-	-	-	n.s.	+	+
Seeder 5	5,8|-	4,2|-	3,5|4,0	4,0|2,8	n.t.	4,1|4,9	3,9|2,9	2,4|-	-	-	n.s.	+	+
Seeder 6	3,8|-	5,1|-	4,6|0,0	4,4|-	n.t.	4,3|-	3,4|-	2,4|-	2,0|-	-	n.s.	+	+
Contact 1	Exp.	-	-	1,3|-	-	-	2,2|-	1,7|-	-	-	-	-	-
Contact 2	Exp.	-	-	-	-	-	-	n.t.	n.t.	n.t.	-	-	-
Contact 3	Exp.	-	-	2,0|-	1,1|-	-	-	n.t.|-	n.t.|-	n.t.|-	-	-	-
Contact 4	Exp.	-	-	1,0|-	-	0,4|-	3,8|-	1,6|-	-	-	-	-	-
Contact 5	Exp.	3,8|-	5,1|-	2,9|-	5,4|-	4,4|-	2,8|-	2,4|-	-	-	-	+	+
Contact 6	Exp.	-	-	-	-	-	-	n.t.	n.t.	n.t.	-	-	-
Drinking Water	-	4,6	-	5,6	5,3	4,5	4	5,1	4,2	-	-		

Subtrial: LITTER

B.

	Days Post-exposure											Serology	
	0	1	2	3	4	5	6	7	8	9	10	14	21
	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL		
Seeder 1	3,6|-	6,1|-	5,3|-	n.t.	n.t.	4,8|-	n.t.	4,2|-	3,8|-	-	n.s.	+	+
Seeder 2	4,1|-	4,1|-	3,6|-	4,6|2,5	5,6|-	2,1|-	-	n.t.	n.t.	-	n.s.	+	+
Seeder 3	5,2|-	4,8|1,4	3,9|-	n.t.	n.t.	4,2|-	4,7|-	3,3|-	-	-	n.s.	+	+
Seeder 4	5,9|-	4,7|-	5,1|-	n.t.	n.t.	3,5|-	4,4|-	3,0|-	3,1|3,7	-	n.s.	+	+
Seeder 5	6,6|-	5,4|-	4,4|6,0	n.t.	n.t.	4,7|5,7	n.t.	-|6,2	3,2|6,8	-|1,5	n.s.	+	+
Seeder 6	4,9|-	5,1|1,3	4,6|-	n.t.	n.t.	3,6|5,2	4,1|6,3	-|3,3	4,1|-	-	n.s.	+	+
Contact 1	Exp.	1,5|-	1,8|-	1,9|2,1	1,6|0,6	1,5|-	-	n.t.	n.t.	n.t.	-	-	-
Contact 2	Exp.	2,2|-	-	1,7|-	2,2|-	4,1|-	5,1|-	4,6|-	4,8|-	4,0|n.t.	-	+	+
Contact 3	Exp.	-	-	2,0|-	2,3|-	6,0|-	5,2|1,8	5,2|-	5,7|1,2	5,6|n.t.	4,9|-	+	+
Contact 4	Exp.	4,3|-	4,7|-	5,1|-	5,7|-	6,0|-	4,2|-	1,8|-	2,7|-	-|n.t.	-	+	+
Contact 5	Exp.	-	-	1,0|-	0,9|-	-	-	2,8|-	3,7|-	2,8|n.t.	5,3|-	+	+
Contact 6	Exp.	-	3,8|-	4,3|-	1,1|-	1,9|-	4,8|-	3,2|-	-|1,1	1,4|n.t.	-	+	+
Drinking Water	-	5	4,4	5,7	4,7	5,4	4,8	5,3	3,6	4,1	-		

H7N1: TRIAL 2

Subtrial: GRID

C.

	Days Post-exposure											Serology	
	0	1	2	3	4	5	6	7	8	9	10	14	21
	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL		
Seeder 1	4,9|-	4,6|-	4,0|-	n.t.	n.t.	2,4|-	4,2|-	2,1|-	3,2|-	-	n.s.	+	+
Seeder 2	6,4|-	4,8|-	4,4|-	n.t.	n.t.	3,3|1,3	4,6|-	-	-	n.t.	n.s.	+	+
Seeder 3	3,8|-	4,7|-	5,2|-	n.t.	5,0|-	-	-	n.t.	n.t.	n.t.	n.s.	+	+
Seeder 4	4,2|-	6,0|0,8	4,4|2,9	n.t.	n.t.	4,2|6,3	n.t.	2,2|5,4	-|4,0	n.t.	n.s.	+	+
Seeder 5	-	5,8|-	5,6|-	n.t.	n.t.	3,9|-	n.t.	3,8|-	-	3,3|-	n.s.	+	+
Seeder 6	2,8|-	3,8|-	3,3|-	n.t.	n.t.	2,8|-	4,3|-	-	-	n.t.	n.s.	+	+
Contact 1	Exp.	2,9|n.t.	4,3|-	3,5|-	n.t.	3,0|-	-	2,7|-	-	1,5|-	1,2|-	+	-
Contact 2	Exp.	2,9|n.t.	1,0|-	2,7|-	3,9|n.t.	4,4|-	3,3|-	-	3,1|1,1	2,8|-	3,5|-	Dead	
Contact 3	Exp.	1,7|n.t.	2,3|-	-|1,8	1,4|-	-	n.t.	n.t.	n.t.	n.t.	n.t.	-	-
Contact 4	Exp.	3,0|n.t.	3,7|-	4,9|-	-	-	n.t.	n.t.	n.t.	n.t.	n.t.	+	+
Contact 5	Exp.	3,0|n.t.	2,4|-	3,7|2,2	n.t.	-|2,3	-|5,0	-|3,8	-	-	-	+	+
Contact 6	Exp.	2,5|n.t.	2,1|-	2,2|3,3	-	2,0|-	-	-	n.t.	n.t.	n.t.	-	+
Drinking Water	3,3	5,8	4,6	5,4	4,3	4,5	5	5,1	5,2	4,7	-		

Subtrial: LITTER

D.

	Days Post-exposure											Serology	
	0	1	2	3	4	5	6	7	8	9	10	14	21
	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL		
Seeder 1	5,4|-	4,4|-	2,7|-	n.t.	n.t.	1,9|2,9	2,8|5,1	n.t.	n.t.|4,4	n.t.|3,5	n.s.	+	+
Seeder 2	5,5|-	4,6|-	3,6|-	n.t.	n.t.	2,2|-	1,5|-	-	n.t.	n.t.	n.s.	+	+
Seeder 3	4,8|-	4,8|1,9	4,8|3,4	n.t.	n.t.	4,9|6,0	n.t.	-|5,2	-|3,3	-	n.s.	+	+
Seeder 4	6,1|-	4,7|-	3,7|1,3	n.t.	n.t.	3,6|-	3,2|-	-	n.t.|-	n.t.|-	n.s.	+	+
Seeder 5	3,6|-	5,0|-	4,7|-	n.t.	n.t.	4,6|3,7	n.t.	1,9|5,2	n.t.|5,0	n.s.	n.s.	Dead	
Seeder 6	5,3|-	3,3|-	4,9|1,0	n.t.	n.t.	2,3|-	-	-	-	n.t.	n.s.	+	+
Contact 1	Exp.	-	1,8|-	2,2|-	-	2,0|2,9	-	-	-	-	-	-	+
Contact 2	Exp.	1,3|-	1,9|-	2,3|-	-	2,4|-	1,7|-	-	-	-	-	±	+
Contact 3	Exp.	-	-	2,3|-	-	2,1|-	2,1|-	-	-|1,5	2,2|-	1,4|-	-	-
Contact 4	Exp.	1,8|-	2,5|-	3,1|-	2,6|-	-	-	-	-	-	-	+	+
Contact 5	Exp.	1,5|-	4,9|-	2,8|5,0	n.t.	4,0|-	n.t.	n.t.	-|4,9	n.t.|5,0	n.t.|4,2	+	+
Contact 6	Exp.	-	-	2,7|-	-	-	n.t.	n.t.	n.t.	n.t.	n.t.	+	-
Drinking Water	4,3	4,2	5,1	5,5	5	4,6	5,1	5	4	-	4,5		

In trial 2, all seeders shed virus until at least 4dpe. One inoculated animal from the grid subtrial was negative by RRT-PCR at 0dpe, but was found positive one day later. Cloacal virus shedding was observed in two seeders from the grid subtrial (Table 2C) and five animals from the litter subtrial (Table 2D). In the grid subtrial, all contact animals were RRT-PCR positive from 1dpe until at least 3dpe. Then, one animal ceased virus shedding, two animals were found positive on two more days whilst the other three animals were found positive by RRT-PCR until at least 7dpe. No blood samples were available from one contact animal from the grid subtrial that died at 10dpe from a non-AIV related cause. For the remaining five contact animals from this group, four animals seroconverted at 14 and/or 21dpe. The one contact animal that did not seroconvert was found RRT-PCR positive for 4 consecutive days but did not shed large amounts of virus. For the litter subtrial, one contact animal was found shedding large doses of virus via the oropharynx and cloaca from 1dpe until the end of the trial and seroconverted. The other contact animals however, were positive at several times, but the amounts of virus shed were clearly smaller. Nevertheless, four of them were found positive on serology (Table 2D). Viral RNA was demonstrated in drinking water from 0dpe until 9 or 10dpe.

In both trials, the number of seeders that shed viruses via the cloaca was higher in the groups housed on litter compared to the groups housed on grid, despite virus shedding at 0dpe being highly comparable for all subtrials. In trial 1, the virus was transmitted more successfully in the group housed on litter. However, no difference regarding the number of contacts becoming infected can be seen between the two housing groups in trial 2.

4.3 Transmission of H5N3 A/Anas platyrhynchos/Belgium/09-884/2008

Only one trial was conducted with this virus. In the grid subtrial, only two seeders shed virus through the oropharynx for 2 or more days. A third animal was found slightly positive by RRT-PCR at 0dpe. Seroconversion was demonstrated in 4 seeders (Table 3A). In the litter subtrial, virus shedding by seeders was comparable; one animal shed virus for 5 days as well whilst two other animals were found positive at 0dpe only. Seroconversion was seen in four seeders as well (Table 3B). In the grid subtrial, one contact animal was found slightly positive by RRT-PCR at 2dpe and none seroconverted (Table 3A). In the litter subtrial, two contact animals were found slightly positive by RRT-PCR. Again, none of the contact animals

seroconverted (Table 3B). In both subtrials, no virus was detected by RRT-PCR in any of the drinking water samples.

Table 3 (next page): Results for transmission experiments with H5N3 A/Anas platyrhynchos/Belgium/09-884/2008 LPAIV. Virus shedding is expressed as $\log_{10}$ EID_{50}eq/ml storage medium for oropharyngeal and cloacal swabs. Light grey squares = OP swab with virus concentration above $10^{1,3}$ EID_{50}eq/ml and CL swab below or equal to $10^{1,3}$ EID_{50}eq/ml or not tested, dark grey squares = CL swab with virus concentration above $10^{1,3}$ EID_{50}eq/ml and OP swab below or equal to $10^{1,3}$ EID_{50}eq/ml or not tested, black squares = OP and CL swabs both containing a virus concentration above $10^{1,3}$ EID_{50}eq/ml. Virus in drinking water is expressed as $\log_{10}$ EID_{50}eq/ml. Immune response at 14 and 21days post exposure as determined by ELISA test. +=positive, ±=doubtful; -=negative; n.t.=not tested; n.s.=no sample; Exp.=Exposure. A=Trial 1-Subtrial 1; B=Trial 1-Subtrial 2; C=Trial 2-Subtrial 1; D=Trial 2-Subtrial 2.

H5N3

Subtrial: GRID

A.

	Days Post-exposure											Serology	
	0	1	2	3	4	5	6	7	8	9	10	14	21
	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL		
Seeder 1	-	-	-	-|n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.s.	-	+
Seeder 2	0,3|-	-	-	-|n.t.	-|n.t.	-|n.t.	n.t.	n.t.	n.t.	n.t.	n.s.	-	±
Seeder 3	-	-	-	-|n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.s.	-	-
Seeder 4	3,6|-	3,4|-	4,0|-	3,8|n.t.	3,5|n.t.	-|n.t.	-|n.t.	-|n.t.	n.t.	n.t.	n.s.	+	-
Seeder 5	2,9|-	2,6|-	-	-|n.t.	-|n.t.	-|n.t.	n.t.	n.t.	n.t.	n.t.	n.s.	+	+
Seeder 6	-	-	-	-|n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.s.	-	-
Contact 1	Exp.	-	-	n.t.|-	n.t.|-	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	-	-
Contact 2	Exp.	-	-	n.t.|-	n.t.|-	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	-	-
Contact 3	Exp.	-	-	n.t.|-	n.t.|-	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	-	-
Contact 4	Exp.	-	0,5|-	-	-	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	-	-
Contact 5	Exp.	-	-	n.t.|-	n.t.|-	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	-	-
Contact 6	Exp.	-	-	n.t.|-	n.t.|-	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	-	-
Drinking Water	-	-	-	-	-	-	-	-	-	-	-		

Subtrial: LITTER

B.

	Days Post-exposure											Serology	
	0	1	2	3	4	5	6	7	8	9	10	14	21
	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL	OP|CL		
Seeder 1	2,1|-	-	-	-|n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.s.	+	-
Seeder 2	2,7|-	-	-	-	-	n.t.	n.t.	n.t.	n.t.	n.t.	n.s.	±	-
Seeder 3	-	-	-	-|n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.s.	-	-
Seeder 4	-	-	-	-	-|n.t.	-|n.t.	-|n.t.	-|n.t.	-|n.t.	-|n.t.	n.s.	-	+
Seeder 5	4,4|-	4,0|-	2,6|-	3,9|-	2,0|-	-	-	-	n.t.	n.t.	n.s.	+	+
Seeder 6	-	-	-	-|n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	n.s.	-	-
Contact 1	Exp.	-	-	n.t.|-	n.t.|-	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	-	-
Contact 2	Exp.	0,3|-	-	n.t.|-	n.t.|-	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	-	-
Contact 3	Exp.	0,2|-	-	n.t.|-	n.t.|-	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	-	-
Contact 4	Exp.	-	-	n.t.|-	n.t.|-	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	-	-
Contact 5	Exp.	-	-	n.t.|-	n.t.|-	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	-	-
Contact 6	Exp.	-	-	n.t.|-	n.t.|-	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.	-	-
Drinking Water	-	-	-	-	-	-	-	-	-	-	-		

4.4 Quantification of Virus Transmission

Figure 2 shows the evolution of the number of contact infected animals per time unit. For the H5N2 experiment, all infections on both floor systems occurred within 3dpe. For the H7N1 experiment, the graphs for the two floor systems have different courses (Figure 2). Quantification of virus transmission was initially carried out for each subtrial separately *(results not shown)*. Since results of subtrials with the same flooring and virus were not significantly different, these subtrials were combined in order to increase the precision of the study.

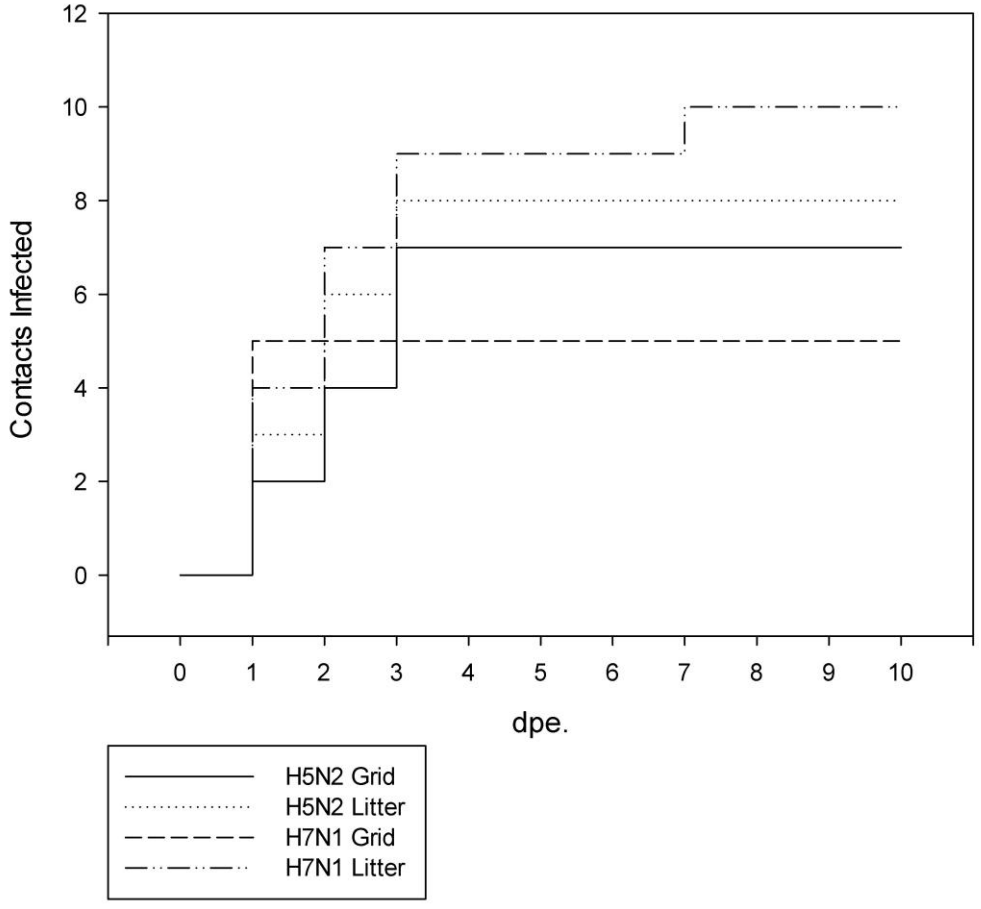

Figure 2: Evolution of the number of contact-infected animals for experiments with H5N2 A/Ch/Belgium/150VB/99 and H7N1 A/Ch/Italy/1067/v99 conducted on two different floor systems.

Virus	Flooring	Contacts Infected	Median Time to Detection	GLM			FS
				Mean I.P.	Contacts, 1/α	Transmission Rate, β	Reproduction ratio, R_0	Reproduction ratio, R_0
H5N2	Grid	7 / 12	2 (1-3)	6,5	[3,93 - 8,92]	0,24 [0,10 - 0,44]	1,54 [0,84 - 2,24]	1,27 [0,43 - 3,50]
H5N2	Litter	8 / 12	2 (1-3)	5,5	[2,36 - 8,64]	0,37 [0,14 - 0,61]	2,04 [0,79 - 3,28]	1,77 [0,55 - 4,14]
H7N1	Grid	5 / 11	1 (1-1)	6,2	[1,73 - 10,67]	0,15 [0,02 - 0,25]	0,93 [0,21 - 1,65]	1,03 [0,27 - 2,82]
H7N1	Litter	9 / 12	2 (1-7)	6,1	[0,45 - 11,75]	0,38 [0,11 - 0,44]	2,32 [0,12 - 4,52]	1,72 [0,68 - 4,14]
H5N3	Grid	0 / 6						0 [0 - 0,72]
H5N3	Litter	0 / 6						0 [0 - 0,72]

Table 4: Quantification of transmission for combined experiments with A/Ch/Belgium/150VB/99 (H5N2), A/Ch/Italy/1067/v99 (H7N1) and A/Anas platyrhynchos/Belgium/09-884/2008 (H5N3) on each floor system, according the generalized linear model and the final size model. Median time to detection is expressed as days post exposure with min & max values between round brackets. Mean infectious period (I.P.) is also expressed in days. Values between square brackets are 95% confidence intervals.

Table 4 summarizes the results for combined transmission experiments and the reproduction ratios estimated according GLM and FS method. Transmission of the H5N2 and H7N1 viruses was successful on all floor types. This table shows that the point estimates of the basic reproduction ratios for the H5N2 experiment are above 1 for both floor systems, suggesting this virus is capable of spreading through a susceptible population. However, the 95% confidence interval calculated for both floor systems was large and not significantly higher than one. Both point estimates for the two floor systems evaluated in this study do not differ a lot and both confidence intervals largely overlap suggesting that there is no important difference in virus transmission between the two floor systems. For the H7N1 experiment, values for R_0 are larger for the subtrials conducted on litter than on grid. However, also in these trials the 95% confidence intervals largely overlap. Since none of the contact animals in the experiments with H5N3 A/Anas platyrhynchos/Belgium/09-884/2008 seroconverted, R_0 for this virus could not be obtained with the GLM method.

Basic reproduction ratios estimated using the Final Size Model are comparable to values for R_0 estimated with the GLM method

and mostly have larger 95% confidence intervals.

5 Discussion

In the present study, the impact of two parameters, virus strain and flooring, on virus transmission was assessed in a series of transmission experiments involving direct contact between inoculated and susceptible SPF chickens. The three selected LPAIVs had different infective characteristics for the host species. The impact of flooring on virus transmission was assessed by dividing each trial in two subtrials that were conducted simultaneously and differed only in the floor system used. Subtrials where animals were housed on a grid were chosen to mimic cage housing or the housing of animals on raised floors on which droppings do not accumulate while subtrials in which the floor of the isolator was covered with litter were used to mimic conditions in an all litter housing system. Several parameters such as infectious period, time of infection and basic reproduction ratio were determined to quantify the impact of these parameters on virus transmission.

To our knowledge, only few studies have quantified transmission of H5 and H7 LPAIVs between chickens in laboratory experiments so far. Van der Goot et al. have estimated R_0 for A/Chicken/Pennsylvania/21525/83 H5N2 LPAIV (van der Goot et al., 2003) and Gonzales et al. have estimated R_0 for A/Chicken/Netherlands/2006 H7N7 LPAIV, A/Turkey/Italy/1067/99 H7N1 LPAIV and have observed no transmission of A/Turkey/Italy/2369/2009 H5N7 LPAIV (Gonzales et al., 2011 and 2012). It is generally assumed that viruses that are well adapted to a certain bird species replicate easily in this species and are shed in large amounts (Swayne et al., 2008; Spekreijse et al., 2011), giving rise to a large infective pressure in the environment, which has been suggested to determine the incidence and the course of infection (Bouma et al., 2009).

The chicken-origin H5N2 virus selected in this study proved to be successfully transmitted between SPF chickens. Preliminary studies had shown that H5N2 A/Ch/Belgium/150VB/99 has a strong infectious potential for SPF chickens and is shed almost exclusively via the oropharynx (G. Claes, unpublished data). The present study demonstrates that cloacal virus shedding among seeders was only seen in a minority of animals and only occurred near the end of the infectious period. Since contact infected birds were mostly already shedding virus

before that time, it can be said that transmission of this virus occurred through oropharyngeal virus shedding by infectious animals and that aerosols, which are proven to be important in LPAIV transmission (Tsukamoto et al., 2007; Okamatsu et al., 2007; Yee et al., 2009), have an important role in the transmission of this virus. The differences in transmission parameters between subtrials with this virus conducted on grid or litter flooring were insignificant and might rather be attributed to biological variation than to an impact of the floor system.

Transmission of H7N1 A/Ch/Italy/1067/v99 from inoculated SPF chickens to susceptible SPF chickens occurred in all trials as well. Regarding the impact of floor system on transmission of this virus, we have observed: i) more contact animals seroconverting, ii) more seeders presenting cloacal virus shedding and iii) infection of contact animals throughout the entire course of the experiment for the litter housing groups. These observations suggest an impact of the floor system on the transmission of this virus. Moreover, the joint reproduction ratio estimated for the subtrials conducted on grid flooring showed that less transmission occurred in this housing condition, compared to the subtrials conducted on litter. However, since the confidence intervals of the reproduction ratios were strongly overlapping, it is impossible to conclude, based on the current results, whether this difference is a true biological difference or rather due to coincidence.

This result might be linked to the substantial affinity of H7N1 A/Ch/Italy/1067/v99 for intestinal epithelia of infected animals. Our results agree with Marché et al. (2010), where cloacal shedding of H7N1 A/Ch/Italy/1067/v99 occurred after a short period (2 or 3 days) of virus replication in the oropharynx or trachea as well (Marché et al., 2010). Therefore, after an initial transmission of virus via aerosols, transmission between animals housed on litter may have been aided by the accumulation of infectious faeces. Contrarily, housing of animals on grid provided a constant discharge of faeces and hereby impeded a further increase of infective pressure.

Regarding the assessment of reproduction ratios for this virus, the 10 days-timeframe used to monitor virus shedding in the animals appeared too short. Virus shedding above $10^{1,3}$ EID_{50}eq/ml was still observed in some animals at 9 or 10dpe, suggesting that virus shedding still continued at 11dpe and that the mean I.P. is underestimated. Basic reproduction ratios for H7N1 A/Ch/Italy/1067/v99 were smaller than basic reproduction ratios that have previously been estimated by Gonzales et al. (2011) for a similar virus from the same LPAIV outbreak

(Gonzales et al., 2011). In both studies however, the criteria that were used to define the status of individuals within the SIR-model were different.

Next to the results presented in this study, both H7N1 and H5N2 strains used have genetic evidence of adaptation to poultry as the length of the stalk of their neuraminidase is reduced. Details can be found in the EpiFlu databank: Isolate id: Epi_isl_74837 (Epiflu databank). Interestingly however, both these poultry-adapted LPAIVs have different virus shedding patterns. Based on the results from this study, the intestinal tropism of LPAIVs should not be considered as the only mechanism for poultry-adaptation. Whether this statement counts for only certain AIV subtypes or whether it represents an intrinsic characteristic for each LPAIV is still to be explained. Other studies might suggest that this replication tropism might be subtype specific as the characteristics of the H5N2 A/Ch/Bel/150VB/99 were highly comparable to the characteristics observed by van der Goot et al. for H5N2 A/Chicken/Pennsylvania/21525/83 LPAIV (van der Goot et al., 2003). Both strains have the same distinct affinity for the upper respiratory tract, are hardly shed via the cloaca and have very similar estimates for R_0.

There is no evidence that H5N3 A/Anas platyrhynchos/Belgium/09-884/2008 was transmitted to the contact animals. A preliminary study had proven this virus to be of low infectivity for poultry since less than 50% of inoculated SPF chickens established virus shedding after oculonasal inoculation with a 10^6 EID_{50} viral dose. Moreover, virus shedding had proven to be very short, mainly oropharyngeal and generally weak (G. Claes, unpublished data). A study by Van Borm et al. (2011), which focused on the genetic properties of this and related viruses, indicated no evidence of adaptation to poultry, such as deletions in the neuraminidase stalk region (Van Borm et al., 2011). Therefore, the virus was incapable of successfully infecting enough inoculated animals to build up the infective pressure required for transmission and/or was too quickly eliminated by the host's immune response to allow virus shedding. The latter probably explains why seroconversion was observed for two seeders in which virus shedding was not detected. In analogous experiments, Gonzales et al. observed no transmission of H5N7 A/Turkey/Italy/2369/2009 LPAIV between layer chickens (Gonzales et al., 2012). These findings corroborate the hypothesis that an adaptation step would be needed for the spread of similar viruses in chicken flocks.

Several fomites have been suggested to contribute to the spread of AIV. For example, it is well known that AIVs can remain infectious in water reservoirs for a considerable amount of time (Rohani et al., 2009; Stallknecht et al., 1990; Leung et al., 2007; Lang et al., 2008; Domanska-Blicharz et al., 2010; Nazir et al., 2010). Some recent animal experiments on AIV transmission have found virus titers in drinking water high enough to cause virus transmission (Achenbach et al., 2011). Therefore, daily water samples from experiments carried out in this study were analyzed for presence of vRNA. Our results show drinking water samples from experiments with H5N2 and H7N1 contained large amounts of vRNA, sometimes ranging as far as 10^5 EID_{50}eq/ml. Additional tests have demonstrated that virus from these samples were viable (data not shown). Since the drinking water was refreshed each time after sampling, it can be assumed that the quantities of vRNA found in the drinking water was a good reflection of the virus shedding or virus charge in the isolator at each time interval. Indeed, for seven out of eight experiments where vRNA was demonstrated in the drinking water, the first day on which virus shedding was demonstrated in the majority of the contact animals coincides with the day on which peak concentrations of vRNA were found in the drinking water. However, whether the increase of vRNA in the drinking water is either the cause or consequence of infection of contact animals is difficult to assess as a possible contamination of oropharyngeal swabs (animal drinking just before sampling) cannot be excluded.

Conducting experiments under laboratory conditions inevitably leads to an artificial environment. Fewer variables are thus involved, which enhances the reproducibility of experiments. On the other hand, the atmosphere created by controlled temperature, air flow and relative humidity may differ from what is appropriate in the field and the sustainability of virus in feces or on surfaces such as the grid floor or wood shavings may be altered (Lowen et al., 2007; Guan et al., 2009). Likewise, the susceptibility of SPF chickens to LPAIVs may be different from that of conventional chicken breeds.

Virus transmission was evaluated according to the Susceptible-Infectious-Recoverd (SIR) model (de Jong and Kimman, 1994). Basic reproduction ratios of this model were estimated using the Final Size and Generalized Linear Model. Estimation of reproduction ratios using the FS method is independent on a latency period. Contrarily, the GLM method does take the latency period into account since each time unit of the experiment is considered instead of just the final state (Velthuis et al., 2002). Because the GLM method makes use of more input data,

the 95%C.I. of R_0 estimated with this method are generally smaller than when the FS method is used.

In transmission experiments that use the SIR model, the outcome is largely dependent on the criterion that is used to define the status of an animal and on the diagnostic assay that is used to detect infection (Dewulf et al., 2002; Comin et al., 2011). Indeed, for many pathogens that cause no or only minor symptoms in diseased animals, it is sometimes difficult to undeniably demonstrate freedom from disease in the flock. Since this is certainly the case for LPAIV, a wide variety of criteria defining an infected contact animal in LPAIV transmission studies are used in literature. Isolation of virus from OP or CL swabs from animals is traditionally regarded a sign of infection (Li et al., 2010; Makarova et al., 2003; Westbury et al., 1981) whilst some studies have included serology in their data analysis (Gonzales et al., 2011 and 2012; van der Goot et al., 2003). In recently conducted LPAIV transmission experiments, real-time RT-PCR is frequently used for determining infection in contact animals (Gonzales et al., 2012; Bertran et al., 2011; Pillai et al., 2010; Yee et al., 2009). In our study, we have assumed that oropharyngeal swabs can be RRT-PCR positive because the animal picked up viruses from the environment just before sampling, which can lead to a positive RRT-PCR result without the animal actually being infected. Therefore, we have used the more conservative approach that assumes that a true virus replication leads to seroconversion and shedding of fairly large doses of virus. For this reason, only animals that seroconverted were considered infectious and a stricter RRT-PCR cut-off value than simply the limit of detection of this method was used to assess the moment of infection, the infectious period and the moment of recovery. Our choice of a $10^{1,3}$ EID_{50}eq/ml cut-off value was based on a small analysis of the results that had shown that, when using this cut-off value, most animals that are considered infected are indeed positive on both RRT-PCR and ELISA test, hereby reducing the number of animals that are positive by RRT-PCR but negative by ELISA or vice versa as much as possible (data not shown).

In this study, we have demonstrated that the floor system in poultry barns might have an impact on transmission of LPAIVs that are replicated in the intestinal tract of infected animals. However, since the observed effect was rather small, this environmental factor should not be considered as a critical factor for LPAIV transmission and other factors should be considered influential as well. Indeed, transmission of LPAIVs must be considered as a

highly complex event and since all these environmental factors act upon the infective pressure, their impact on transmission, and thus the risk of the virus to become highly pathogenic, should not be underestimated.

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Chapter 4:

An experimental model to analyze the risk of introduction of a duck originated H5 low pathogenic avian influenza virus in poultry through close contact and contaminative transmission

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1 Summary

Aquatic wild birds are often carriers of LPAIVs. If H5 and H7 LPAIVs are transmitted to poultry and have the opportunity to circulate, a HPAIV may arise. Contact with aquatic wild birds is one of the most important ways in which these LPAIVs can be introduced into poultry flocks. In this study, the transmissibility of a duck originated H5 LPAIV between ducks and chickens was analyzed in a series of animal experiments, using different transmission routes. Results indicate that the outcome of virus intake by chickens exposed to infectious ducks depends on the way the virus is presented. Drinking water contaminated by faeces proved to be the most efficient route by which the virus can be transmitted to chickens. The results from this study also suggest that some duck originated H5 LPAIVs may be introduced to poultry but do not have the potential to become established in poultry populations.

2 Introduction

Influenza A is a highly diverse virus and is therefore classified according to the two glycoproteins that are presented on the virus membrane, the Hemagglutinin (HA) and Neuraminidase (NA). Since infected poultry may develop a wide variety of symptoms, Avian Influenza Viruses (AIVs) are additionally classified according to their virulence in poultry (Alexander, 2007; Webster et al., 1992). Highly Pathogenic Avian Influenza Viruses (HPAIV) cause severe sickness and high mortality rates in affected poultry holdings. Outbreaks of these viruses may lead to serious epidemics with disastrous consequences that can affect a large region (Alexander, 2007). To date, HPAIVs have mainly been found among the H5 and H7 subtypes. Avian influenza viruses that cause no or minor symptoms in poultry are classified as Low Pathogenic Avian Influenza Viruses (LPAIV). However, infections with LPAIV may result in more severe symptoms if they are combined with poor air quality in poultry barns or secondary infections (Halvorson et al., 2003). Low Pathogenic Avian Influenza Viruses can be found among HA subtypes 1-16 (Fouchier et al., 2005). Whereas LPAIV outbreaks are usually of limited economic importance, a spontaneous insertion of basic amino acids at the HA0 cleavage site may give rise to a highly pathogenic variant of the

strain (Alexander et al., 2007). This was witnessed in some past H5 and H7 LPAIV outbreaks in Pennsylvania (1983), Mexico (1994-1995) and northern Italy (1999-2000) (Capua et al., 2000; Bean et al., 1985; Horimoto et al., 1995).

Avian influenza viruses can be introduced in commercial poultry holdings or smallholder flocks in numerous ways. Human activity or movements from other mechanical vectors such as rodents, passerines or other animals may cause such passive introduction without being infected (Olsen et al., 2006). Direct contact with pigs or humans infected with avian influenza has been observed as a possible route for introduction of AI as well (Mohan et al., 1981; Wood et al., 1997). However, direct contact with infected waterfowl is considered to be the most important route for the introduction of LPAI to poultry (Alexander, 2007). Indeed, for most HPAI-outbreaks in poultry that have been reported during the past 5 decades, it has been shown that a wild bird LPAIV had been introduced to the index case and then mutated into an HPAIV (Koch and Elbers, 2006). Since the beginning of AI surveillance studies in the early 1970s, a tremendous amount of LPAI viruses have been isolated in different wild bird species (Alexander and Brown, 2009; Lupiani and Reddy, 2009). Among these, wild aquatic birds, particularly anseriformes and charadriiformes, are now known to be the natural reservoir of LPAIV (Olsen et al., 2006). Therefore, the possibility of direct or indirect contact between poultry and wild birds is considered a major risk for the introduction of the virus and the subsequent emergence of an HPAIV, if the virus is allowed to spread (Koch and Elbers, 2006; Alexander and Brown, 2009). However, this natural route of LPAIV introduction into poultry has never been observed closely, and important information on this occurrence is lacking. Therefore, studying the transmission of LPAIVs from wild birds to poultry in experimental conditions may provide more insight into the dynamics of this event and on the exact route by which the virus is transmitted.

In this study, a wild bird originated H5N3 LPAIV was at first phenotypically characterized in SPF chickens and Pekin ducks. Then, the introduction of the virus from infective Pekin ducks to susceptible SPF chickens was studied in a series of transmission experiments targeting direct virus transmission through i) close contact between infected ducks and chickens and ii) contaminative transmission.

3 Methods

3.1 Viruses

All experiments were conducted with LPAIV H5N3 A/Anas platyrhynchos/09-884/2008. The virus was isolated from a cloacal swab of a mallard duck that was sampled in La Hulpe (Belgium) in December 2008 as part of the Belgian long-term wild bird monitoring programme (Van Borm et al., 2011). The swab was found positive by qRRT-PCR, and the virus was isolated in 9-day-old embryonated SPF chicken eggs. For this study, a second passage of the virus was diluted in sterile phosphate-buffered saline to obtain an inoculum of 10^7 EID_{50}/ml.

3.2 Animals

One-day old white pekin ducks (Anas platyrhynchos) were purchased from a local producer (Wijverkens pluimvee, Halle, Belgium). Specific-pathogen free chicken eggs were purchased from Lohman-Valo (Cuxhaven, Germany) and hatched in our facilities, under biosafety level 3 (BSL-3) conditions. All animals were housed in BSL-3 isolators (type: HM1500, Montair Process Technology B.V., Kronenberg, the Netherlands) until the end of the experiment. The inner floor surface of the isolators measures 80 x 150 cm and the height of the isolator is 72 cm. The isolator floor consisted of a stainless steel grid covered with a plastic grid to allow more grip for the animals. In the transmission experiments, half of the grid area was covered with plastic, to prevent faeces from passing through. The walls and ceiling of the isolator are made of stainless steel and two walls contain acrylic glass with rubber gloves allowing manipulation of the animals. The animals received feed from a stainless steel feeder and tap water from a plastic automated bell drinker, unless stated otherwise. A negative air pressure of 45 ± 5 m^3/hour was maintained during the entire course of the experiment. Each animal experiment was conducted under the authorization and supervision of the Biosafety and Bioethics Committee at the VAR, following national and European regulations.

3.3 Experimental design

3.3.1 SPF chicken Infection Experiment (Experiment #1)

Twelve SPF chickens were oculo-nasally inoculated with 100µl of the inoculum. Virus shedding was then followed by collecting oropharyngeal (OP) and cloacal (CL) swabs at 1, 3, 6 and 10 days post inoculation (dpi). Immune response was assessed by collecting blood samples at 7, 10, 14 and 21 dpi.

3.3.2 Transmission Experiments involving Close Contact (Experiment #2)

This experiment consisted of three trials (2a, 2b, 2c). Trials 2a and 2b each started with a group of six pekin ducks which were oculo-nasally inoculated with 100µl of the inoculum and then placed in an isolator. Trial 2c started with a group of three pekin ducks which were inoculated and placed in a third isolator. The day of inoculation is hereafter referred to as day -1. In all trials, OP and CL swabs were collected from the inoculated pekin ducks at day 0, after which each group of ducks was transferred to an isolator where 6 susceptible SPF chickens were housed. Then, OP and CL swabs were collected from every duck and chicken at days 1, 2, 3, 4, 6, 10, 14, 18 and 21. At the same times the animals were sampled, floor swabs and drinking water samples were collected to assess the environmental infection pressure. Blood samples from all animals were collected at 14 and 21 days post inoculation/exposure (corresponding to days 13 and 20 for pekin ducks and 14 and 21 for SPF chickens). In the results section of this manuscript, this experiment is presented in two parts: the assessment of the infectivity of H5N3 for pekin ducks (experiment 2 – part 1) and the transmission experiment between ducks and chickens involving close contact (experiment 2 – part 2).

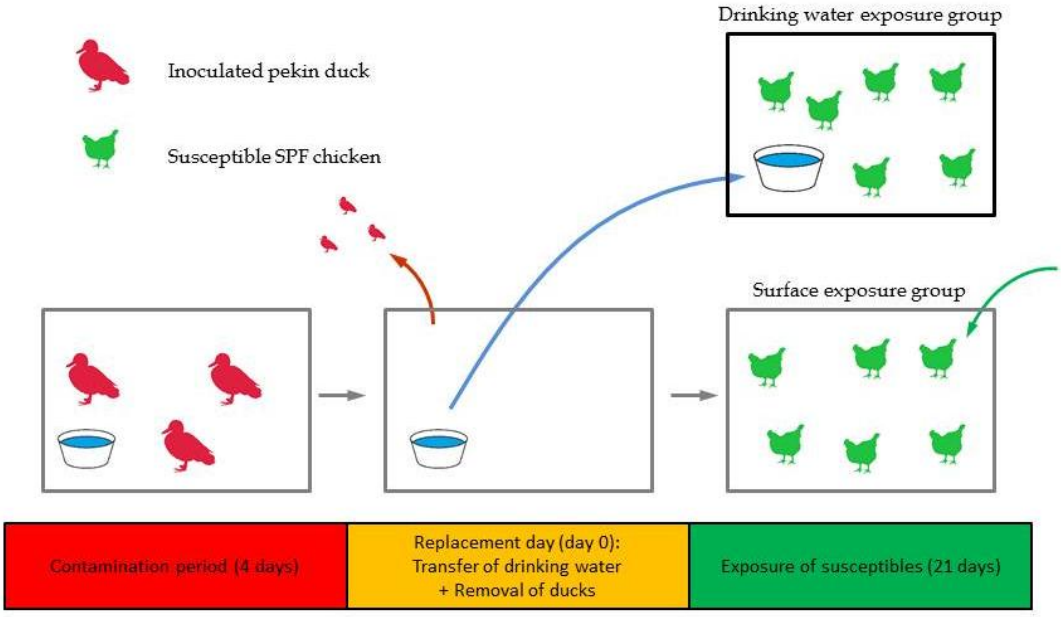

Figure 1: Experimental design of direct transmission experiments involving contaminative transmission. Three inoculated pekin ducks were housed in an isolator for four days. At the end of this four-day period, the ducks were removed from the isolator. Then, the drinking bowl was placed in a different isolator where six susceptible SPF chickens were housed (drinking water exposure group); and six other susceptible SPF chickens were placed in the isolator where the ducks were previously housed (surface exposure group). Virus transmission to SPF chickens from both groups was then monitored during a period of 21 days.

3.3.3 Transmission Experiments involving Contaminative Transmission (Experiment #3)

This experiment consisted of two trials (3a and 3b), which were replicates. Each trial consisted of two groups of six susceptible SPF chickens, the drinking water exposure (DWE) group and the surface exposure (SE) group, the names referring to the fomite to which these chickens were exposed. The trials were designed as follows (Figure 1): three pekin ducks were inoculated at day -4 and housed in an isolator for four days, until day 0. Instead of using the automated bell drinker that was used in all other experiments, the ducks received drinking

water in a 2,5 liter polypropylene drinking bowl that was replenished daily. At day 0, also referred to as the replacement day, the ducks were removed from the isolator; the drinking bowl was placed in the isolator housing the DWE group and the chickens from the SE group were placed in the isolator where the ducks were previously housed. Chickens of the DWE group were thus exposed to contaminated drinking water and chickens of the SE group were thus exposed to (primarily faecal) contaminated dry surfaces such as isolator walls, floor, feeder and feed. An automated bell drinker was installed in the SE group to provide non-contaminated drinking water. Since contamination was induced by the same three inoculated pekin ducks for both the DWE and SE group, a comparison of the importance of these fomites for LPAIV transmission was made possible. Oropharyngeal and cloacal swabs were collected from all susceptible SPF chickens at days 1-5, 7, 10 and 14. The environmental infection pressure was assessed by taking floor swabs and drinking water samples at days 1- 5, 7, 10, 12 and 14. Blood samples were collected from all chickens at days 14 and 21.

3.4 Sample handling

Animal and floor swabs were immediately immersed in 1,5ml of a storage medium (Brain-Hearth-Infusion Broth enriched with a mixture of antibiotics containing gentamycin, kanamycin, penicillin and streptomycin (BHI+AB)), after which the sample was briefly vortexed to release swab material and the cotton was discarded. Drinking water samples (1,5ml) were poured in 1,5ml of a double concentrated BHI+AB storage medium, to yield the same concentration of medium and antibiotics in drinking water samples as the one present in swab samples. All samples were stored at -80°C, awaiting further analysis. Blood samples were allowed to coagulate at room temperature after which the serum was harvested and stored at -20°C until testing for antibodies was conducted.

3.5 Detection and quantification of viral RNA in samples using one-step real-time RT-PCR

Samples were allowed to reach room temperature and viral RNA (vRNA) was semi-automatically extracted from 50µl sample material using a KingFisher magnetic particle processor and the MagMaxTM AI/ND-96 VRNA Kit (Ambion Inc., Austin, Texas), according to the manufacturer's protocol. A total of 25µl reaction volume (containing 2µl of purified RNA) was prepared using the Quantitect Probe RT-PCR kit (QiagenGmBH, Hilden,

Germany) and amplification of the matrix gene was carried out in a Biosystems 7500 real time PCR cycler (Applied biosystems, Lennik, Belgium) (Van Borm et al., 2007). In each run, a series of eight 1:10 dilutions of synthetic matrix RNA was run simultaneously to calculate the number of RNA copies per ml sample medium. Animals were considered positive by qRRT-PCR when at least one swab sample taken throughout the course of the experiment contained at least 10^0 copy of vRNA per reaction volume, thus corresponding to $10^{2,7}$ vRNA copies/ml sample medium.

3.6 Serology

Serum samples were tested for the presence of antibodies directed towards the viral nucleoprotein with the IDScreen influenza A antibody competition ELISA kit (Idvet, Montpellier, France). All tests were conducted according to the manufacturer's instructions. In the data analysis, serum samples with a sample-to-negative ratio greater or equal to 0,5 were considered negative, and S/N ratio less than 0,5 were considered positive. Animals were considered positive by serology if at least one serum sample was found positive by NP-ELISA.

3.7 Virus titration using chicken embryo fibroblasts (CEF)

Quantification of AIV in water samples and floor swabs was done using a microtitre endpoint titration in primary cultures of chicken embryo fibroblasts (CEF). Final cell suspensions of $2x10^5$ CEF/ml were prepared in L15/Leibovitz+McCoy medium enriched with Gentamycin and Glutamin and allowed to adhere to the wells of a 96 microtitre plate for two days in the presence of inactivated fetal bovine serum. A more detailed protocol can be found elsewhere (Stallknecht et al., 1990). The samples were diluted in L15/Leibovitz+McCoy medium enriched with gentamycin, glutamin and TPCK trypsin and incubated. The wells were examined for cytopathic effect with light microscopy. Fifty per cent tissue culture infectious dose per ml sample medium ($TCID_{50}$/ml) was calculated using the method described by Reed & Muench (1938) (Reed and Muench, 1938).

3.8 Statistical analysis

The transmission of LPAIV *H5N3 A/Anas platyrhynchos/09-884/2008* by close contact (experiment 2) was modeled in a stochastic susceptible-infectious (SI) transmission model.

We assumed unequal infectivities and susceptibilities for ducks and chickens, and considered our study population to be heterogeneous. Theoretically, four different transmission parameters can thus be considered to play a part in experiment 2; transmission from ducks to chickens (β_{dc}), transmission between chickens (β_{cc}), transmission from chickens to ducks (β_{cd}) and transmission between ducks (β_{dd}), analogous to Velthuis et al. (2003) (Velthuis et al., 2003). Since all ducks were inoculated at the beginning of the experiment, only β_{dc} and β_{cc} were considered in the statistical analysis. Likewise, in a previous study investigating transmission of the same virus between chickens, β_{cc} could not be determined since transmission did not occur (Claes et al., 2013). Therefore, it was assumed that $\beta_{cc} = 0$ and that all infected chickens became infected by duck-chicken virus transmission, quantified by β_{dc}. The transmission parameter β_{dc} and its 95% confidence interval was estimated with a Generalized Linear Model in SPSS 19, using a complementary log-log function and the offset function: $\ln(I_d \Delta t/N)$, where I_d represents the number of infectious ducks and N represents the total number of animals at the beginning of the time interval Δt. Susceptible chickens were considered infected if anti-AIV antibodies were detected in blood serum at either 14 or 21 days post exposure. The time of infection was determined as the first day on which an OP swab from an infected chicken was found positive by qRRT-PCR.

Comparing overall quantities of vRNA in OP and CL swabs between experiments was done by calculating median area under the curve (AUC) values (Brown et al., 2009; Spekreijse et al., 2011). Hereto, quantities of vRNA in OP and CL swabs of each individual were plotted over time and the AUC was calculated in Microsoft Excel 2010 (Microsoft corporation, Redmond (WA), United States of America), using the following formula:

$$AUC = \sum_{i=1}^{n}(t_i - t_{i-1})q_i + \frac{(t_i - t_{i-1})(q_i - q_{i-1})}{2},$$

where q_i is the number of vRNA copies or $TCID_{50}$ per ml storage medium for the sample at time t_i. Only animals that were positive by qRRT-PCR for at least one swab sample were considered. AUC values were thus calculated for each individual and the median, 25th and 75th percentiles are shown in the results.

Relationships between vRNA quantities in duck and chicken OP and CL swabs and environmental samples were assessed with an analysis of variance (ANOVA), which was

carried out in SPSS version 19 (SPSS Inc., Armonk, New York). A $\log_{10}(1+x)$ transformation was carried out.

4 Results

4.1 Experiment 1: infectivity of H5N3 A/Anas platyrhynchos/09-884/2008 for SPF chickens

At 1 dpi, viral RNA was detected in OP swabs from 5/12 chickens, of which three chickens were still found positive at 3 dpi (Figure 2A). At 6 and 10dpi, vRNA was no longer detected in OP swabs (Figure 2A). CL swabs were all negative, except for 1 swab taken at 3dpi from a chicken that was also found positive for OP shedding (Figure 2B). An overall estimation of vRNA quantities shed by each chicken throughout the entire course of the experiment was made by calculating the area under the curve (AUC) of vRNA quantities found in each sample plotted over time. These results show that the oropharyngeal viral shedding in inoculated SPF chickens is the strongest, whilst cloacal virus shedding is almost negligible (Table 1). Seroconversion, however, was observed in more chickens than virus shedding was. Indeed, anti-AIV antibodies were demonstrated in serum samples from 9/12 inoculated SPF chickens at both 14 and 21 dpi (Table 2).

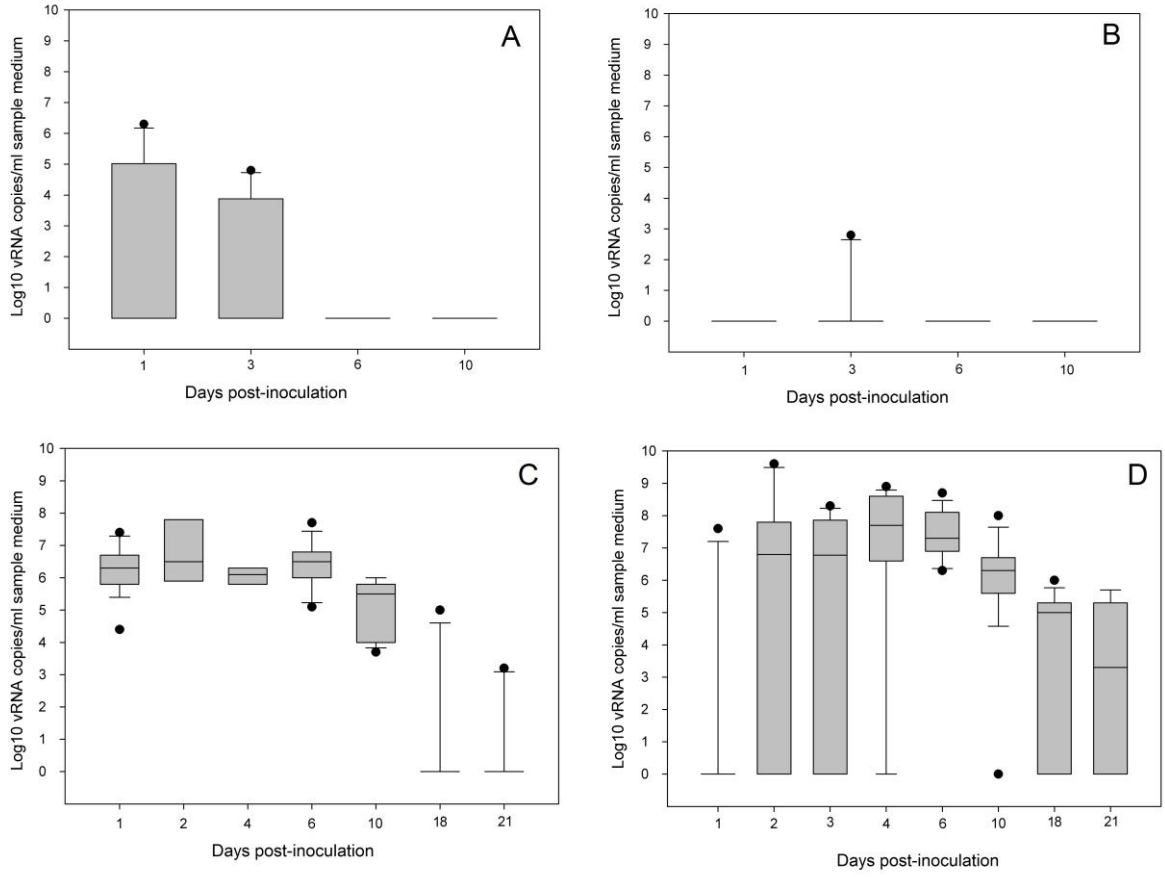

Figure 2: Virus shedding patterns for SPF chickens and pekin ducks inoculated with H5N3 A/Anas platyrhynchos/09-884/2008. Log_{10} vRNA copies/ml sample medium are presented for Oropharyngeal SPF chickens swabs (A), Cloacal SPF chicken swabs (B), Oropharyngeal pekin duck swabs (C) and cloacal pekin duck swabs (D). Negative samples are represented as 10^0.

4.2 Experiment 2 – part 1: infectivity of H5N3 A/Anas platyrhynchos/09-884/2008 for pekin ducks

All fifteen pekin ducks (6 from trial 2a, 6 from trial 2b and 3 from trial 2c) showed OP and CL virus shedding; OP virus shedding started at 1dpi whilst the onset of CL virus shedding varied (Figures 2C and 2D). One duck from trial 2c already showed cloacal shedding at 1dpi, although this commenced at 2dpi for all ducks from trial 2a and the two remaining ducks from trial 2c. The onset of cloacal virus shedding was more delayed in trial 2b: 2/6 ducks at 3dpi, 1/6 ducks at 4dpi en 2/6 ducks at 6dpi (samples from 5dpi were not analyzed). Overall estimation of the routes and intensity of virus shedding throughout the infectious period shows a completely different profile for pekin ducks than for SPF chickens. Whilst virus

shedding in SPF chickens is primarily through the oropharynx, cloacal virus shedding is the strongest virus shedding route in ducks. Moreover, the overall intensity of virus shedding was higher in ducks than in SPF chickens (Table 1). Seroconversion was seen in all inoculated pekin ducks at 14dpi. However, the presence of antibodies in this species did not appear as long-lasting since only 12/15 pekin ducks remained positive by NP-ELISA at 21 dpi (Table 2).

Table 1: Overview of the total amount of viral RNA found in oropharyngeal (OP) and cloacal (CL) swabs from chickens and ducks throughout the entire course of each trial. Log_{10} of the median and the interquartile range (IQR) of the areas under the curve that were calculated for each animal separately is shown for each trial. n=the number of animals that were positive by qRRT-PCR at least once, and on which data for AUC calculation is based.

Experiment Number	Species	Exposure type	median AUC [IQR]			
			OP	*n*	CL	*n*
1	SPF chicken	Inoculated	5,5 [4,9 - 5,7]	*5*	3,2 n/a	*1*
2a	Pekin duck	Inoculated	7,7 [7,5 - 7,9]	*6*	8,5 [8,1 - 8,9]	*6*
2b	Pekin duck	Inoculated	7,4 [7,1 - 7,6]	*6*	8,8 [8,5 - 9,1]	*6*
2c	Pekin duck	Inoculated	7,1 [7,0 - 7,7]	*3*	9,2 [8,9 - 9,6]	*3*
2a	SPF chicken	Close contact	7,4 [7,2 - 7,4]	*6*	6,9 [6,8 - 7,1]	*6*
2b	SPF chicken	Close contact	7,2 [7,0 - 7,5]	*6*	7,5 [7,0 - 8,4]	*6*
2c	SPF chicken	Close contact	6,2 [5,9 - 6,3]	*6*	5,3 [5,2 - 5,6]	*6*
3a	SPF chicken	Drinking water	5,4 [5,1 - 5,5]	*6*	2,7 [2,6 - 3]	*2*
3a	SPF chicken	Floor	5,4 [5,1 - 5,6]	*6*	4,5 [4,3 - 4,7]	*6*
3b	SPF chicken	Drinking water	n/a	*0*	n/a	*0*
3b	SPF chicken	Floor	n/a	*0*	n/a	*0*

4.3 Experiment 2 – part 2: transmission experiment between ducks and chickens involving close contact

Virus shedding and seroconversion in inoculated pekin ducks have been discussed before. In all three trials, all susceptible SPF chickens were positive by qRRT-PCR. In fact, all OP and most CL swabs taken from these chickens were positive by qRRT-PCR for several consecutive days starting at 1dpe for trial 2a, 3dpe for trial 2b and 1dpe for trial 2c. These

time points each coincided with the onset of CL virus shedding that was observed in pekin ducks (vide supra). The profile of vRNA detection in OP and CL SPF chicken swabs was different from what was observed in experiment 1 (figure 3). Not only were more CL swabs found positive within 10 days after exposure (25/30 for trial 2a; 16/30 for trial 2b and 14/24 for trial 2c), but also the difference in vRNA quantities for the two swab types was smaller (Table 1). Moreover, the amounts of vRNA found in both OP and CL swabs were many times larger than those found in experiment 1, as witnessed by the AUC values shown in table 1. Fewer SPF chickens were positive by serology than by qRRT-PCR (Table 2). Based on the duration, intensity and routes of virus shedding, the chickens that seroconverted were not readily distinguishable from those that did not. In the two trials where 6 inoculated pekin ducks were used, anti-AIV antibodies were detected in 5/6 SPF chickens (trials 2a and 2b). In the trial where only 3 inoculated pekin ducks were used, anti-AIV antibodies were detected in 3/6 SPF chickens (trial 2c). For the three trials combined, β_{dc} was estimated at 0,30 (95% C.I.: [0,18 – 0,52]).

Table 2: Overview of NP-ELISA serology results for all trials conducted in the present study. dpi=days post inoculation; dpe=days post exposure.

Experiment Number	Species	Exposure type	Number of Seeders	Anti-AIV antibodies at 14dpi/dpe	Anti-AIV antibodies at 21dpi/dpe
1	SPF chicken	Inoculated	n/a	9/12	9/12
2a	Pekin duck	Inoculated	n/a	6/6	5/6
2b	Pekin duck	Inoculated	n/a	6/6	4/6
2c	Pekin duck	Inoculated	n/a	3/3	3/3
2a	SPF chicken	Close contact	6 ducks	5/6	5/6
2b	SPF chicken	Close contact	6 ducks	5/6	5/6
2c	SPF chicken	Close contact	3 ducks	3/6	3/6
3a	SPF chicken	Drinking water	3 ducks	3/6	3/6
3a	SPF chicken	Floor	3 ducks	0/6	0/6
3b	SPF chicken	Drinking water	1 duck	0/6	0/6
3b	SPF chicken	Floor	1 duck	0/6	0/6

Large quantities of vRNA were demonstrated in drinking water samples and floor swabs from all trials. In trial 2a and 2c, vRNA was first detected in these samples at 1dpe whereas in trial 2b (where duck cloacal virus shedding was delayed), vRNA was first detected in drinking water at 3dpe and on the floor of the isolator at 2dpe. To assess the relationship between vRNA in swabs from inoculated ducks, drinking water samples and floor swabs, an analysis of variance (ANOVA) was carried out. These results indicated significant associations between vRNA in duck CL swabs - drinking water ($p<0,01$) and duck CL swabs - floor swabs ($p<0,01$). However, no significant relationship was found between vRNA in duck OP swabs - drinking water ($p=0,395$) and duck OP swabs - floor swabs ($p=0,197$). For SPF chickens, the same analysis indicated a significant relationship between vRNA in CL swabs – drinking water ($p<0,01$) and CL swabs – floor swabs ($p=0,023$) but also between vRNA in OP swabs - drinking water ($p<0,01$) and OP swabs – floor swabs ($p<0,01$).

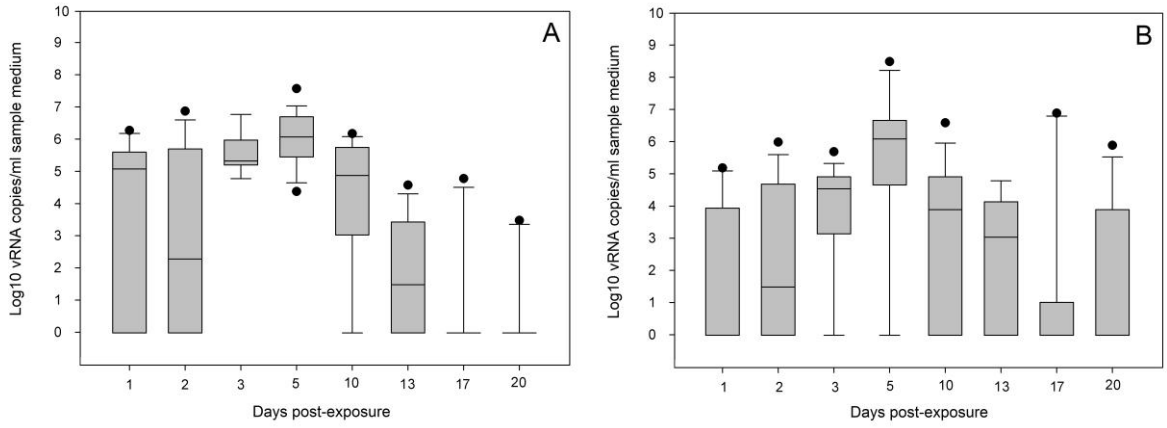

Figure 3: Presence of viral RNA in oropharyngeal and cloacal swabs from SPF chickens that were exposed in close contact to pekin ducks inoculated with H5N3 A/Anas platyrhynchos/09-884/2008. Log_{10} vRNA copies/ml sample medium are presented for oropharyngeal SPF chickens swabs (A) and cloacal SPF chicken swabs (B). Negative samples are represented as 10^0.

4.4 Experiment 3: transmission experiment between ducks and chickens involving contaminative transmission

4.4.1 Trial 3a

In trial 3a, all three inoculated pekin ducks were successfully infected. At the replacement day, the drinking water was visually contaminated with bird faeces and contained $10^{8,6}$ vRNA copies/ml drinking water. Likewise, the floor swab of the isolator contained $10^{8,4}$ vRNA copies/ml storage medium (Table 3).

For each susceptible SPF chicken from the DWE group, one or several OP swabs were positive by qRRT-PCR. In total, 23/48 OP swabs, analyzed between 0 and 14dpe were found positive by qRRT-PCR. Contrarily, a total of only 2/48 CL swabs, coming from two different chickens, were found positive during the same time period. Results from AUC calculation show that the quantities of vRNA found in the OP swabs throughout the entire course of the trial were larger than those found in CL swabs, which concerned only traces of vRNA (Table 1). The AUC values for both swab types are comparable to what was observed in experiment 1. However, we observed that positive samples often alternated with negative samples throughout the trial and that the evolution of the number of vRNA copies per time interval was unpredictable, which is in contradiction to the observations made in experiment 1. Sera

from three chickens were found positive at 14 and 21 dpe (Table 2). Consequently, 50% of susceptible SPF chickens were positive by qRRT-PCR without showing seroconversion, which contradicts what was observed in experiment 1.

Table 3: Transmission experiments involving contaminative transmission, trial 3a: assessment of the environmental contamination. Overview of the number of vRNA copies and 50% tissue culture infectious dose ($TCID_{50}$) in drinking water samples (drinking water exposure group) and floor swabs (surface exposure group). Results are expressed as log_{10} vRNA copies/ml sample medium or log_{10} $TCID_{50}$/ml sample medium. An overview of the total amount of vRNA copies and $TCID_{50}$ in samples obtained between the day of exposure and 7 days post exposure (dpe) is given by calculation of the areas under the curve (AUC). + = positive sample (titer not determined); - = negative sample.

dpe	Drinking Water Exposure Group Drinking water		Surface Exposure Group Floor swabs	
	viral RNA (log_{10})	CEF Viability (log_{10} $TCID_{50}$/ml)	vRNA copies (log_{10}/ml)	CEF Viability (log_{10} $TCID_{50}$/ml)
0	8,6	6,2	8,4	3,6
1	8,4	5,8	6,1	2,6
2	7,8	3,5	5,8	-
3	7,8	3,4	5,5	-
4	7,5	2,6	5,9	2,5
7	8,4	4,3	5,5	2,5
12	6,0	+	5,7	-
14	6,3	+	5,3	-
AUC (0-7dpe)	9,0	6,4	8,4	3,7

In the SE group, all chickens were positive by qRRT-PCR as well. In this exposure group, 28/48 OP swabs, analyzed between 0 and 14dpe, were positive. A total of 16/48 CL swabs were found positive during that same period, which is much more than what was observed in the DWE group. Quantities of vRNA were larger in OP swabs than in CL swabs. Compared to the DWE group, OP swabs contained similar quantities of vRNA, but CL swabs contained significantly larger quantities of vRNA (Table 1). Positive swabs were often alternated with

negative swabs as well. However, none of the SPF chicken serum samples had detectable antibodies directed against viral NP (Table 2).

4.4.2 Trial 3b

In trial 3b, only one of the three inoculated ducks was successfully infected; at the time of removing the ducks from the trial and establishing the drinking water exposure and surface exposure groups, no vRNA was detected in the drinking water, which had a clean appearance, and the floor swab contained only $10^{3,5}$ vRNA copies/ml storage medium. After exposure, all swabs taken from the susceptible SPF chickens were negative by qRRT-PCR for both exposure groups and none of the susceptible SPF chickens seroconverted.

4.4.3 Assessment of environmental infection pressure

In the DWE group from trial 3, further follow-up of the drinking water showed that vRNA was still present for at least 14 more days and that the quantities of vRNA declined slowly. Testing these samples for virus viability revealed that all drinking water samples contained viable virus and that the titers declined along with the amounts of vRNA (Table 3). In this trial's SE group, follow-up of vRNA in floor swabs showed that quantities of vRNA had decreased seriously by 1dpe and then remained more or less constant until 14dpe at a level which was +/- 3 $\log_{10}$ below the initial concentration of vRNA. Testing virus viability showed that only some floor swabs contained viable virus and that the virus titers of these samples were generally low (Table 3). A comparison between vRNA quantities and $TCID_{50}$-titers in drinking water and floor samples collected during the first week post exposure was made possible by calculating the AUC values for these samples (Table 3). These results show that 1 $TCID_{50}$ in drinking water samples corresponded to roughly 400 vRNA copies, whereas 1 $TCID_{50}$ in floor swabs corresponded to roughly 50.000 vRNA copies.

5 Discussion

In this study, we studied the mechanisms of introduction of a duck originated H5N3 LPAIV to poultry. The strain H5N3 A/Anas platyrhynchos/09-884/2008 was selected for this study because characterization had previously revealed that it is part of a highly dynamic population of wild bird LPAIVs that are circulating among wild birds in Belgium. Also, genetic characterization of the virus, carried out by Van Borm et al (2011) has indicated that no

additional glycosylation sites are present in the HA and that the length of the NA stalk is not reduced, indicating that this strain is most likely not adapted to poultry (Van Borm et al., 2011). Results from our assessment of the infectivity of this virus in SPF chickens and pekin ducks provide additional proof that this strain is phenotypically adapted to ducks and that a high infection dose may be necessary to cause active infection in SPF chickens (Swayne and Slemons, 2008). Moreover, we observed that the profile of infectivity was different in pekin ducks than in SPF chickens. Indeed, we observed seroconversion together with virus shedding in 5/12 inoculated SPF chickens and seroconversion without virus shedding in 4/12 oculonasally inoculated SPF chickens. The remaining three inoculated SPF chickens exhibited neither immune response nor virus shedding. The most important virus shedding route for inoculated SPF chickens was through the oropharynx whilst cloacal virus shedding was negligible. Virus shedding by the cloacal route was much stronger in ducks than in SPF chickens and was always associated with seroconversion.

In a second step, in order to assess the introduction potential of H5N3 A/Anas platyrhynchos/09-884/2008 to poultry by its natural host, we conducted a series of transmission experiments with inoculated pekin ducks and susceptible SPF chickens. From these results, it was shown that co-housing of susceptible SPF chickens and inoculated pekin ducks led to seroconversion in a similar percentage of SPF chickens compared to oculo-nasal inoculation. However, in these experiments, all SPF chickens were positive by qRRT-PCR for both OP and CL swabs, even in those that did not seroconvert. This discordancy may possibly be explained by the occurrence of localized virus replication which is detectable in the oropharynges and cloacae and which the chickens were able to clear with a T-cell mediated response, without developing a systemically measurable humoral immune response. However, to our knowledge, no scientific account exists on the occurrence of localized influenza virus replication without the development of antibodies in veterinary medicine. Alternatively, some qRRT-PCR results for chicken swabs may have come from the ingestion of virus from the environment rather than from a replicative infection. Since we observed a strong contamination of the isolators with infective duck faeces, it is possible that scratching and pecking the floor or just lying down caused large doses of virus coming from the environment to end up in the chickens' beaks or cloacae. Unfortunately, the impact of this environmental contamination on our results is impossible to estimate. Combining the two possibilities, we

assumed that the positive swabs obtained from SPF chickens reflected both virus shedding and virus exposure. The existence of a significant relationship between OP and CL chicken swabs and environmental samples might therefore indicate that a higher environmental contamination leads to a higher exposure and possibly higher virus shedding. Furthermore, the significant relationship between duck CL swabs and environmental samples indicates the importance of cloacal virus shedding by infectious ducks for the build-up of environmental infection pressure.

Since the occurrence of an LPAIV infection without the development of antibodies is not certain and since the use of serology reduces the possibility of incorrectly interpreting truly non-infected SPF chickens as infected, we decided to consider the immune response of chickens as a decisive factor for our model. The estimates we obtained for β_{dc} indicate that close contact between a single duck infected with H5N3 A/Anas platyrhynchos/09-884/2008 and fully susceptible chickens may result in 0,3 new infections/day. Since a previous study suggests that transmission of this virus between chickens does not occur (Claes et al., 2013), we can conclude that this LPAIV can be transmitted from wild birds to poultry through close contact, but that subsequent circulation is unlikely. Other H5/H7 LPAIVs of wild bird origin however, have been shown to have a strong infectious potential for chickens (Marché et al., 2012). Therefore, it can be expected that such wild bird originated LPAIVs may have the ability to circulate within chicken populations after introduction. Conducting similar interspecies transmission experiments as the one presented in the present study, but with a wild bird originated H5/H7 LPAIV which has better infectious potential for poultry or which is closely related to a poultry adapted strain may provide clarity on this matter.

It should be kept in mind that the experimental design of our transmission experiment involving close contact created an artificial environment which may have substantially forced transmission and that the results must be somehow mitigated. Indeed, in existing situations where contact between poultry and wild birds may occur, wild ducks and chickens do not tend to come in such close contact with each other for such a long period of time. An observational study carried out by Welby et al. has demonstrated ducks visiting smallholder poultry flocks may repose inside the holding, eat and make use of the drinking water for only a few minutes up to a couple of hours (article in preparation). This leads to faecal contamination of the soil,

the surroundings of the feeding systems and the drinking water whereas close contact might be rather limited because of more space. Since LPAIV-infected ducks tend to shed virus primarily through the intestinal tract (Vandalen et al., 2010) and since environmental contamination has been postulated to play an important role in several transmission experiments (Claes et al., 2013; Vandalen et al., 2010; Achenbach and Bowen, 2011; Rohani et al., 2009; Joh et al., 2009), we designed and conducted our animal experiment to force the system and examine the dynamics of LPAIV introduction through the most similar wild-bird induced environmental contamination.

Two types of environmental transmission were considered; transmission through contaminated drinking water and transmission through contaminated surfaces. Results from this experiment proved that drinking water that is contaminated by faeces from three LPAIV-shedding ducks can lead to detection of vRNA in all, and seroconversion in 50% of chickens exposed to it. These results prove that drinking water contaminated by wild birds is an efficient route for introduction of LPAIVs into poultry holdings. Moreover, since ducks may shed up to 10^9 EID_{50}/g faeces (World health organization, 2006), the initial virus concentration of $10^{6,2}$ $TCID_{50}$/ml reported for drinking water in the present study can reasonably be reached with a small degree of faecal contamination.

When chickens were exposed to contaminated surfaces, vRNA was detected in the oropharynges and cloacae but seroconversion was not observed. Therefore, this route of transmission is probably less effective than drinking water, likely because water is a more virus-friendly environment. Indeed, it is well-known that AIVs persist well in humid environments (Stallknecht et al., 1990; Brown et al., 2007; Domanska-Blicharz et al., 2010; Nazir et al., 2010; Leung et al., 2007), whereas survival in faeces or on surfaces may be compromised, especially at low humidity (De Benedictis et al., 2007; Chumpolbanchorn et al., 2006; Lu et al., 2003; Shortridge et al., 1998). Therefore, we additionally carried out an endpoint-titration on CEF to estimate $TCID_{50}$/ml sample medium for environmental samples. This test considers live virus instead of vRNA, which may also come from defective virus, and thus enables assessing virus viability in drinking water or on surfaces, for this study. Since these results indicate that the proportion of vRNA copies / $TCID_{50}$ per ml is much larger for the SE group than for the DWE group, it can be concluded that a large proportion of

vRNA in the former is in fact derived from defective viruses which did not participate in the build-up of infection pressure (Guan et al., 2009).

Alternatively, water may well be a better vehicle for infecting chickens with wild bird-originated LPAIVs than surface contamination is. Indeed, besides being ingested, water droplets can also be inhaled when drinking, which leads to virus being directly delivered to the respiratory epithelium, similar to what happens in an intranasal or intratracheal inoculation. On the other hand, the primary way through which virus on faecal contaminated feed or surfaces can be taken in by chickens is through ingestion. The virus is thus only delivered to the oral, pharyngeal or oesophageal epithelium, which are less reactive than the respiratory epithelium (Hirabayashi et al., 1990). Finally, the survival of the virus in the drinking water containing different (amounts of) disinfectants should be investigated further, as this could represent an additional preventive measure.

We have demonstrated that LPAIV-shedding ducks may build-up an infection pressure that is strong enough to cause intake of large amounts of virus in exposed chickens. Whilst a highly sensitive method such as qRRT-PCR was effective in detecting exposure and environmental contamination, this method did not appear useful for assessing if virus intake led to a true infection in chickens or not. Therefore, in order to avoid overestimation of virus transmission in experiments involving a strong degree of environmental contamination, we recommend the use of other methods for determining infection in animals. The use of serology could be useful; however this method may be less sensitive as localized virus replication may not always induce systemic immune response. We additionally provided evidence that drinking water contaminated with faeces from LPAIV-shedding ducks alone, may cause successful infection of poultry with a wild-bird LPAIV. However, since the virus used in the present study is not easily transmitted between chickens (Claes et al., 2013), the chances of such an event leading to circulation of the virus among poultry can be limited. It is therefore possible that some of these introduction events remain unnoticed. Other wild-bird originated LPAIVs however, may have good infective characteristics for poultry (Swayne et al., 2008) and may thus be more prone to be transmitted in chickens. If such a virus is introduced efficiently, this may lead to the establishment of the virus in poultry and to the possible emergence of a HPAIV.

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Chapter 5:

Extended transmission of two H5/H7 low pathogenic avian influenza viruses in chickens

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1 Summary

Transmission experiments are useful for investigating the mechanisms of LPNAI transmission. In this study, the hypothesis that inoculation-infected chickens are more infectious than contact-infected chickens was tested. To this end, extended transmission experiments with one H5N2 and one H7N1 LPAIV which had previously been characterized in a series of standard transmission experiments were conducted in SPF chickens. For the H5N2 LPAIV, the infectivity of contact-infected chickens was similar to the infectivity of inoculated chickens. Despite results from a previous study suggested the H7N1 LPAIV strain to be similarly infectious to SPF chickens as the H5N2 LPAIV, the acquisition of contact-infected chickens proved more difficult for the H7N1 LPAIV. It was assumed that this might have been a consequence of the length and timing of the exposure period. In conclusion, for LPNAIVs that first seemed equally infectious, the short-term transmissibility may vary considerably.

2 Introduction

Avian influenza viruses (AIVs) may infect many different bird species. In wild aquatic birds, the natural reservoir of AIVs, AIV infections are mainly subclinical and the viruses are circulating freely without causing harm to the hosts (Webster et al., 1992; Olsen et al., 2006). In poultry species however, AIVs may cause a variety of symptoms. Therefore, two pathotypes of AIV are considered, based on the symptoms the virus causes in chickens. In poultry, most AIVs produce subclinical infections or signs of a localized infection such as respiratory disease with general depression and a drop in egg production, which may or may not be aggravated by opportunistic infections (Nili and Asasi, 2002; Halvorson et al., 2003; Swayne and Pantin-Jackwood, 2006). These AIVs are typically classified as low pathogenic avian influenza viruses (LPAIVs). As a result of mutations, LPAIVs belonging to the H5 and H7 subtypes (LPNAIVs) may evolve into highly pathogenic avian influenza viruses (HPAIVs) which cause a systemic disease and very high mortality rates, often up to 100%. These outbreaks may lead to large economic disasters (Swayne, 1997; Stegeman et al., 2004). It is currently widely accepted that HPAIVs emerge by mutation from LPNAIVs when they are circulating in poultry (Koch and Elbers, 2006).

In order to install surveillance & control programs directed towards a rapid detection of LPNAIVs that are circulating in poultry (European Commission, 2005), reliable scientific

data on the transmission of these viruses is required. Transmission experiments allow for the studying of the effect of a single factor on LPNAI transmission and have been used for studying the modes of LPNAI transmission (Van der Goot et al., 2003; Saenz et al., 2012; Vandalen et al., 2010; Achenbach and Bowen, 2011; Claes et al., 2013a; Claes et al., 2013b; Okamatsu et al., 2007; Yee et al., 2009). With this information, decision makers can direct surveillance and control strategies to focus more on those sectors of the poultry industry that are at risk, hereby increasing the odds of detecting LPNAIVs more quickly and improving the allocation of resources (Welby et al., 2010). Additionally, estimating LPNAIV transmission parameters provides insight in the velocity of LPNAI spread in poultry flocks, which can then be used for setting the specific requirements of surveillance programs and their feasibilities (Gonzales et al., 2011; 2012).

Transmission experiments are mainly conducted by exposing susceptible animals to experimentally inoculated animals for a certain amount of time or until the final state of the experiment is reached. Such experiments are typically referred to as standard transmission experiments. Since higher LPNAIV inoculation doses may lead to chickens excreting higher amounts of virus during a longer time period (Capua et al., 2010; Zarkov and Bochev, 2008; Lu and Castro, 2004), it can be reasonably assumed that the use of inoculated instead of naturally infected animals may lead to an overestimation of transmission. Alternatively, extended transmission experiments can be applied (Velthuis et al., 2007). In these experiments, inoculated animals are used to create a first generation of contact-infected animals, which are then brought into contact with susceptibles. It can be assumed that these contact-infected animals might more closely resemble naturally infected animals (Bouma et al., 1997). To our knowledge, such extended transmission experiments have not yet been conducted to model the transmission of LPNAIVs in chickens. In the present study, we have conducted extended transmission experiments with two chicken originated LPNAIVs; one H5 and one H7 LPAIV. By estimating the basic reproduction ratio (R_0), which is essentially the average number of susceptible individuals that are infected by one typical infectious individual during its entire infectious period in a fully susceptible population (Diekmann et al., 1990), it was assessed if virus transmission from contact-infected chickens to susceptible chickens differs importantly from virus transmission from inoculated to susceptible chickens.

3 Materials & Methods

3.1 Viruses

Two LPNAIVs were used in the present study. LPAIV *H5N2 A/Ch/Belgium/150VB/99* was isolated in 1999 by the Veterinary and Agrochemical Research institute. The virus was isolated from chickens in a mixed backyard poultry holding with about 100 chickens and 20 ducks. The first clinical signs such as depression, diarrhea and respiratory distress appeared 10 days after a few chickens were bought from a dealer at a local market (Meulemans et al., 2000). A second egg passage of this virus was used for inoculation of the animals.

LPAIV *H7N1 A/Ch/Italy/1067/v99* was isolated by the Istituto Zooprofilattico Sperimentale. This virus was isolated from chickens during the 1999 LPAI epidemic in northeastern Italy (Capua et al., 2000). A fourth egg passage of this virus was used for inoculation of the animals.

3.2 Animals

Experiments were conducted with specific pathogen-free (SPF) chickens. Eggs were delivered by Lohmann-Valo (Germany) and hatched at own facilities. Chickens were housed in biosafety level-3 isolators (type: HM1500, Montair Process Technology B.V., The Netherlands) from the day of hatching until the end of the experiment. The isolators have a floor surface of 1,2 m^2 and the internal volume measures 0,9 m^3. The floor of the isolators was covered wood shavings (Agrospan Houtkrullen, Vividerm, Belgium). A negative air pressure of 45±5m^3/h was maintained. Each animal experiment was conducted under the authorization and supervision of the Biosafety and Bioethics Committee at VAR, following national and European regulations.

3.3 Experimental design

Two extended transmission experiments were conducted. Each experiment consisted of two trials, which were replicates. In experiment 1 (trials 1&2), LPAIV *H5N2 A/Ch/Belgium/150VB/99* was used. In experiment 2 (trials 3&4), LPAIV *H7N1 A/Ch/Italy/1067/v99* was used.

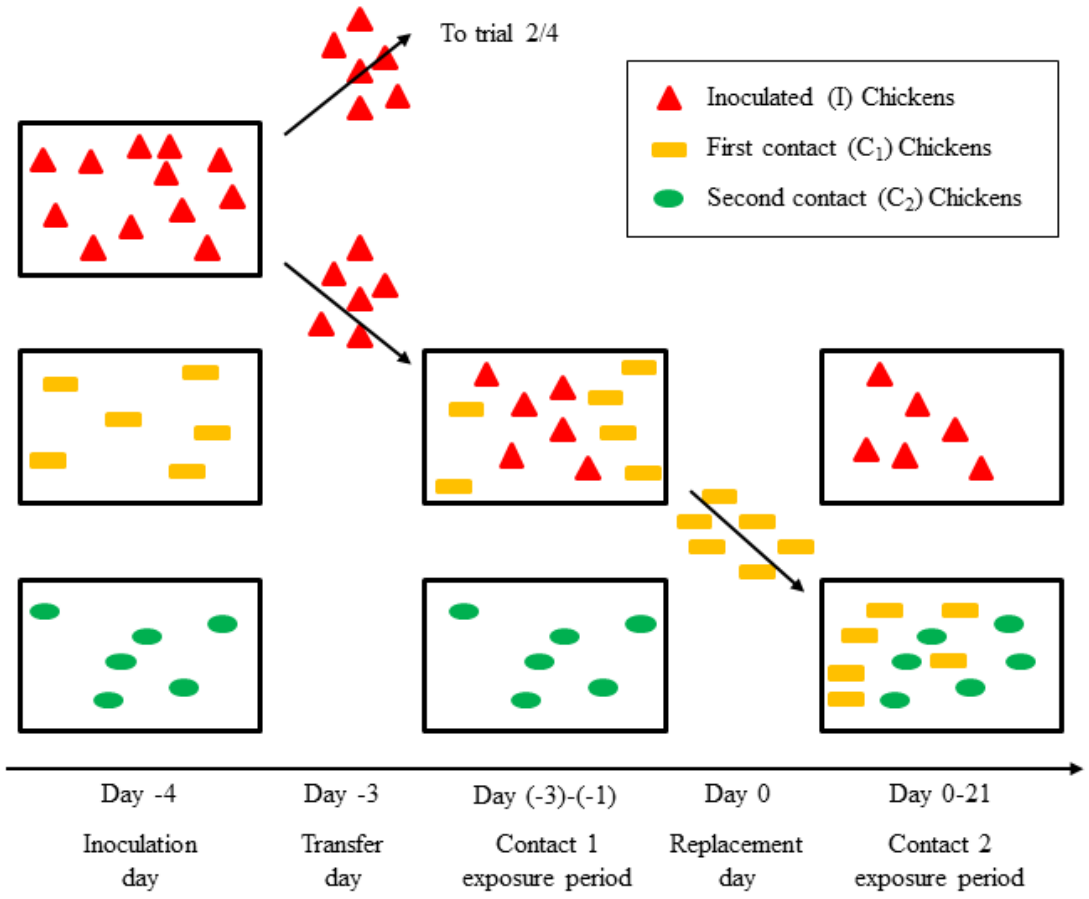

Figure 1: Experimental design of extended transmission experiments conducted in the present study. For each of the two experiments, twelve 5 week old SPF chickens were inoculated at day -4. The next day, these I-chickens (red triangles) were randomly separated in two groups of six chickens, allocated to one of the two trials and moved to a different isolator which contained six susceptible animals (C_1-chickens; yellow rectangles). After three days of close contact between I-chickens and C_1-chickens (contact 1 exposure period), the C_1-chickens were moved to another isolator, again containing six susceptible animals (C_2-chickens; green ovals). C_1- and C_2-chickens were then housed together until the experiment was ended, at day 21 (contact 2 exposure period).

At the beginning of each experiment, twelve 5 week old SPF chickens were oculonasally inoculated with a virus dose of 10^6 EID_{50}/100µl. This day is referred to as the inoculation day, or day-4. At day -3, the transfer day, these twelve inoculated chickens (I-chickens) were randomly separated in two groups of six and allocated to one of the two trials compiling the experiment. This way, both trials of each experiment started with inoculated chickens that

were inoculated at the same time and with the same inoculum. Each group of six I-chickens was then moved to a different isolator which contained six susceptible animals, hereafter referred to as first contact chickens (C_1-chickens). The I- and C_1-chickens were housed together for three days, until the replacement day (day 0). On the replacement day, the C_1-chickens were moved to another isolator which again contained six susceptible animals, hereafter referred to as second contact chickens (C_2-chickens). C_1- and C_2-chickens were then housed together until the experiment was ended, at day 21 (Figure 1).

3.4 Sampling

Blood samples were collected from I-chickens, prior to the onset of the experiment and at 14 and 21 days post inoculation (dpi). For the assessment of virus transmission, blood samples were collected from C_1- and C_2-chickens prior to the onset of the experiments and at 7, 10, 14 and 21 days post exposure (dpe). Blood samples were allowed to coagulate, after which sera were harvested and stored at -20°C, awaiting further analysis.

Assessment of virus shedding was done by taking oropharyngeal and cloacal swabs at critical time points only, to reduce stress for the animals as much as possible. I-chickens were sampled on the transfer day (day -3) and again sampled on the replacement day (day 0). The C_1-chickens were sampled on day 0 and again on day 4. The C_2-chickens were swabbed on days 1,3 and 7. Swabs were immediately immersed in brain-hearth-infusion broth enriched with a mixture of antibiotics (10^6 U/l penicillin G, 2 g/l streptomycin, 1 g/l gentamycin sulfate and 66 ml/l kanamycin sulfate 100x). Sample tubes were briefly vortexed to release swab material after which the cotton was discarded. Samples were then stored at -80°C, awaiting further analysis.

3.5 Sample analysis

Blood sera were tested for presence of antibodies directed towards the AIV nucleoprotein with IDScreen influenza A antibody competition ELISA kit (IDvet, France). The test was conducted according to the manufacturer's instructions. Serum samples with a sample-to-negative (S/N) ratio greater or equal to 0,5 were considered negative, and S/N ratios smaller than 0,5 were considered positive. Sera were also tested for antibodies directed towards the homologous antigen, using a hemagglutination inhibition (HI) assay. HI assays were performed according to OIE-recommendations, using homologous antigen (Office

International des Epizooties, 2013). Samples with a HI-titer equal to or above 16 were considered positive and the assay's detection limit was 4096.

Swabs were tested for presence of viral RNA (vRNA) using a one-step real-time reverse transcription–polymerase chain reaction (RRT-PCR). Viral RNA was semi-automatically extracted from 50 µl thawed sample material using a KingFisher magnetic particle processor and the MagMax™ AI/ ND-96 Viral RNA kit (Ambion Inc., USA). A total of 25µl reaction volume (containing 2µl of purified RNA) was prepared using the Quantitect Probe RT-PCR kit (QiagenGmBH, Hilden, Germany) and amplification of the matrix gene was carried out in a Biosystems 7500 real time PCR cycler (Applied biosystems, Lennik, Belgium) (Van Borm et al., 2007). Samples with a cycle threshold value greater than or equal to 40 were considered negative. With each run, a series of 1:10 dilutions of synthetic matrix RNA was included to calculate the number of vRNA copies in each sample. A series of 1:10 dilutions of the stock solution of each virus was analyzed to create a calibration curve from which EID_{50} equivalents per ml (EID_{50}eq/ml) sample medium was calculated. Results were finally expressed as EID_{50}eq/ml sample medium.

3.6 Statistical analysis

Animals were considered infected if at least one serum sample was found positive by NP-ELISA. Virus transmission between C_1- and C_2-chickens was assessed according to the Susceptible-Infectious-Recovered (SIR)-model (Velthuis et al., 2007). For an estimation of the basic reproduction ratio (R_0), the maximum likelihood estimator was used, according to following formula:

$R_0 = max \prod_{i=1}^{n} Prob(x_i \,.\, R_0 | N.S_0.I_0)$,

where R_0 is the basic reproduction ratio, x_i is the number of contact-infected animals, N is the total number of animals, S_0 is the number of susceptible animals at the beginning of the experiment and I_0 is the number of infectious animals at the beginning of the experiment. I_0 was determined as the number of C_1-chickens found positive by RRT-PCR at day 0. Ninety-five percent confidence intervals (95% C.I.) were constructed symmetrically around the estimate of R_0 (de Jong and Kimman, 1994; Bouma et al., 2000). No basic reproduction ratios were estimated for virus transmission between I- and C_1-chickens, since the exposure was interrupted before the end-state was reached.

4 Results

4.1 Experiment 1: Extended transmission of H5N2 A/Ch/Belgium/150VB/99

4.1.1 Trial 1

All six I-chickens were successfully infected. Virus shedding was observed in every I-chicken at least once throughout the contact 1 exposure period. OP virus shedding was present in 5/6 I-chickens at day -3 and again 5/6 I-chickens at day 0. Cloacal virus shedding was witnessed in 4/6 I-chickens, all at day 0 only (Table 1). Immune responses were present in all I-chickens as well (Table 2). HI-assay indicates HI-titers at day 21 ranged between 128 and 4096 (Figure 2).

At the replacement day, 5/6 C_1-chickens proved to be shedding virus, via the OP route only. At day 4, OP virus shedding was observed in 5/6 C_1-chickens, one of which showing CL virus shedding as well (Table 1). At the end of the trial, all C_1-chickens were found to have seroconverted. One of them however, had a transient immune response which was only detected by NP-ELISA, at day 14 (Table 2).

OP virus shedding was observed in one C_2-chicken at day 3 and four C_2-chickens at day 7. No cloacal virus shedding was observed. The same four virus-shedding C_2-chickens and an additional fifth C_2-chicken, which was not found to be shedding virus at day 1, 3 or 7, were found positive by NP-ELISA (Table 3).

4.1.2 Trial 2

OP virus shedding was detected in 3/6 I-chickens at day -3 and 5/6 I-chickens at day 0. Cloacal virus shedding was detected in one animal, at day 0 only (Table 1). All I-chickens developed an immune response (Table 2). HI-titers at day 21 were generally lower than in trial 1, ranging between 128 and 512 (Figure 2).

All C_1-chickens were found to be shedding virus at day 0 and day 4. Cloacal virus shedding was not observed (Table 1). By the end of the experiment, all C_1-chickens had developed an immune response (Table 2).

Oropharyngeal virus shedding was observed in five C_2-chickens, one already at day 1, the other animals at day 3 and day 7 (Table 1). Cloacal virus shedding was not observed. The same animals that demonstrated virus shedding were also found to have seroconverted (Table 2).

Table 1: qRRT-PCR results for experiments carried out in this study

Virus	Trial	Chicken[a]	Sample type[b]	Number of pos. samples/total number of samples tested (median virus shedding [IQR])[c] Day number						
				-4	-3	0	1	3	4	7
H5N2	Trial 1	I	OP	Inoculation	5/6 (3,7 [3,2-3,7])	5/6 (4,2 [4 - 4,8])				
			CL		0/6	4/6 (4,4 [4,3 - 4,9])				
		C_1	OP		Exposure	5/6 (3,6 [3,6 - 3,8])	n.t.	n.t.	5/6 (2,7 [2,3-3,9])	n.t.
			CL			0/6			1/6 (5,9)	
		C_2	OP			Exposure	0/6	1/6 (3,8)	n.t.	4/6 (2,9 [1-3,8])
			CL				0/6	0/6		0/6
	Trial 2	I	OP	Inoculation	3/6 (3,4 [3,2-3,8])	5/6 (4 [3,7 - 4,7])				
			CL		0/6	1/6 (3,7)				
		C_1	OP		Exposure	6/6 (4,4 [4,2 - 4,6])	n.t.	n.t.	6/6 (3,8 [3,1-3,9])	n.t.
			CL			0/6			0/6	
		C_2	OP			Exposure	1/6 (2,7)	5/6 (3,8 [2,8-3,9])	n.t.	4/6 (3,4 [1,9-3,9])
			CL				0/6	0/6		0/6
H7N1	Trial 3	I	OP	Inoculation	5/6 (4,7 [2,8-4,9])	4/6 (4,3 [4,2 - 4,8])				
			CL		0/6	1/6 (5)				
		C_1	OP		Exposure	0/6	n.t.	n.t.	n.t.	n.t.
			CL			0/6				
		C_2	OP			Exposure	0/6	n.t.	n.t.	n.t.
			CL				0/6			
	Trial 4	I	OP	Inoculation	4/6 (4,1 [2,7-4,7])	4/6 (4,4 [4,2 - 5,3])				
			CL		0/6	0/6				
		C_1	OP		Exposure	0/6	n.t.	n.t.	n.t.	n.t.
			CL			0/6				
		C_2	OP			Exposure	0/6	n.t.	n.t.	n.t.
			CL				0/6			

[a] I=Inoculated chicken, C_1=First contact chicken, C_2=Second contact chicken; [b] OP=Oropharyngeal swab, CL=Cloacal swab; [c] Median EID_{50}eq/ml sample medium; IQR: interquartile range; n.t.=not tested

4.1.3 Differences in immune response intensities

Figure 2 represents the HI-titers observed in sera from animals that were found positive by HI assay with the homologous H5N2 virus. Results of both trials combined, an independent samples t-test showed no significant difference between the intensity of the log_2HI-titers in I-chickens and C_1-chickens at 14 days (p=0,14) and a borderline significant difference at 21 days (p=0,05) post inoculation/exposure. No significant difference between the intensity of the log_2HI-titers in C_1-chickens and C_2-chickens was observed at 14 days (p=0,10) and at 21 days (p=0,23) post exposure. The intensity of the log_2HI-titers in I-chickens and C_2-chickens at 14 days and at 21 days was significantly different (p=0,01 and p=0,03).

4.1.4 Quantification of transmission

A reproduction ratio was estimated for virus transmission from C_1-chickens to C_2-chickens. Results from both trials were combined and the R_0 was estimated at 2,11 [0,85-6,15].

Table 2: Overview of NP-ELISA and HI-assay results for all trials conducted in this study. The number of individuals found positive by NP-ELISA (before the hyphen) and by HI-assay (after the hyphen) are presented for blood samples collected at 7, 10, 14 or 21 days post inoculation/exposure.

Virus	Trial	Chicken[a]	Days post infection/exposure (Animals pos. by NP-ELISA - Animals pos. by HI assay)				Total
			7	10	14	21	
H5N2	Trial 1	I	nt	nt	6-6	6-6	6/6
		C_1	4-1	5-5	6-5	5-5	6/6
		C_2	2-0	2-2	5-2	4-4	5/6
	Trial 2	I	nt	nt	6-6	6-6	6/6
		C_1	2-0	6-5	6-6	6-6	6/6
		C_2	2-1	5-5	5-5	5-5	5/6
H7N1	Trial 3	I	nt	nt	6-6	6-6	6/6
		C_1	0-0	0-0	0-0	1-0	1/6
		C_2	0-0	0-0	0-0	0-0	0/5
	Trial 4	I	nt	nt	5-5	5-5	5/6
		C_1	1-0	1-1	1-1	1-1	1/6
		C_2	0-0	0-0	1-0	0-0	1/6

[a]I= Inoculated chicken, C_1= First contact chicken, C_2= Second contact chicken; n.t.=not tested

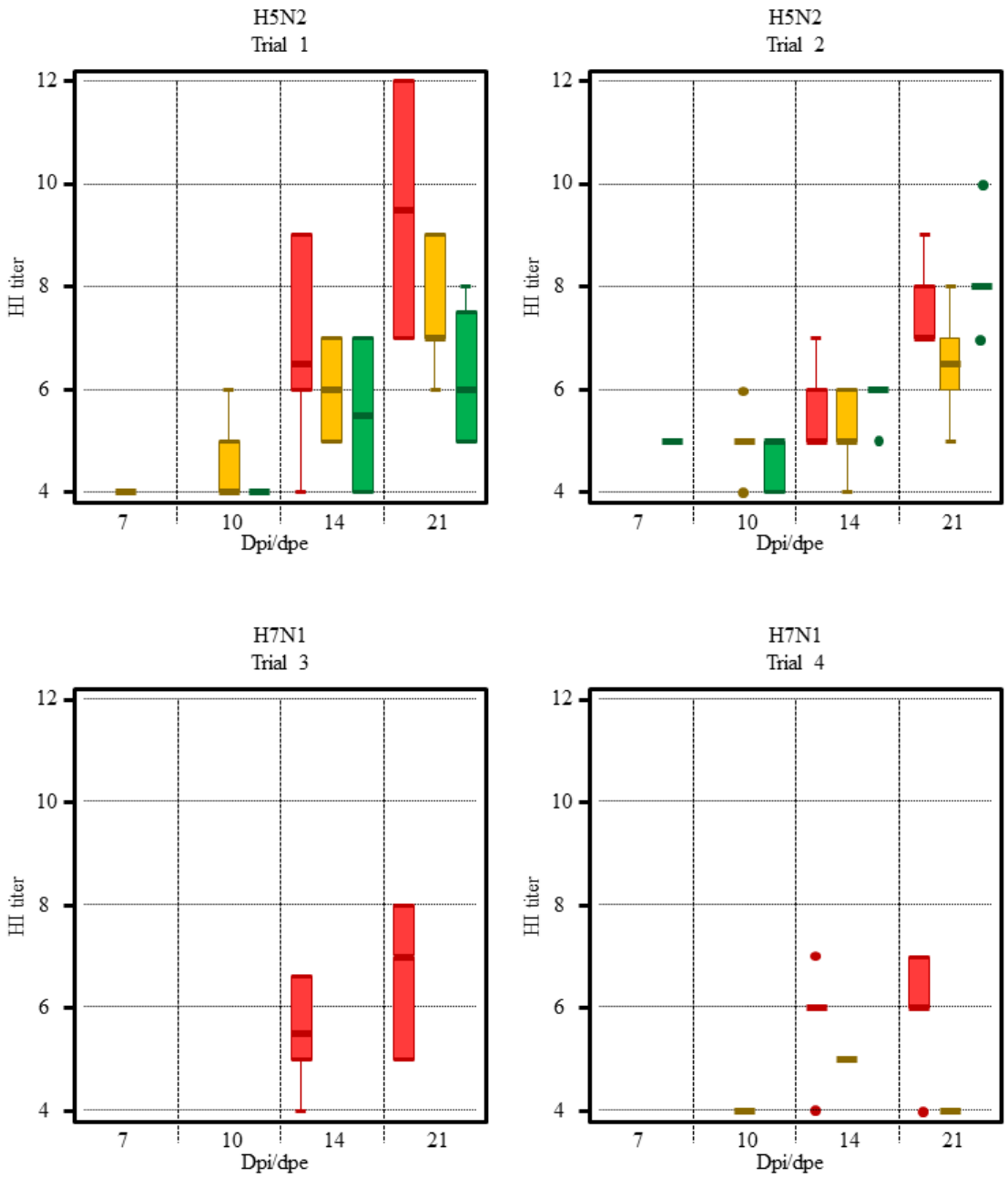

Figure 2: Schematic presentation of HI-titers found in serum samples obtained at 14 and 21 days post inoculation for I-chickens (red boxes) and 7, 10, 14 and 21 days post exposure for C_1- and C_2-chickens (yellow and green boxes). The thick line inside the boxes represents the median value, the beginning and end of the boxes represent the 25th and 75th percentiles and the whiskers represent the 5th and 95th percentiles. Outliers are represented as circles. HI titers are represented as $\log_2$ values; dpi=days post inoculation; dpe=days post exposure.

4.2 Experiment 2: Extended transmission of H7N1 A/Ch/Italy/1067/v99

4.2.1 Trial 3

OP virus shedding was detected in 5/6 I-chickens at day -3 and 4/6 I-chickens at day 0. Cloacal virus shedding was witnessed in one I-chicken, at day 0 only (Table 1). All I-chickens seroconverted (Table 2).

At day 0, none of the C_1-chickens showed virus shedding (Table 1). An immune response was detected in only one C_1-chicken, at 21dpe only, a result that was borderline positive (Table 2). None of the C_2-chicken swabs were found to contain vRNA at day 1 (Table 1). Therefore, and because virus shedding was not observed in C_1-chickens, no further swabs were analyzed. None of the C_2-chickens were found to have developed an immune response (Table 2). One C_2-chicken died from a non-influenza related cause at day 10.

4.2.2 Trial4

Viral RNA was detected in OP swabs from 4/6 I-chickens at day -3 and day 0. CL virus shedding was not observed (Table 1). Five I-chickens were found to have seroconverted.

None of the C_1-chickens were found positive by RRT-PCR at the day of transfer (day -3). Immune response was seen in only one C_1-chicken (Table 2), HI-titers in sera derived from this animal were low (Figure 2).

In C_2-chickens, virus shedding was not detected at day 1 (Table 1). One C_2-chicken was found borderline positive by NP-ELISA alone, at 14dpe, whilst immune response was absent in all other C_2-chickens (Table 2).

4.2.3 Quantification of transmission

A reproduction ratio was estimated for virus transmission from C_1-chickens to C_2-chickens. The joint R_0 was estimated at 0,73 (95% C.I.: [0,03-12,55]).

4.3 Discussion

The transmission of LPAIVs in poultry is traditionally examined through standard transmission experiments. In the present study, we tested the hypothesis that inoculation-infected chickens are more infectious than contact-infected chickens. To this end, we conducted extended transmission experiments with two LPNAIVs which we had previously used in a series of standard transmission experiments, LPAIV H5N2 A/Ch/Belgium/150VB/99 and LPAIV H7N1 A/Ch/Italy/1067/v99 (Claes et al., 2013a). In

analogy with that study, we considered chickens infected if antibodies against the virus were detected by NP-ELISA, even if this immune response was transient.

In the extended transmission experiment conducted with H5N2 A/Ch/Belgium/150VB/99 LPAIV (experiment 1), virus transmission was seen from contact-infected SPF chickens to susceptible chickens. A comparison of the results obtained from this experiment with results obtained in a previous study by Claes et al. (2013a) suggests that SPF chickens that are inoculated with this virus are approximately equally infectious as contact-infected SPF chickens. Indeed, the joint reproduction ratio we estimated in the present study was found to be only slightly larger than the R_0 that was previously estimated for the same virus, in a standard transmission experiment (1,77 [0,55-4,14]: (Claes et al., 2013a)). Furthermore, the EID_{50} equivalents we detected in swabs from C_1-chickens are similar to those detected in swabs from inoculated chickens in standard transmission experiments (Claes et al., 2013a). However, a possible difference in virus shedding could have been missed since swab samples from only two days were analyzed in the present study. For these reasons, it can be assumed that the infectivity of SPF chickens inoculated by a single oculonasal administration of a $10^6 EID_{50}$/dose of this virus, as described in Claes et al (2013a), does not differ importantly from the infectivity of chickens that have become infected as the result of a three-day exposure to infectious chickens under the current circumstances. Whether this means that a natural infection with this virus in the field can be confidently reproduced with the considered inoculation method remains uncertain, however; the assessment of infectivity is based on a limited set of data and the R_0 estimate has a fairly large 95% C.I. We additionally investigated if differences in immune response intensities can be seen in I-, C_1- and C_2-chickens. In this regard, a difference can only be assumed from the graphical representation of HI-assay results obtained for trial 1 (figure 2), whilst an independent samples t-test does not point towards a general trait. Arguably, the intensity of the immune response might be related to the stronger intestinal virus replication observed in this trial and is not a good indicator for estimating the infectivity of animal, for this virus.

For extended transmission experiments with H7N1 A/Ch/Italy/1067/v99 LPAIV, the R_0 estimate was smaller than the one estimated before, in a standard transmission experiment (1,72 [0,68-4,14]: (Claes et al., 2013a)). However, since this R_0 estimate falls within the then estimated 95% C.I., the observed low degree of virus transmission could be due to a normal variation in transmission (Van der Goot et al., 2003). Moreover, very little virus transmission was observed during the contact 1 exposure period, which resulted in few C_1-chickens being infectious at the start of the contact 2 exposure period. As a consequence, the 95% C.I. for the

thus obtained R_0 estimate was very wide, which decreases the value of this estimate for drawing a conclusion.

The low degree of virus transmission during the contact 1 exposure period was unanticipated. Compared to virus shedding data obtained previously, in Claes et al. (2013a) (Claes et al., 2013a), virus shedding during the contact 1 exposure period seems not to have been unusually low. Therefore, we believe that the low number of infected C_1-chickens was due to the timing of the contact 1 exposure period, which may have been too short or may have been planned too soon after inoculation. Since the first three days following inoculation is generally characterized by an OP virus shedding for this virus (Claes et al., 2013a; Gonzales et al., 2012; Marché et al., 2010), the C_1-chickens were not exposed to infectious fecal matter. Despite oral LPAI transmission has been recognized in chickens in several studies, fecally shed LPAIV has been suggested to increase virus transmission (Claes et al., 2013a; Yee et al., 2009). It can thus be assumed that the infection dose required for transmission was not attained by oral virus shedding between 1 and 4 dpi alone.

Since chickens inoculated with H5N2 A/Ch/Bel/150VB/99 LPAIV were able to successfully transmit the virus within three days after becoming infected, it can be suggested that this virus is capable of rapidly spreading throughout a susceptible chicken population. Therefore, the fact that it was isolated on only one occasion (Meulemans et al., 2000) makes this virus particularly interesting. Considering the conditions in which the virus was obtained, spread to other backyard flocks connected through the same dealer could be assumed (Meulemans et al., 2000). However, since no other cases were detected, it is possible that the virus died out spontaneously because the available number of susceptible individuals in backyard holdings is generally small and because Belgian backyard poultry holdings rarely have off-farm movements (Van Steenwinkel et al., 2011). Additionally, transmission to commercial poultry may not have occurred because in Belgium, backyard poultry holdings can be considered to be epidemiologically isolated from commercial poultry holdings (Van Steenwinkel et al., 2011; Bavinck et al., 2009). It is hereby suggested that potentially dangerous LPNAIVs may be present in backyard poultry holdings and that introduction from backyard to industrial poultry holdings depends on the contact structures between the two sectors. Backyard poultry holdings are not included in the Belgian active AI surveillance program, which may indeed not be necessary. However, special focus on those holdings that are connected to both the rural and industrial poultry sector may prove very useful (Cecchinato et al., 2011).

In conclusion, for LPNAIVs with similar characteristics to the strains tested in the present study, the hypothesis that inoculation-infected chickens are more infectious than contact-

infected chickens could not be confirmed. Additionally, our results show that the timing of the exposure period may influence transmission, even for LPNAIVs that transmit well. This finding may be of limited importance for industrial poultry holdings, but could suggest that some poultry adapted LPNAIVs are less likely to be transmitted at bird gatherings where the exposure time can be short, like street markets or live bird markets.

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Chapter 6:

Evaluation of Four Enzyme-Linked Immunosorbent Assays for the serological survey of Avian Influenza in Wild Bird Species

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1 Summary

Wild birds that reside in aquatic environments are the major reservoir of Avian Influenza Viruses (AIV). Since this reservoir of AIV forms a constant threat for poultry, many countries have engaged AIV surveillance. More and more commercial Enzyme Linked Immunosorbent Assays (ELISA) are available for serological surveillance but these tests are often developed and validated for use in domestic poultry. However, for a correct interpretation of ELISA test results from wild bird sera, more information is needed.

In present study, four ELISA test kits (ID-Vet IDScreen®, IDEXX FlockChek™ AI MultiS-Screen Ab Test Kit, Synbiotics FluDETECT™BE and BioChek AIMSp) were compared for the serological analysis of 172 sera from mallard, mute swan and canada goose. Samples were selected based on ID-Vet IDScreen-results to obtain a balanced number of positive and negative samples. A total of 92 sera from experimentally infected Specific Pathogen Free (SPF) chickens and Pekin ducks were included in the tests as well for validation purposes.

Cohen's kappa statistics and Spearman correlation coefficients were calculated for each combination of 2 tests and for each bird species. Test agreement for mallard sera varied from poor to moderate while test results for Canada goose and swan sera agreed from fair to almost perfect. The best agreement was obtained with sera from experimentally infected SPF chickens and Pekin ducks. This study shows that some care must be taken before using NP-ELISA for the testing of sera from wild birds and that more reliable validation studies should be considered before their use in the serologic surveillance of wild birds.

2 Introduction

Avian Influenza Viruses (AIV) are found in many wild bird species all over the world. Wild birds that reside in aquatic environments such as Anseriformes and Charadriiformes are the major reservoir of the virus (Munster and Fouchier; 2009; Olsen, 2006). Because wild birds represent a constant source of introduction of AIV in poultry, many countries have engaged active AIV surveillance in wildlife (Alexander, 2007; Commission of the European Communities, 2006). Indeed, a better understanding of the circulation of influenza in wildlife is needed for the improvement of biosafety measures in poultry holdings and for the design of targeted surveillance programs.

Assessment of virus circulation in the avifauna can be performed with the help of virological and serological methods, which both have their benefits and limitations. Isolation of AIV

from swabs from wild birds provides information on the prevalence of AIV infections in the population at the time of sampling. However, positive results with this method can only be obtained when animals are sampled during the phase of active virus shedding. Since AIV-shedding can be very short (Webster et al., 1992), this implies that evidence of infection with AIV can easily be missed. Contrarily, anti-AIV antibodies can be demonstrated in bird sera several weeks or months after exposure to the virus, depending on the bird species (Hoye et al., 2011). Therefore, serology gives more information on past AI-exposure. Indeed, seroprevalence forms a better picture of the extent to which the virus has spread throughout the population during the past weeks or months, notwithstanding it does not provide information on when the infection took place or the number of times an individual has been infected (Charlton et al., 2009).

Hemagglutination inhibition (HI) and Agar-gel immunodiffusion (AGID) assay are currently used as reference tests for anti-AI antibody detection in sera from domestic and wild animals. However, these tests are not suitable for the analysis of large numbers of samples. Hemagglutination inhibition assay does not allow detection of antibodies against all Hemagglutinin (HA) subtypes in a single test, implying that each serum has to be tested against each subtype, which is very costly and time-consuming (Perez-Ramirez et al., 2010; Starick et al., 2006). The AGID test on the other hand does allow detection of antibodies against all avian influenza viruses in one test, but results obtained with this test are not reliable for sera from animals that do not consistently produce precipitating antibodies, such as ducks (Charlton et al., 2009; Higgins, 1989), and is prone to misinterpretation due to the subjective reading of the results (Spackman et al., 2008; Sulivan et al., 2009).

Recently, several commercial (Brown et al., 2009; Song et al., 2009) or in-house (de Boer et al., 1990; Jin et al., 2004; Shafer et al., 1998; Starick et al., 2006; Zhou et al., 1998), competitive or blocking ELISA's have been developed. These polyvalent assays are developed to detect antibodies directed towards the antigenically conserved nucleoprotein of Influenza A Viruses (Spackman et al., 2008) and are therefore theoretically suited for testing sera from multiple species. Moreover, they are often found to be more reliable (Sullivan et al., 2009) and equally or more sensitive (Beck and Swayne, 2003; Marché and van den Berg, 2010; Spackman et al., 2008; Zhou et al., 1998) than classical serological assays, what represents a considerable advantage for the analysis of wild bird sera, since these species sometimes have weak humoral responses to AIV-infection (Kida et al., 1980).

Unfortunately, the validation of these tests for the serosurveillance of wild birds is complicated by the absence of positive or negative reference sera (Marché and van den Berg,

2010). Hence, comparative studies assessing the possible impact of the used ELISA kit on seroprevalence studies are needed (Lebarbenchon et al., 2011). In this study, four commercially available multispecies NP-ELISA test kits were compared using wild bird sera. The agreement and association between results have been measured in a species-specific approach.

3 Materials & Methods

3.1 Serum samples

For the present study, 172 wild bird serum samples were obtained from wild birds caught during the Belgian 2009 active surveillance program. During this surveillance program, a total of 2596 swabs (1187 oropharyngeal and 1749 cloacal) from 35 species and 846 serum samples from Canada geese (Branta canadensis; n=522), mallards (Anas platyrhynchos; n=27) and mute swans (Cygnus olor; n=297) were taken from birds captured between January and December 2009 at various locations throughout Belgium. Thirty-three swabs were found positive by real-time RT-PCR, from 10 of which it was possible to isolate a virus. Forty-nine Canada goose, 17 mallard and 114 mute swan sera were found positive by NP-ELISA (IDScreen influenza A antibody competition ELISA kit, Idvet, Montpellier, France). For the present study, seventy-three Canada goose and seventy-two Mute swan sera were selected by stratified sampling, using the surveillance results. The samples were at first divided in two strata (positive and negative serum samples) and then randomly selected. This way, approximately equal numbers of positive and negative samples were obtained. Due to the small number of available samples, all mallard samples (n=27) were selected.

Also, 92 poultry sera that were obtained from a recently carried out animal experiment were included. These included 62 sera from SPF chickens and 30 sera from Pekin ducks. The SPF chicken sera were obtained at 21 days post infection (dpi.) from chickens either inoculated (n=12) with H5N3 A/Anas platyrhynchos/09-272/08 or with H7N1 A/Ch/Italy/1067/v99 low pathogenic avian influenza (LPAIV) or exposed (n=50) to animals inoculated with these viruses. The Pekin duck sera included 9 sera from naïve Pekin ducks and 21 sera obtained at 21 dpi. from Pekin ducks inoculated with H5N3 LPAIV A/Anas platyrhynchos/09-272/08. The SPF chicken and Pekin duck sera were all selected according the same sampling method as described above, based on previous HI assay results from the animal experiments.

All sera were stored at -20°C and allowed to reach laboratory temperature prior to testing.

3.2 ELISA test kits

All samples were tested with 4 commercially available antibody detection ELISA kits, all directed towards antibodies against the nucleoprotein (NP). All tests were conducted according to the manufacturer's instructions.

ELISA-1 is the IDScreen influenza A antibody competition ELISA kit (Idvet, Montpellier, France). This ELISA kit works in a blocking ELISA (bELISA) format although its name suggests a competitive format. Since this is the same test as the one used in the wild bird surveillance program, the results from this program were used in this study for the wild bird sera. The dilution step instructed different dilutions for chicken sera and for sera from mallard and goose. An incubation period of 1 hour was applied. Serum samples with a sample-to-negative (S/N) ratio greater or equal to 0,5 were considered negative, S/N ratio between 0,45 and 0,50 were considered doubtful and S/N ratio less than or equal to 0,45 were considered positive. In the data analysis, all doubtful samples were considered positive.

ELISA-2, the AI MultiS-Screen Ab ELISA kit (Idexx, Westbrook, ME, United States), is a bELISA as well. The dilution step was the same for all bird species. Samples with an S/N ratio smaller than 0,5 were considered AI antibody positive.

ELISA-3 is Flu Detect BE Avian Influenza Virus Antibody Test Kit, cELISA (Synbiotics, Kansas City, MO, United States). Samples with an S/N ratio smaller than 0,6 were considered positive.

ELISA-4 is the only competitive ELISA (cELISA) included in the study, the Type A Influenza Multi species Antibody Test Kit (AI MSp) (BioChek, Reeuwijk, The Netherlands). Samples with a sample-to-negative ratio of 0,6 or less were considered positive.

3.3 Statistical analysis

For each bird species (wild birds and experimentally infected birds), percentage agreement was calculated between each ELISA and the three other ELISA's. Additionally, Cohen's Kappa statistics were calculated to measure the strength of this agreement. Interpretation of the Cohen's Kappa coefficient (κ) was done according to the divisions described by Landis and Koch: κ values below 0,00 indicate poor; 0,00-0,20 slight; 0,21-0,40 fair; 0,41-0,60 moderate; 0,61-0,80 substantial and 0,81-1,00 almost perfect agreement (Landis and Koch, 1977). Next to this qualitative approach, the amount of variation between results was measured using Spearman's rank correlation test. This test does not assume linear relationship between results and is less sensitive to strong outliers than Pearson correlation coefficient. Common guidelines for interpreting Spearman's rank correlation coefficients (r_s) are the

following: below 0,4: weak; 0,40-0,70: moderate and above 0,70 strong association (Jarvis et al., 2011; Satoh et al., 2007; Sweeney et al., 1995). All calculations were done in SPSS Statistics 19 (SPSS Inc., Armonk, NY, United States).

4 Results

For mute swan, SPF chicken and Pekin duck sera, the number of sera that were found positive by each ELISA kit was highly comparable whilst more variation was observed for Canada goose and mallard sera (table 1). Kappa coefficients show that agreement between the assays strongly depends on the bird species. Generally, a better agreement was observed for sera obtained under experimental conditions (table 2). Spearman's rank correlation coefficients (table 3) give additional information on the distribution of the S/N ratios. Scatterplots are shown for Canada goose, mallard and mute swan sera (figure 1).

For SPF chicken sera, ELISA 1, 2 and 3 found more sera positive than the HI assay. However for Pekin duck sera, ELISA 2 and 4 found more sera positive than the HI assay. Interestingly, ELISA 2 and 4 were the only assays that found all sera from inoculated Pekin ducks positive and all sera from naïve Pekin ducks negative.

Table 1: Number of sera found positive by each ELISA test kit and the HI assay.

	Canada goose n=73	Mallard n=27	Mute swan n=72	SPF chicken n=62	Pekin duck n=30
ELISA 1	36	17	36	35	18
ELISA 2	36	21	40	34	21
ELISA 3	12	2	42	38	15
ELISA 4	21	22	31	27	21
HI Assay	n/a	n/a	n/a	30	20

ELISA 1 - ELISA 2. ELISA 1 and 2 agreed substantially to almost perfectly for Canada goose, mute swan, SPF chicken and pekin duck sera. For mallard sera, the kappa coefficient indicated only a fair agreement between the assays. However, the r_s value for this species indicated a moderate association. This contradiction can be explained by the fact that ELISA 2 found 6 more sera positive than ELISA 1, which had S/N values not far above the threshold

value for both tests. On the other hand, two strong outliers which were both positive by ELISA 1 and negative by ELISA 2 were observed (figure1A).

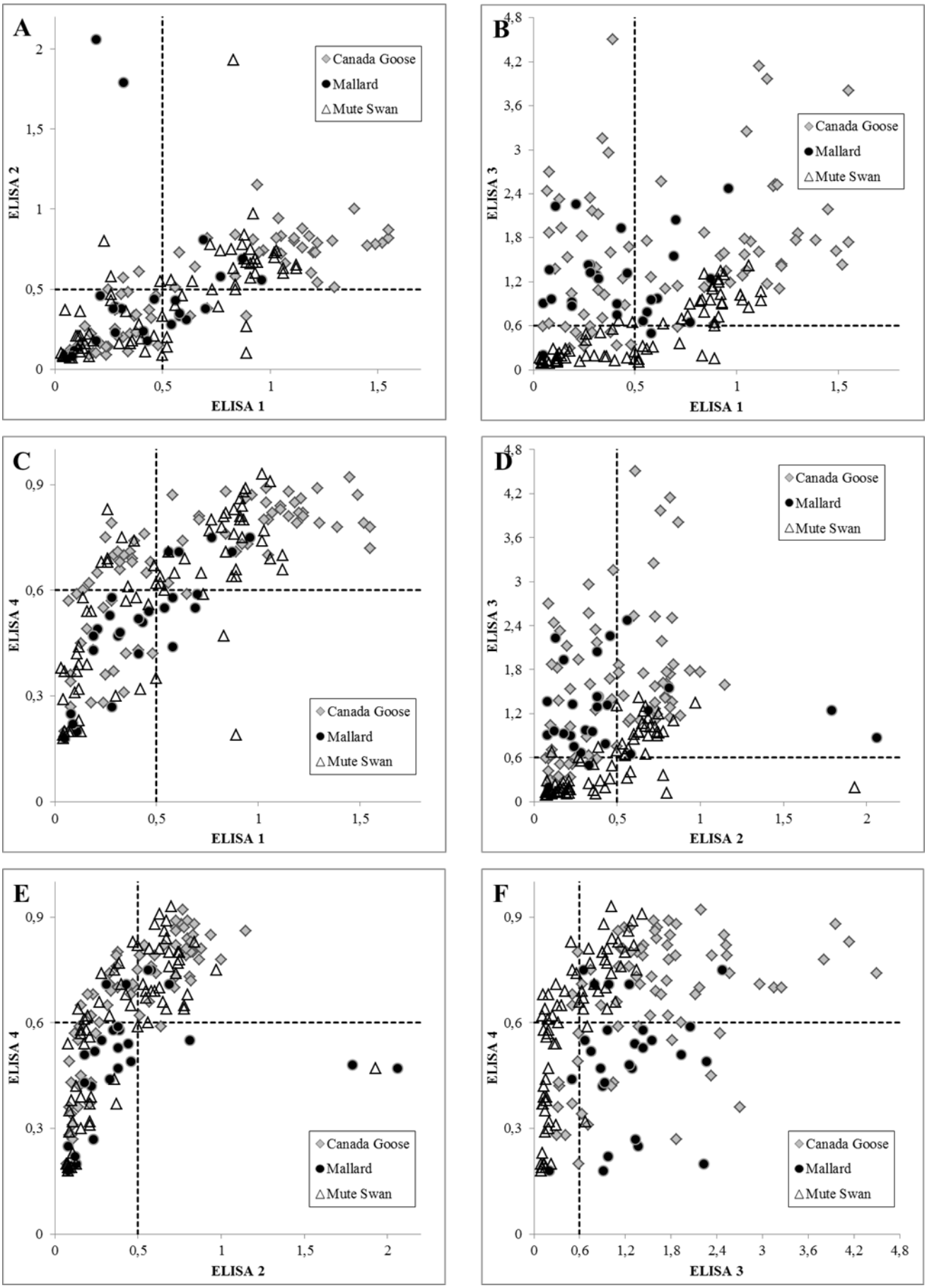
Table 2: Cohen's kappa coefficient and percentage agreement (in brackets) of each ELISA with the other ELISAs, stratified per species.

	Canada Goose n = 73	Mallard n = 27	Mute Swan n = 72	SPF Chicken n = 62	Peking Duck n = 30
ELISA 1					
ELISA 2	0,84 (92%)	0,31 (70%)	0,72 (86%)	0,97 (98%)	0,78 (90%)
ELISA 3	0,28 (64%)	-0,03 (37%)	0,72 (86%)	0,83 (92%)	0,67 (83%)
ELISA 4	0,53 (77%)	0,56 (81%)	0,64 (82%)	0,75 (87%)	0,78 (90%)
ELISA 2					
ELISA 3	0,34 (67%)	0,05 (30%)	0,77 (89%)	0,8 (90%)	0,6 (80%)
ELISA 4	0,53 (77%)	0,43 (81%)	0,59 (79%)	0,71 (85%)	1 (100%)
ELISA 3					
ELISA 4	0,43 (79%)	0,04 (26%)	0,59 (79%)	0,66 (82%)	0,6 (80%)

ELISA 1 - ELISA 3. These two ELISA's agreed substantially to almost perfectly for mute swan, Pekin duck and SPF chicken sera, for which S/N ratios were strongly associated. For the Canada goose and especially mallard sera, much less sera were positive by ELISA 3 than by ELISA 1. Kappa coefficients for these species indicate fair to poor agreement and figure 1 shows that the data points are widely dispersed on the scatter plot.

ELISA 1 – ELISA 4. ELISA 1 and 4 agreed moderately to substantially for all species (table 2). In most cases where disagreement between the assays was observed, S/N values were close to the threshold value (figure 1C), supporting the strong association (i.e. elevated r_s values) between the results (table 3).

Figure 1 (next page): Sample-to-negative control ratios for each combination of ELISA test kits for sera from Canada goose, mallard and mute swan. A: ELISA 1-ELISA 2, B: ELISA 1-ELISA 3, C: ELISA 1-ELISA 4, D: ELISA 2-ELISA 3, E: ELISA 2-ELISA 4, F: ELISA 3-ELISA 4 Threshold values are indicated by the dotted lines.


A
ELISA 2
ELISA 1
Canada Goose
Mallard
Mute Swan
B
ELISA 3
ELISA 1
C
ELISA 4
ELISA 1
D
ELISA 3
ELISA 2
E
ELISA 4
ELISA 2
F
ELISA 4
ELISA 3

ELISA 2 – ELISA 3. ELISA 2 agreed moderately with ELISA 3 for mute swan, SPF chicken and Pekin duck sera (table 2). Much less mallard and Canada goose sera were found positive by ELISA 3 than by ELISA 2. Agreement for these two species was poor to fair. Spearman's correlation coefficient showed no significant association between S/N ratios obtained for mallard sera and weak association between S/N ratios obtained for Canada goose sera. Indeed, the scatterplot in figure 1D shows a wide dispersion of the data points for these two species.

ELISA 2 – ELISA 4. ELISA 2 and 4 agreed substantially to almost perfectly for sera from SPF chickens and Pekin ducks, but agreed moderately for all wild bird field sera. Clearly more Canada goose and mute swan sera were positive by ELISA 2 than by ELISA 4, but figure 1E shows that some sera over which the assays disagreed had S/N ratios close to both assays' threshold value and that the intersection of the two cut-off lines lies just below and to the right of the data points instead of in a central position (which was the case for assays with better agreement ratios), explaining the high r_s values despite a relatively weak agreement between the test kits.

ELISA 3 – ELISA 4. These ELISA kits agreed substantially for SPF chicken sera and moderately for Canada goose, mute swan and Pekin duck sera. Results were weakly associated for Canada goose sera, but since both assays found a large proportion of sera from this species negative, kappa coefficient and especially agreement rate were relatively high, illustrating the necessity of observing data distribution when comparing ELISA tests. Only a slight agreement was seen for mallard sera and S/N ratios were weakly associated for this species.

Table 3: Spearman's Rank Correlation coefficient and p-values for each ELISA with the other ELISAs, stratified per species.

	Canada Goose		Mallard		Mute Swan		SPF Chicken		Peking Duck	
	r_s	*p*	r_s	*p*	r_s	*p*	r_s	*p*	r_s	*p*
ELISA 1										
ELISA 2	0,84	*p<0,01*	0,57	*p<0,01*	0,75	*p<0,01*	0,91	*p<0,01*	0,83	*p<0,01*
ELISA 3	0,29	*p=0,01*	0,05	*p=0,79*	0,83	*p<0,01*	0,87	*p<0,01*	0,74	*p<0,01*
ELISA 4	0,79	*p<0,01*	0,85	*p<0,01*	0,76	*p<0,01*	0,89	*p<0,01*	0,95	*p<0,01*
ELISA 2										
ELISA 3	0,37	*p<0,01*	0,16	*p=0,43*	0,75	*p<0,01*	0,85	*p<0,01*	0,61	*p<0,01*
ELISA 4	0,85	*p<0,01*	0,67	*p<0,01*	0,78	*p<0,01*	0,85	*p<0,01*	0,86	*p<0,01*
ELISA 3										
ELISA 4	0,39	*p<0,01*	0,14	*p=0,50*	0,82	*p<0,01*	0,87	*p<0,01*	0,75	*p<0,01*

5 Discussion

The development of blocking and competition ELISA has contributed greatly to the establishment of AIV serosurveillance in many bird species. Nowadays, multi-species anti-NP ELISAs are widely used for AIV-surveillance (Brown et al., 2010a and 2010b; Dalessi et al., 2007; De Marco et al., 2003; Owoade et al., 2006; Perez-Ramirez et al., 2010). However, while many research on the performance of these tests for the diagnosis of AIV in domestic poultry is carried out, very little is known about the performance of these tests for the screening of wild bird sera. Indeed, as reference sera from poultry can easily be obtained, test sensitivities and specificities can accurately be determined and test protocols can be adjusted to meet the requirements of AIV-diagnosis in these species. On the contrary, since reference sera from wild birds are difficult to obtain and the assessment of AI-seroprevalence in these species has other requirements, the performance of NP-ELISAs for AIV-surveillance in wild birds needs to be investigated more thoroughly.

In this study, results of each one of four commercially available ELISA kits were compared in a qualitative (Cohen's kappa statistics) and quantitative (Spearman's rank correlation coefficient) way with results from the three other kits, using wild bird sera and sera from animal experiments. These pairwise comparisons showed that results obtained for NP-ELISA based serosurveillance of wild birds are largely influenced by the choice of the ELISA test kit and the bird species. Results obtained with ELISA 1 and ELISA 2 were mostly comparable, although these tests clearly disagreed over many mallard sera. Results obtained with ELISA 4 were to a lesser extent comparable with ELISA 1 and 2. ELISA 3 often gave clearly different results than the other ELISAs, especially for Canada goose and mallard sera. Interestingly, differences between ELISA test kit results are not seen to such extent when analyzing sera from experimentally infected animals. Indeed, the differences in agreement between SPF chicken and Pekin duck sera were generally smaller than the differences in agreement for the wild bird sera. Possibly, biological differences between birds may explain the variation in reactivity observed in this study. The possibility of aspecific reactions which background cannot be investigated due to the lack of negative reference wild bird sera. Another explanation might be linked to the age of the animals at sampling. The sera from experimentally infected animals were obtained between 4 and 9 weeks of age, whilst most animals sampled during the wild bird surveillance programme were adults that might have been exposed to other infectious agents besides Influenza A viruses during their lifetime.

Calculating the association between results obtained for each species allowed an assessment of the cut-off values recommended by the manufacturers. In some cases, e.g. the comparison of results for mallard sera obtained with ELISA 1 and 2, κ was rather low whilst r_s still indicated a moderate association between the obtained S/N ratios. For this species, samples that tested negative by ELISA 1 and positive by ELISA 2 all had S/N ratios close to the threshold values of each assay. Once again, the absence of negative reference sera makes the establishment of a sound threshold value difficult. Since results obtained with ELISA 3 for Canada goose and mallard sera had both low κ and r_s, the reason for disagreement with the other ELISA kits for these species is not an incorrect threshold value, but rather biological or technical problems with this assay.

In conclusion, since the outcome of the analyses depends largely on the ELISA test that is used and on the bird species under consideration, results obtained with NP-ELISA tests for the analysis of wild bird sera should be cautiously interpreted.

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Chapter 7:

General discussion

Much investigation on the transmission of highly pathogenic avian influenza has been carried out, whereas the dynamics of low pathogenic avian influenza transmission is studied far less intensively. However, since LPNAIVs may evolve into HPAIVs, understanding the whereabouts, dynamics and the intra- and interspecies transmission of these viruses is crucial for understanding HPAIV emergence. Therefore, research on LPNAIVs can present the basis on contemporary and future avian influenza control & surveillance.

Whereas the control of HPAIV used to be directed towards a swift and stringent stamping-out of outbreaks, the emphasis has now shifted towards preventing the emergence of HPAIV. Hereto, surveillance programs directed to an early detection of LPNAIVs in poultry and the monitoring of LPNAIVs in wild birds have been implemented. However, as this is a relatively new approach, some important scientific knowledge which could further improve the design of these surveillance programs is still lacking.

In this thesis, transmission experiments that were carried out to investigate the impact of a selection of parameters on the transmission and circulation of LPNAIV strains in chickens are described. The results also provide a scientific background for assessing the threat for the poultry industry posed by LPNAI isolates.

1 Studying LPNAIV transmission

1.1 Experimental design aspects

Transmission experiments have mostly been used for examining the effect of vaccination on LPNAI transmission (de Jong and Kimman, 1994; van der Goot et al., 2005; van der Goot et al., 2007). In this thesis, we demonstrated that transmission experiments can also be employed to investigate the impact of housing conditions on LPNAI transmission, to identify new risk factors and to broaden the empirical knowledge on the transmission of LPNAIVs between chickens and between ducks and chickens (chapters 3-5).

Accordingly, future transmission experiments can be designed to investigate the impact of other factors that may possibly play a role in the transmission of LPNAIVs. For example, differences in susceptibility regarding poultry species, breed and age can be investigated by means of transmission experiments. Also, the impact of relative humidity, air flow, temperature, biochemical characteristics of drinking water and other environmental/management factors can be examined (Lowen et al., 2007).

1.1.1 Study population and strain infectivity

A first important consideration in the design of transmission experiments is the interaction between the study population and the selected LPNAI strain. Indeed, the ability for an LPNAIV to replicate may vary for different host species (Swayne and Slemons, 2008). Logically, good transmissibility can be expected if the animal species in the study population is the same species as the one from which the LPNAIV was originally isolated, and a weak transmissibility can be expected if the virus was originally isolated from a different, non-related species. However, this is not always true; from appendix 1 and 2, it can be seen that several characterization experiments have witnessed a good virus replication, although the bird species in the study population was not related to the species from which the virus was originally isolated (appendix 1 and 2).

Since no knowledge on the transmissibility of the selected LPNAI strains was available at the beginning of our experiments, we did not know whether to expect a strong or a weak virus transmission. Therefore, to avoid having either no transmission, or either full transmission to all susceptible animals, a 50:50 ratio of infected and susceptible animals was chosen. This is generally considered a good compromise, if preliminary knowledge is lacking. However, making use of different infected:susceptible ratios should be considered, if knowledge on the transmissibility of the virus is available (Velthuis et al., 2007).

1.1.2 Acquisition of infectious animals

The route by which infection of animals is achieved may also influence the outcome of transmission experiments. All LPNAI transmission experiments that are cited in this thesis involve exposing susceptible birds to inoculated birds i.e.: standard transmission experiments. In chapters 3 and 4, we also made use of this type of transmission experiments.

Because it is often assumed that inoculated animals are exposed to fairly large infection doses, it is believed that these animals may shed more virus than naturally infected animals. Therefore, the transmission in standard transmission experiments may be artificially enhanced (Velthuis et al., 2007). We conducted extended transmission experiments to evaluate if LPNAIV transmission still occurs if the infection dose is closer to that experienced in a natural infection (chapter 5).

Since extended transmission experiments and standard transmission experiments gave analogous results for reproduction ratios estimated for the H5N2 A/Ch/Bel/150VB/99 LPAIV and since virus shedding quantities did not differ much between inoculated and contact animals in both types of transmission experiments with this virus, we concluded that the use of standard transmission experiments for this virus in chapter 3 had not led to an artificially

enhanced transmission. Indeed, the inoculation dose and method used in this thesis for the H5N2 A/Ch/Bel/150VB/99 LPAIV appears to be comparable to the natural infection dose experienced by SPF chickens that are exposed to infectious SPF chickens from 1dpi until 4dpi, under the conditions described in this thesis. To what extent a different experimental setting would have influenced the outcome of this study is impossible to estimate, but it can be assumed that experimental inoculation as described in this thesis is a good alternative to natural infection for this virus. Results must not be over-generalized, however: if similar experiments would be conducted with LPNAIVs that are more/less infectious than H5N2 A/Ch/Bel/150VB/99 LPAIV, it is possible that larger differences are obtained.

Unfortunately, our extended transmission experiments with H7N1 A/Ch/Italy/1067/v99 failed to cause infection in C_2-chickens. Since this was most likely due to a problem in the timing of exposure of the C_1-chickens to the I-chickens, we could not formally prove if an extended transmission experiment would improve the model for this virus (chapter 5). Therefore, no conclusions on this behalf can be drawn for this virus. Ideally, the experiment would have needed to be repeated, exposing the C_1-chickens to the I-chickens for a few more days, to ensure that infection takes place. Indeed, the time-span over which a certain infection dose is applied may determine the result of exposure to a pathogen (Pujol et al., 2009).

1.2 Analytical aspects

Through statistical analysis of the results of the transmission experiments, the effects of environmental factors or intervention strategies on virus transmission can be determined and quantified. Transmission of AIVs is mostly analyzed by using an SIR-model (Table 1).

However, fitting data from transmission experiments with LPNAIVs in an SIR-model can pose some difficulties. Whilst some methods for estimating the R_0 with an SIR-model allow using more input data to give a more precise quantification of transmission, these input data may in return be influenced by the chosen diagnostic assays and their limitations (Comin et al., 2011; Dewulf et al., 2002; Mundt et al., 2009; Velthuis et al., 2003). This means that even a well-developed statistical model which theoretically enables an objective description of transmission can still be influenced by subjective reading of test results, the selection of cut-off values, etc.

1.2.1 Determining infection

According to the OIE, infection with an HPAIV can be confirmed with the isolation of the virus, detection of specific viral RNA or the detection of specific antibodies (Senne, 2007). For LPNAIVs however, no such definition is available, nor can a researcher rely on visual signs of infection. As a result, a variety of criteria for determining infection in animals are used in the available literature on small-scale LPAIV transmission experiments (table 1).

Furthermore, since the infectivity of LPNAIVs is smaller than that of HPAIVs, the intake of small amounts of virus does not automatically imply that the animal will become infected and infectious to other animals (Swayne and Slemons, 2008). Since we assumed that highly sensitive methods like RRT-PCR can detect the vRNA from taken viruses in OP/CL swabs from uninfected animals, we believe that using this method for determining infection in animals may lead to false positive results. This assumption was further endorsed by several observations in which very small amounts of vRNA were detected in OP/CL swabs originating from animals that did not develop an antibody-mediated immune response. Therefore, we considered animals infected only if anti-AIV antibodies were detected in blood serum during the course of the experiment or at the end of the experiment. Analysis of blood serum was done with NP-ELISA since this method is generally considered to be more sensitive than HI-assay (Beck and Swayne, 2003). We additionally investigated the reliability of the selected NP-ELISA kit and found it to be highly reliable for the analysis of SPF chicken sera (chapter 6; Marché et al., 2010). On the downside, considering only seroconverted animals as infected may be a fairly conservative analysis of virus transmission since it excludes animals that became infected and did not develop an immune response, or animals that became infected and developed only local or cellular immune response. Unfortunately, this is poorly recorded in the existing literature, so this hypothesis cannot be endorsed. However, it can be hypothesized that the absence of an antibody-mediated immune response in chickens exposed to LPNAIVs indicates low virus replication, which means that virus shedding is low as well. In conclusion, whereas RRT-PCR may currently be the most sensitive and fastest method for diagnosing LPNAI in field samples, its results must be carefully interpreted in transmission experiments. Furthermore, since this test detects vRNA coming from both defective and live viruses, an overconfident adoption of RRT-PCR results may additionally lead to an incorrect picture of the environmental infection pressure, as suggested in chapter 4 of this thesis and endorsed by results by Guan et al. (2009) (Guan et al., 2009).

Table 1: Reproduction ratios estimated in small-scale LPNAIV transmission experiments cited in this thesis.

		Virus[a]	Species[a]	R_0[b]	[95% C.I.]	Criterion for infection	Reference
LP	H5N2	A/Ch/Bel/150VB/99	SPF Ch	1,3	[0,4 - 3,5]	Serology	Chapter 3
LP	H5N2	A/Ch/Bel/150VB/99	SPF Ch	1,5	[0,8 - 2,2]	Serology	Chapter 3
LP	H5N2	A/Ch/Bel/150VB/99	SPF Ch	1,8	[0,6 - 4,1]	Serology	Chapter 3
LP	H5N2	A/Ch/Bel/150VB/99	SPF Ch	2,0	[0,8 - 3,3]	Serology	Chapter 3
LP	H5N2	A/Ch/Bel/150VB/99	SPF Ch	2,1	[0,9 - 6,2]	Serology	Chapter 5
LP	H5N2	A/Ch/PA/21525/83	SPF Ch	1,1	[0,5 - 1,9]	Virus isolation	Van der Goot et al., 2003
LP	H5N2	A/Ch/PA/21525/83	SPF Ch	0,6	[0,2 - 1,3]	Serology	Van der Goot et al., 2003
LP	H5N3	A/Anas pl/09-884/2008	SPF Ch	0,0	[0,0 - 0,7]	Serology	Chapter 3
LP	H5N3	A/Anas pl/09-884/2008	SPF Ch	0,0	[0,0 - 0,7]	Serology	Chapter 3
LP	H7N1	A/Ch/Italy/1067/v99	SPF Ch	1,0	[0,3 - 2,8]	Serology	Chapter 3
LP	H7N1	A/Ch/Italy/1067/v99	SPF Ch	0,9	[0,2 - 1,7]	Serology	Chapter 3
LP	H7N1	A/Ch/Italy/1067/v99	SPF Ch	1,7	[0,7 - 4,1]	Serology	Chapter 3
LP	H7N1	A/Ch/Italy/1067/v99	SPF Ch	2,3	[0,1 - 0,4]	Serology	Chapter 3
LP	H7N1	A/Ch/Italy/1067/v99	SPF Ch	0,7	[0,0 - 13,8]	Serology	Chapter 5
LP	H7N1	A/Tu/Italy/1067/99	SPF Ch	4,0	[1,7 - 11,0]	RRT-PCR	Gonzales et al., 2011
LP	H7N1	A/Tu/Italy/1067/99	SPF Ch	3,8	[1,3 - 6,3]	RRT-PCR	Gonzales et al., 2011
LP	H7N1	A/Ch/Italy/1279/99	Turkey	15,3	[11,8 - 19,7]	RRT-PCR	Saenz et al., 2012
LP	H7N7	A/Ch/Neth/2006	Layer Ch	0,8	[0,4 - 1,8]	RRT-PCR	Gonzales et al., 2012
LP	H7N7	A/Ch/Neth/2006	Layer Ch	0,7	[0,0 - 1,7]	RRT-PCR	Gonzales et al., 2012

[a] Ch=Chicken, Anas pl=Anas platyrhynchos, Tu=Turkey

[b] Multiple basic reproduction ratios for the same virus refer to separate analyses of the same experiment or replicates which may or may not have been conducted in different circumstances

1.2.2 Quantifying transmission

In this thesis, two methods for quantifying LPNAIV transmission according to the SIR-model are used. In chapters 3 and 4, we have used the GLM; in chapters 3 and 5, we have used the FS model. Whereas the FS model ignores many data, the more elaborate GLM uses data from every time interval separately. Therefore, the GLM is a more powerful method for estimating differences in transmission between two treatment groups than the FS method is (Velthuis, 2002).

Comparing the R_0 estimates obtained in chapter 3, no important difference between the FS and the GLM method can be seen. Therefore, both methods may be confidently used in quantifying LPNAI transmission. However, we observed smaller 95% confidence intervals

for R_0 estimated with the GLM method. Therefore, differences between groups might theoretically become more apparent using this method.

An important advantage of the GLM method is that an adapted method of it can be used for estimating β in heterogeneous groups of animals (Velthuis et al., 2003), whereas the FS method can only be used in populations that are homogeneous. Using this method, we were able to quantify the transmission of a LPNAIV isolated from a wild bird (WB-LPNAIV) from ducks to chickens (chapter 4).

2 Risk factors associated with enhanced LPNAIV circulation in or attracting LPNAIV to poultry holdings

In order to prevent the emergence of HPAIVs, active surveillance programs must be able to detect LPNAIVs that are circulating among poultry as soon as possible. Since 2012, Belgium uses a risk-based sampling approach (Animal health and veterinary laboratories agency, 2013). The current Belgian active surveillance program consists of sampling all poultry holdings containing more than 200 animals of the species duck, goose, turkey, guinea fowl, pheasant, partridge, chicken (excluding broiler chickens) and meat pigeon once/year. Additionally, sampling is conducted a second time in holdings that are situated in high risk areas, as defined by the FASFC (Federal authority for safety of the food chain, 2013a), holdings where animals have outdoor access and in holdings raising turkeys, geese and ducks (Federal authority for safety of the food chain, 2013b). However, for a thorough risk-based active surveillance program in poultry, the possibility of including more risk factors or the necessity of further adapting this surveillance program needs to be investigated; a study by Welby et al. (2010) has concluded that further increasing the sampling frequencies in holdings located in high risk areas or concentrating sampling during migration of wild birds could theoretically improve the current active surveillance program (Welby et al., 2010). Other member states of the European Union that have used a risk-based sampling approach during the 2012 active surveillance in poultry include Bulgaria, Denmark, Finland, France, Germany, Italy, Luxembourg, The Netherlands and United Kingdom (Animal health and veterinary laboratories agency, 2012). The sampling regimes are diverse, however. Member states target different holdings in their risk-based sampling designs. Risk factors that are considered in other member states' programs and not in the Belgian program include: trade activities, timing of sampling, reactive sampling, presence of water bodies on poultry premises, mixed

poultry species holding where one of the species is waterfowl, etc (European commission, 2012; 2013; Animal health and veterinary laboratories agency, 2013). The decision to include certain risk factors in future risk-based surveillance strategies can be based on epidemiological surveys, expert opinions or experiments. In this thesis, we investigated if the type of housing system, the water supply and the raising of mixed poultry species should be considered as risk factors for the Belgian active LPNAI surveillance in poultry.

2.1 Implementing housing systems in risk-based LPNAIV surveillance

Laying hens are commonly housed in either cage-based (furnished cages) or floor-based housing systems. The advantages and disadvantages of these housing systems regarding social behavior, ease of management, costs, production, metabolic disorders, foot lesions and infectious diseases have been extensively discussed (Dumas et al., 2011; Duncan, 2001; Elson, 2010; Kreienbrock et al., 2003; Madelin and Wathes, 1989; Rodenburg et al., 2005; Tauson et al., 1999). Studies on the impact of the type of housing on pathogen prevalences have also been conducted, mostly focusing on S*almonella spp*. However, these studies have found no conclusive evidence that either type of housing (cage-based or floor-based) is predisposing for infection with *Salmonella spp* in laying hens (Pieskus et al., 2008; Van Hoorebeke, 2010).

In this thesis, transmission experiments were conducted to investigate the impact of the type of chicken housing on the transmission of LPNAIV. Quantification of transmission was carried out to analyze the data as objectively as possible. We observed a difference in LPNAIV transmission between chickens housed on a grid and chickens housed on a floor covered with wood shavings (chapter 3). Whilst at a first glance, the differences in R_0 estimates for the two floor types might appear rather small, it must be considered that, for reproduction ratios between 1 and 4, small changes in R_0 may result in dramatic changes in the final size of an outbreak (de Jong, 1995). Therefore, it can be concluded that housing chickens on a floor covered with litter such as wood shavings might indeed be a risk factor for LPNAIV. However, the differences we observed were not statistically significant and therefore, it is possible that these differences were due to biological variations in transmission. To provide further insights on this matter, additional research is needed. This does not necessarily involve conducting further animal transmission experiments, but could also make use of epidemiological data from LPNAI outbreaks. Since a possible impact of the type of poultry house on LPNAIV transmission might also come from other characteristics such as the ease with which the equipment can be cleaned and the fact that between-cage transmission

can slow down the spread of the virus throughout the poultry house (Elbers et al., 2004; Yee et al., 2009), results from such an investigation could be readily adopted by authorities. In existing literature on AIV outbreaks, the type of poultry houses involved is only rarely discussed. If listed, it mostly concerns HPAI outbreaks (Tsukamoto et al., 2007). We have now demonstrated that acquiring information on the type of poultry houses that are involved in LPNAI outbreaks may give interesting results and may enable deciding if this parameter should be considered in risk-based LPNAIV surveillance.

2.2 The role of water supply in LPNAI introduction

We demonstrated that the transmission of LPNAIVs from virus-shedding ducks to poultry may occur through fecally contaminated drinking water, without the need of close contact between the animals. Moreover, our results suggest that even a small amount of duck feces deposited in drinking water can result in the transmission of LPNAI to chickens. In poultry holdings where the drinkers are directly accessible to visiting wild birds (poultry holdings with outdoor access), WB-LPNAIVs can be readily transmitted to the poultry through fecal contamination of the drinking water. However, other poultry holdings may also be at risk for introducing WB-LPNAIVs through water consumption. Belgian poultry holdings use water from different sources (tap water, ground water and, to a lesser extent, rainwater, surface water and recovered water) for drinking water, cleaning of equipment or cleaning of the poultry houses between production rounds (D'hooghe et al., 2007; Coulier, 2011). The water that is used must meet certain standards, which are mostly aimed at reducing fecal contamination (Diergezondheidszorg Vlaanderen, 2013). Often, chlorine or peroxide is added to improve water quality, and sometimes disinfecting UV-lamps are installed (Coulier, 2011). Whilst these measurements may be effective in reducing the risk of LPNAIV introduction by this route (De Benedictis et al., 2007; Faust et al., 2012; Leung et al., 2007; Stallknecht et al., 1990), the possibility remains that, in case of a sub-optimal functioning or a breakdown of the disinfecting installations, LPNAIVs are introduced via drinking or cleaning water. For the index farm of the Chilean 2002 HPAIV epizootic, it is suggested that the use of drinking water from a pond that was frequented by wild birds was the route by which the progenitor LPNAIV was introduced (Koch and Elbers, 2006; Max et al., 2006). Since the risk of virological contamination is different for the different sources of water, it may be advisory to consider the type of water supply and/or water decontamination methods for the design of risk-based sampling strategies.

2.3 Type of poultry holding

2.3.1 Mixed poultry holdings

The presence of multiple bird species on one farm is generally considered as one of the most important risk factors for LPNAIV-infections. Especially those farms where contact between domesticated aquatic birds and land-based poultry is possible, may act as bridging farms for the introduction of WB-LPNAIVs to poultry (Koch and Elbers, 2006).

In this thesis, the transmissibility of a H5N3 WB-LPAIV from ducks to chickens housed in close contact was demonstrated (Chapter 4). Interestingly, this virus had previously been found to be poorly infectious to SPF chickens, without transmission in our model (Chapter 3). The fact that it afterwards proved to have the ability to be transmitted from pekin ducks to SPF chickens suggests that WB-LPNAIVs which can be maintained among domestic aquatic birds may be, depending on the intensity of the contact (close contact, airborne and waterborne transmission), repeatedly/continuously transmitted to chickens that are raised in the same holding. In addition, the possibility exists that during the course of the experiment, the virus acquired adaptation to circulation in SPF chickens. This hypothesis could be confirmed by conducting an intraspecies transmission experiment with the virus that was obtained from the contact-infected SPF chickens and estimating if transmission is enhanced. Arguably, genetically comparing the virus that was isolated from chickens with the original virus might reveal some markers for adaptation. This could prove that these interspecies transmissions may form a perfect starting point for the LPNAIV to establish itself in chickens and might also enable an assessment of some early markers of adaptation.

Regarding the extrapolation of these results to the field situation, it must be considered that the experimental designs presented in this thesis represent forced models that often favored transmission. Therefore, it is not certain if the above described interspecies transmission will occur as easily in the field. Still, the combined results of chapters 3 and 4 suggest that this is more likely to occur in a mixed holding than in a non-mixed holding.

In addition, some WB-LPNAIVs are intrinsically found to be more capable of infecting chickens than the WB-LPNAIV that was used in this thesis (appendix 1 and 2). For example, a H7N1 virus that was isolated from a common shellduck (A/Tadorna tadorna/Belgium/3441-P3/09) was characterized as such during the course of this thesis (Marche et al., 2012). If such a WB-LPNAIV is introduced to domestic aquatic birds from a mixed holding, transmission to and subsequent establishment in chickens is very likely to occur.

2.3.2 Backyard poultry holdings

Combining the infectious characteristics of the H5N2 A/Ch/Bel/150VB/99 LPAIV with the circumstances under which this virus was isolated provides information on the involvement of backyard poultry holdings in LPNAI outbreaks.

Since we demonstrated that this virus is well adapted to gallinaceous poultry (Chapters 3 and 5), the reason why this virus was isolated on only one occasion becomes intriguing. It can be hypothesized that a widespread distribution from the index case to other holdings did not occur: i) because the virus had been introduced from the wild bird reservoir to this farm and was detected before it could spread to other poultry holdings, or ii) because virus transmission remained restricted to rural poultry farms and did not spread to industrial poultry farms. Indeed, several studies suggest that backyard poultry holdings are of limited importance for introducing LPNAIVs to industrial poultry holdings (Bavinck et al., 2009; European Food Safety Authority, 2005; Gonzales et al., 2010). Backyard poultry holdings are therefore not included in the Belgian active AI surveillance program. Since Belgian backyard poultry holdings are found to be mostly small and epidemiologically isolated, with mostly no off-farm movement of birds (Van Steenwinkel et al., 2011), it can be assumed that a few small-scale outbreaks of H5N2 A/Ch/Bel/150VB/99 LPAIV may have occurred in some backyard flocks that could be linked to the dealer that had been in contact with the index holding, but which remained unnoticed or undiagnosed. Because of the limited connectivity between the affected flocks and industrial poultry holdings, the virus may have spontaneously died out in the rural sector before transmission to industrial poultry holdings occurred. Therefore, the hypothesis that rural and industrial poultry holdings can be considered as two separate poultry compartments may be justified.

However, LPNAI epidemics involving both rural and industrial poultry holdings have occurred in Italy (Cecchinato et al., 2011). It is additionally experienced that such situations makes disease control exceedingly difficult (Terregino, 2010). Therefore, it may be too dangerous to completely exclude backyard poultry holdings from the active AI surveillance programs. Alternatively, identifying the holdings that act as a bridge between backyard and industrial poultry may prove useful. However, a country specific approach to this matter may be necessary since important differences in poultry farming exist among countries (Gonzales et al., 2010).

2.4 Extrapolating experimental results to field situations

Work with LPNAIVs is tied to strict biosafety measures. Therefore, all experiments conducted for this thesis were carried out in BSL-3 isolators. Whilst our experiments form a scientific background for the design of a risk-based active LPNAI surveillance in the Belgian poultry industry, some remarks concerning the extrapolation of these results to the poultry industry need to be made.

The floor surface of the BSL-3 isolators used in this thesis measures only 1,2m². Therefore, the study populations were rather small, which might pose some difficulties in extrapolating results to commercial poultry farming. Therefore, we assured that the animal densities in our experiments were as close as possible to those generally maintained in commercial poultry holdings. Additionally, by analyzing our data as a frequency-dependent transmission model, the transmission rate β represents the number of new infections caused by one infectious individual, per unit of time (McCallum, 2001). This implies that our β estimates are not affected by flock size and can, theoretically, be extrapolated to larger population sizes. A recent study by Saenz et al. (2012) has concluded that transmission rates estimated according to this model are indeed not influenced by the size of the transmission experiment (Saenz et al., 2012).

For all our experiments, we have made use of specific pathogen-free layer chickens. We initially chose this breed because it is generally believed that these chickens are more susceptible to low infectious pathogens, magnifying transmission and amplifying the effects from altering certain parameters; if a LPNAIV is not transmitted between SPF chickens, it can be assumed that it will certainly not be transmitted among conventional layer chickens. However, in a later stage of the thesis, infection experiments conducted with conventional layer chickens showed no difference in susceptibility between SPF and conventional layer chickens in our experimental conditions (unpublished results). Whilst it remains uncertain if a more natural infection dose or infection route would lead to similar results, it should not be expected that differences in LPNAI susceptibilities between SPF chickens and conventional layer chickens are big.

Throughout the experiments, the BSL-3 isolators maintained an internal negative air pressure of 45±5m³/h. Since general directions to poultry farmers advice an air flow of 0,5-3,6 m³/h.kg (Proefbedrijf voor de veehouderij), it can be assumed that our experimental conditions

possibly led to a faster dehydration of feces, water drops, etc. It has been demonstrated that relative humidity and temperature may influence the transmission of human influenza virus between guinea pigs significantly (Lowen et al., 2007). Therefore, it can be assumed that these ambient parameters might have also influenced our experiments but their magnitude is difficult to estimate since the impact of climatological conditions on the transmission of LPAIV between birds has not been examined.

3 The control of LPNAI outbreaks

Whilst HPAI outbreaks are traditionally dealt with by eliminating infected flocks in combination with a number of additional measurements to prevent spread of the disease (van den Berg and Houdart, 2008), various types of strategies are used to control LPNAI outbreaks. Since 2005, when LPAIVs belonging to the H5 and H7 subtypes have become OIE-listed diseases, multiple LPNAI outbreaks in poultry have been reported. Whilst many of these outbreaks have been controlled by stamping-out, vaccination has been used as a tool to control or eradicate the virus in more complicated outbreaks (Swayne, 2011). Indeed, besides stamping out, the European guidelines for the control of an LPNAI outbreak provide room for an adapted approach, if necessary. The national competent authorities can opt for additional or other control measures, from quarantine to immediate culling and, exceptionally, vaccination if the virus has become endemic.

However, vaccination may allow the virus to circulate unperceived and may also increase the genetic drift of the virus (Webster and Hulse, 2004; Webster et al., 2006). Therefore, the decision to proceed to this type of control measure must be carefully considered. Member states of the European Union must present this decision accompanied by a specific surveillance program to the European commission for approval (Capua and Marangon, 2007; Cecchinato et al., 2010; European Commission, 2000; 2007; 2008).

Whether these alternative control measures will be implicated is based on risk analysis and will depend on the animal species affected, the localization of the farm in a densely populated poultry area (DPPA), the distance to the nearest slaughterhouse and possible transportation methods, the degree of biosecurity on the affected farm(s), the risk of the virus spreading to other holdings, the possibility to treat poultry products, public health concerns and socio-economic concerns (Commission of the European Communities, 2006; Federale overheidsdienst volksgezondheid veiligheid van de voedselketen en leefmilieu, 2008).

3.1 Estimating the potential risk of LPNAI isolates found in poultry

Whereas genotyping LPNAI isolates provides information on the pathogenicity and the phylogenicity, phenotypic analysis by means of infection and/or transmission experiments enables assessment of the transmissibility of the virus. With this information, the seriousness of LPNAI outbreaks can be estimated, enabling authorities to act more efficiently in case of re-emergence of the same virus.

Using the formula:

$$p_{outbreak} = 1 - (\frac{1}{R})^{I_0}$$

, where I_0 is the number of infectious individuals at the beginning of the event, results from a LPNAI transmission experiment can be used to estimate the chance of a LPNAI leading to an outbreak (Dohoo et al., 2009). From our results, it can be concluded that the introduction of one chicken infected with H7N1 A/Ch/Italy/1067/v99 LPAIV (R_0 estimates ranging between 0,76 - 2,32) in a homogenous susceptible flock has a 0,0% - 56,9% chance of resulting in an outbreak. Likewise, the introduction of one individual infected with H5N2 A/Ch/Bel/150VB/99 LPAIV (R_0 estimates ranging between 1,27 - 2,11) in a homogenous susceptible flock would have a 21,2% - 52,6% chance of resulting in an outbreak. It needs to be emphasized, however, that this is under the assumption that the contact structures and contact intensities between infectious and susceptible chickens is equal to the experimental settings. It is likely that under field conditions these contacts will be less intense, animals can be exposed to smaller doses and the temporal exposure length can be much longer. Therefore, the obtained estimates can be seen as worst case estimates rather than average estimates.

From this result, it can be suggested that H5N2 A/Ch/Bel/150VB/99 LPAIV was potentially an even more dangerous virus than H7N1 A/Ch/Italy/1067/v99 LPAIV (chapter 5), although it did not lead to an epidemic, contrarily to the H7N1 in Italy. Arguably, timely detection of H5N2 A/Ch/Bel/150VB/99 LPAIV and differences in contact structures between poultry holdings in Belgium and poultry holdings in Italy might represent some of the main reasons why no large-scale outbreak emerged in Belgium.

Since 2006, two cases of LPNAIV in poultry were detected in Belgium; one mixed poultry holding infected with an H5N2 LPAIV and one breeder geese holding that was infected with an H5 LPAIV of which the NA subtype could not be determined. In both holdings, stamping out in combination with quarantine was applied to control the outbreak as quickly as possible, with successful results (Marché et al., 2013). In recent years, LPNAIVs of the subtypes

H5N1, H5N2, H5N3, H7N7 and H7N1 have been encountered in poultry holdings across Western Europe, Denmark en the United Kingdom on several occasions (World animal health information database, 2013). As in the two Belgian cases, culling of the animals is the most used strategy to control the outbreaks. Culling of the infected holding with limited further measures can be justified if an epidemiological investigation suggests that the virus was introduced from the wild bird reservoir, instead of another infected poultry holding. This is the case for holdings that are found to be epidemiologically isolated, and if a direct link between the infected holding and the wild bird reservoir can be assumed (Koch, 2013a and 2013b).

To the contrary, if epidemiological investigation suggests that spread of the virus between poultry holdings has already occurred, like in this year's H7N7 LPAI outbreak in Germany and the Netherlands (World animal health information database, 2013), dealing with the outbreak becomes more complicated. In these cases, full phenotypic characterization of the virus involved could be useful in establishing control measures and in judging if emergency vaccination programs should be installed.

3.2 Estimating the potential risk of LPNAI isolates found in wild birds

As demonstrated in many studies, the infectivity of LPNAI isolates to bird species may vary widely (appendix 1 and 2). To assess the infectivity of LPAI isolates, Swayne et al. (2008) have developed a methodology in which the 50% mean infectious dose of a strain for a particular bird species is determined. With this method, it was very clearly illustrated that, broadly speaking, WB-LPAIVs have, in contrast to poultry originated LPAIVs, a low degree of infectivity for chickens and other gallinaceous poultry (Swayne and Slemons, 2008).

In this thesis, we also observed a poor infectivity of H5N3 A/Anas Platyrhynchos/Belgium/09-884/2008 for SPF chickens. However, we demonstrated that this virus may, despite its low degree of infectivity, be transmitted from ducks to chickens through close contact or through contact with contaminated drinking water. On the other hand, the lack of transmission between chickens suggests that subsequent circulation of this virus by chicken-chicken transmission is highly unlikely. Therefore, it can be assumed that many WB-LPNAIVs may be introduced to chickens through the above described routes of introduction, whilst it can reasonably be assumed that many of these viruses will afterwards die out spontaneously. The yearly Belgian active LPNAIV surveillance in poultry mostly reveals a number of holdings positive by serology whilst further efforts for isolating the virus itself are often unsuccessful (Animal health and veterinary laboratories agency, 2006; 2007; 2008;

2009; 2010; 2011; 2012). Such cases are not considered outbreaks. Indeed, a positive serology merely indicates past exposure to LPNAIV and does not indicate if infection is still ongoing. If no live virus or vRNA can be found, it can be assumed that infection has spontaneously died out. Hence, the consideration that these cases do not indicate active LPNAI infection appears to be correct. Forcing the issue, it can be assumed that many LPNAI introduction events remain unnoticed, especially since many LPNAIV and non-H5/H7 LPAIV infections do not lead to clinical symptoms in poultry (Liu et al., 2003; Morales Jr. et al., 2009).

Some WB-LPNAIVs however, may behave differently and appear to be highly infectious to chickens (appendix 1 and 2). Therefore, the introduction of such LPNAIVs may lead to sustained transmission in chickens or other poultry species and may more easily give rise to the establishment of new LPNAIVs in poultry. Consequently, it can be assumed that only those wild bird LPNAIVs with an increased infectivity to chickens form a realistic threat to the poultry industry, whilst the less infectious ones are perhaps not as dangerous and need to acquire adaptation before circulation in poultry species can occur. However, since differences in transmissibility between low-infectious and highly-infectious wild bird LPNAIVs has not yet been investigated, this statement should be considered as a stimulus for further research.

These conclusions illustrate the importance of LPNAIV surveillance in wild birds. Knowing the characteristics of these viruses and their prevalences in wild birds enables a better estimation of the dangers that may come forth from an introduction to poultry. Unfortunately, the current tests for detection of anti-AIV antibody responses are mainly developed to be used on poultry sera and often yield unreliable results for wild bird sera (Chapter 6; Charlton et al., 2009; Higgins, 1989; Perez-Ramirez et al., 2010; Spackman et al., 2008; Starick et al., 2006; Sullivan et al., 2009). Since no good alternatives have been developped yet, increased funding of research in this domain will prove particularly useful in establishing wild bird surveillance programs that give reliable results. Likewise, phenotypical characterization of new WB-LPNAIV isolates must be continued to estimate the potential hazard of the LPNAIVs that are circulating in the wild. Additionally, since some highly infectious WB-LPNAIVs are proven to lack the traditional markers of adaptation, it can be assumed that adaptation should be able to be linked to other genetic markers (Marche et al., 2012). It may therefore be advisory to direct further research towards the identification of possible markers of adaptation, so molecular characterization can readily indicate if new wild bird LPNAI isolates should be considered dangerous or not.

3.3 LPNAI replicative characteristics and relevance for control

The characteristic of being able to replicate in two systems (respiratory and intestinal tract) instead of one could suggest increased transmission. In this thesis, we sought to identify if differences in virus shedding routes are important characteristics for estimating the threat posed by LPNAIVs.

In wild birds, LPNAIVs are replicated in large amounts in the intestinal tract and transmission is believed to occur through the oro-fecal route with water acting as an essential fomite (Vandalen et al., 2010). To the contrary, intestinal replication of LPNAIVs is less pronounced in chickens, suggesting that LPNAI transmission occurs differently in this species (Fouchier et al., 2009). The LPNAIVs that were used in this thesis proved to be replicating preferentially in the respiratory tract, whilst intestinal replication was clearly less common. In addition, many other LPNAIV transmission and characterization studies in chickens have witnessed stronger virus replication in the respiratory tract than in the intestinal tract, regardless of the serotype of the LPNAIV (appendix 1 and 2). Whilst in past LPNAI-suspicions, mainly cloacal swabs were taken in an attempt to detect the virus (Marché et al., 2013); our results suggest that circulating LPNAIVs are more likely to be detected in chickens when oropharyngeal swabs are taken.

Our results also suggest that oral is the leading route for LPNAI transmission in chickens and intestinal replication might not be determinative. Therefore, the importance of cloacal virus shedding for the assessment of the risk of LPNAI isolates to become widespread may need to be reconsidered. However more research is needed to endorse this hypothesis.

Furthermore, in chapter 5, we observed a strong intestinal replication for H5N2 A/Ch/Bel/150VB/99, which is highly contradictory to the results that were previously observed for this virus in chapter 3. Similarly, great variations in virus shedding routes have been observed among replicates of the same experiment before (Van der Goot et al., 2003). Therefore, when preferential tropism of a LPNAIV for the respiratory or intestinal tract is supposed in chickens, it is possible that this is largely influenced by biological variation or the presence of supershedders. Arguably, conclusions regarding preferential tropism for the respiratory or the intestinal tract of a particular strain need to be somewhat mitigated, especially if they are based on a limited set of trials. Alternatively, the distribution of LPNAIV replication between the respiratory and intestinal tract may be strain-independent or at least only partially influenced by the strain (Post et al., 2013). Possibly, the susceptibility of the host and the degree to which it is infected by the virus is a more important factor, meaning

that the higher the exposure dose experienced by a susceptible individual, the more intestinal replication of the virus will occur.

4 Recommendations & future perspectives

In this thesis, transmission experiments were used to study the introduction and circulation of LPNAIVs in poultry holdings and to investigate the role of possible risk factors for LPNAIV. Based on the obtained results, we can recommend the following guidelines:

1 Since the type of poultry housing system might have an impact on LPNAIV transmission, it is recommended that this parameter is included in the description of LPNAI outbreaks. This should enable further assessing if this parameter should be included as a risk/protection factor in the risk-based surveillance program.

2 Contact between wild birds and poultry is confirmed to be a major risk factor for LPNAIV. Regarding this risk factor, the type of water supply and more specifically, the possibility that drinking water is contaminated by wild birds, should also be considered in a risk-based LPNAI surveillance program.

3 Based on transmission experiments, mixed poultry farms housing domesticated waterfowl and gallinaceous birds are confirmed to be a major risk factor for LPNAIV and must be considered as 'hot spots' for surveillance activities (both passive and active).

4 Most of the currently commercially available multispecies NP-ELISA kits can be considered reliable for the screening of poultry sera for LPNAIV. However, some of these kits may give unreliable results when used for screening of sera from wild aquatic birds. To improve the reliability of active LPNAI surveillance in wild birds, either the current test protocols of these kits need to be re-evaluated in respect to sera from these animals, or more reliable alternatives must be sought for.

5 Altogether, our results suggest that the intrinsic properties of LPNAIVs to adapt to gallinaceous poultry can be highly variable. Therefore, the need for early detection of these viruses through syndromic surveillance systems becomes apparent. In this context, the preferential use of oral swabs for gallinaceous and cloacal swabs for aquatic birds should be recommended for detection of the virus or vRNA. Furthermore, this observation emphasizes the necessity of further developing the current early warning systems to provide detailed information on these viruses.

In addition, the research results presented in this thesis may stimulate further research efforts. These could focus on the following topics:

1. In this thesis, the obtained results concerning the impact of housing conditions on LPNAIV transmission in poultry houses are not convincing enough to decide if this parameter should be included in a risk-based sampling design. To decide if our hypothesis should be abandoned or not, further research would have to be conducted:
    a. Now that enough knowledge on the transmission of the viruses used in this study is available, similar transmission experiments using different ratios of infectious:susceptibles can be designed. These could theoretically lead to R_0 estimates that are easier to interpret and that would better reveal differences.
    b. Alternatively, an epidemiological investigation on the type of housing systems involved in LPNAI outbreaks could be conducted. This option may be preferable since it would consider all characteristics involved in cage-based and floor-based housing systems instead of just the flooring.
2. In chapter 4, the possibility of the H5N3 LPAI acquiring adaptation to circulation in chickens was not investigated. It would be interesting to consider following research efforts concerning the micro-evolution of this virus:
    a. Analyzing the genome sequences of the virus that is isolated from contact-infected chickens in this experiment: Comparison with the genome sequences of the original strain could possibly point towards ongoing adaptation of the virus to chickens and would also enable an assessment of some early markers of adaptation.
    b. Conducting further transmission experiments between chickens with the virus that was isolated from contact-infected chickens in this study: if an increased transmission, compared to results observed in chapter 3, are observed, this would indicate adaptation to chickens.
    c. Investigate how easy adaptation steps may take place in WB-LPNAIVs that are intrinsically incapable of circulating in chickens. This would allow assessing the threat posed by such low-infectious WB-LPNAIVs, compared to WB-LPNAIVs that are intrinsically more capable of transmission in chickens.
3. Concerning the role of water that is contaminated by wild birds in the introduction of LPNAIVs, following research should be considered:
    a. Investigate which water sources are used and which kinds of water decontamination measures are applied by Belgian poultry holdings.

   b. Investigate if the current requirements for water used in poultry holdings are adequate in preventing the introduction of LPNAIVs in poultry holdings.

4. In this thesis, no important differences were observed between the infectivity of inoculated SPF chickens and contact-infected SPF chickens.
   a. Arguably, the CID_{50} of the H5N2 LPAIV used in this thesis is close to the inoculation dose that we used. If additional extended transmission experiments were conducted with LPNAIVs with a more different CID_{50}, it is possible that a difference in infectivity between inoculated and contact-infected chickens could still be observed.
   b. For the H7N1 LPAIV, a different protocol for obtaining contact-infected chickens could be considered.
5. No clear-cut definition of infection with LPNAIV exists for chickens. Since this has its implications on the interpretation of infection studies and transmission studies, fundamental research concerning the following topics would be interesting:
   a. The possibility of LPNAI infections occurring in chickens without the build-up of an immune response, or with the build-up of a local or cellular immune response in absence of an antibody-mediated immune response.
   b. The possibility of LPNAI infections leading only to an immune response, but not leading to virus shedding.
   c. Investigate to what extent taken vRNA can lead to positive RRT-PCR results without the sampled animal being truly infected.
6. Likewise, some questions regarding respiratory or intestinal tropism of LPNAIVs remain unanswered:
   a. Investigating the role of cloacally shed virus in LPNAI transmission between chickens: Is cloacal virus shedding able to cause transmission in chickens? Especially considering that the environment in which chickens are housed is different from the environments where wild aquatic birds live.
   b. What are the determinants for cloacal virus shedding in chickens? Is this a (partially) virus-related characteristic or mainly determined by host factors? Does the route of infection, the infection dose or the type of exposure (short-term exposure to large infection doses or long-term exposure to small doses) have an impact on the occurrence of cloacal LPNAI shedding in chickens?
7. Investigate if adapting the current test protocols for commercially available multispecies ELISA kits could lead to more reliable results in wild bird

serosurveillance, or investigating alternative assays for LPNAI serosurveillance in wild birds.

5 Appendix

Appendix 1: H5 LPAIV shedding routes in chickens: a summary of H5 LPAIV shedding routes observed in the literature that is cited in this thesis. The percentage of animals that was found positive for oropharyngeal (OP) or cloacal (CL) by either virus isolation or RRT-PCR is shown. Dom.=domestic; WB=wild bird; n.s.=not specified

Subtype	Virus name	Origin	OP	CL	Reference
H5N2	A/Avian/NY/31588/00	Dom.	100%	100%	(Pillai et al., 2010)
H5N2	A/Avian/NY/31588/00	Dom.	100%	100%	(Pillai et al., 2010)
H5N2	A/Chicken/Guatemala/270475/03	Dom.	100%	25%	(Pillai et al., 2008)
H5N2	A/Chicken/Ibaraki/1/05	Dom.	100%	30%	(Okamatsu et al., 2007)
H5N2	A/Chicken/PA/13	Dom.	100%	100%	(Pillai et al., 2010)
H5N2	A/Chicken/PA/13	Dom.	100%	100%	(Pillai et al., 2010)
H5N2	A/Chicken/PA/13609/93	Dom.	70%	29%	(Mundt et al., 2009)
H5N2	A/Chicken/PA/13609/93	Dom.	100%	0%	(Lee et al., 2004)
H5N2	A/Chicken/Pennsylvania/21525/83	Dom.	100%	60%	(Post et al., 2013)
H5N2	A/Chicken/Pennsylvania/83	Dom.	100%	0%	(Van der Goot et al., 2003)
H5N2	A/Chicken/Pennsylvania/83	Dom.	100%	0%	(Van der Goot et al., 2003)
H5N2	A/Chicken/Pennsylvania/83	Dom.	100%	20%	(Van der Goot et al., 2003)
H5N2	A/Chicken/Pennsylvania/83	Dom.	100%	n.s.	(Van der Goot et al., 2003)
H5N2	A/Chicken/Pennsylvania/83	Dom.	0%	0%	(Van der Goot et al., 2003)
H5N2	A/Chicken/Pennsylvania/83	Dom.	60%	0%	(Van der Goot et al., 2003)
H5N2	A/Chicken/Pennsylvania/83	Dom.	100%	0%	(Van der Goot et al., 2003)
H5N2	A/Chicken/Pennsylvania/83	Dom.	40%	0%	(Van der Goot et al., 2003)
H5N2	A/Duck/ME/151895-7A/02	Dom.	92%	75%	(Pillai et al., 2010)
H5N2	A/Duck/ME/151895-7A/02	Dom.	100%	33%	(Pillai et al., 2010)
H5N2	A/Duck/NJ/117228-7/01	Dom.	92%	83%	(Pillai et al., 2010)
H5N2	A/Duck/NJ/117228-7/01	Dom.	67%	66%	(Pillai et al., 2010)
H5N2	A/Duck/NY/185	Dom.	100%	67%	(Pillai et al., 2010)
H5N2	A/Duck/NY/185	Dom.	67%	0%	(Pillai et al., 2010)
H5N2	A/Emu/NY/12716/94	Dom.	100%	67%	(Pillai et al., 2010)
H5N2	A/Emu/NY/12716/94	Dom.	100%	100%	(Pillai et al., 2010)

Appendix 1 (continued)

H5N2	A/Pheasant/MD/4457/98	Dom.	36%	13%	(Pillai et al., 2010)
H5N2	A/Pheasant/MD/4457/98	Dom.	13%	0%	(Pillai et al., 2010)
H5N2	A/Pheasant/NJ/1355/98	Dom.	100%	92%	(Pillai et al., 2010)
H5N2	A/Pheasant/NJ/1355/98	Dom.	100%	100%	(Pillai et al., 2010)
H5N2	A/Turkey/CA/8651-C/04	Dom.	100%	67%	(Pillai et al., 2010)
H5N2	A/Turkey/CA/8651-C/04	Dom.	100%	0%	(Pillai et al., 2010)
H5N2	A/Turkey/CA/D0208651-C/02	Dom.	20%	0%	(Lee et al., 2004)
H5N2	A/Turkey/MN/10734-2/95	Dom.	100%	44%	(Pillai et al., 2010)
H5N2	A/Turkey/MN/10734-2/95	Dom.	100%	66%	(Pillai et al., 2010)
H5N3	A/Chicken/TX/167280-4/02	Dom.	100%	13%	(Pillai et al., 2010)
H5N3	A/Chicken/TX/167280-4/02	Dom.	80%	14%	(Mundt et al., 2009)
H5N3	A/Chicken/TX/167280-4/02	Dom.	100%	0%	(Lee et al., 2004)
H5N3	A/Chicken/TX/167280-4/02	Dom.	25%	0%	(Pillai et al., 2010)
H5N7	A/Turkey/Italy/2369/2009	Dom.	25%	0%	(Gonzales et al., 2012)
H5N1	A/Mallard/Italy/3401/05	WB	33%	0%	(Post et al., 2013)
H5N1	A/Muteswan/MI/451072/06	WB	20%	0%	(Mundt et al., 2009)
H5N1	A/Muteswan/MI/451072-2/06	WB	63%	25%	(Pillai et al., 2010)
H5N1	A/Muteswan/MI/451072-2/06	WB	63%	25%	(Spackman et al., 2007)
H5N1	A/Muteswan/MI/451072-2/06	WB	25%	0%	(Pillai et al., 2010)
H5N1	A/Muteswan/MI/451072-2/06	WB	25%	0%	(Spackman et al., 2007)
H5N2	A/Duck/ME/151895-7A/02	WB	20%	40%	(Lee et al., 2004)
H5N2	A/Goose/Belgium/19432-2/08	WB	33%	25%	(Marché et al., 2013)
H5N2	A/Mallard/MN/182742/98	WB	100%	89%	(Pillai et al., 2010)
H5N2	A/Mallard/MN/182742/98	WB	100%	66%	(Pillai et al., 2010)
H5N2	A/Parrot/CA/406032/04	WB	100%	0%	(Pillai et al., 2010)
H5N2	A/Parrot/CA/406032/04	WB	100%	0%	(Pillai et al., 2010)
H5N2	A/Parrot/California/6032/04	WB	100%	0%	(Pillai et al., 2008)
H5N2	A/Parrot/California/6032/04	WB	100%	25%	(Pillai et al., 2008)
H5N2	A/Parrot/California/6032/04	WB	100%	25%	(Pillai et al., 2008)
H5N3	A/Mallard/MN/479/00	WB	89%	25%	(Pillai et al., 2010)
H5N3	A/Mallard/MN/479/00	WB	67%	0%	(Pillai et al., 2010)
H5N3	A/Mallard/WI/42/75	WB	75%	25%	(Pillai et al., 2010)

Appendix 1 (continued)

H5N3	A/Mallard/WI/42/75	WB	75%	50%	(Pillai et al., 2010)
H5N3	A/RuddyTurnstone/NJ2242/00	WB	100%	92%	(Pillai et al., 2010)
H5N3	A/RuddyTurnstone/NJ2242/00	WB	100%	100%	(Pillai et al., 2010)
H5N5	A/Mallard/MN/3	WB	56%	92%	(Pillai et al., 2010)
H5N5	A/Mallard/MN/3	WB	66%	100%	(Pillai et al., 2010)
H5N7	A/RuddyTurnstone/DE/2046/01	WB	100%	25%	(Pillai et al., 2010)
H5N7	A/RuddyTurnstone/DE/2046/01	WB	100%	66%	(Pillai et al., 2010)
H5N9	A/RuddyTurnstone/DE/85/03	WB	100%	50%	(Pillai et al., 2010)
H5N9	A/RuddyTurnstone/DE/85/03	WB	100%	33%	(Pillai et al., 2010)

Appendix 2: H7 LPAIV shedding routes in chickens: A summary of H7 LPAIV shedding routes observed in the literature that is cited in this thesis. The percentage of animals that was found positive for oropharyngeal (OP) or cloacal (CL) by either virus isolation or RRT-PCR is shown. Dom.=domestic; WB=wild bird

Subtype	Virus name	Origin	OP	CL	Reference
H7N1	A/Chicken/Italy/1067/99	Dom.	100%	67%	(Post et al., 2013)
H7N1	A/turkey/Italy/1067/99	Dom.	100%	80%	(Gonzales et al., 2011)
H7N1	A/turkey/Italy/1067/99	Dom.	60%	60%	(Gonzales et al., 2011)
H7N1	A/turkey/Italy/1067/99	Dom.	100%	100%	(Gonzales et al., 2011)
H7N1	A/turkey/Italy/1067/99	Dom.	100%	80%	(Gonzales et al., 2011)
H7N1	A/turkey/Italy/1067/99	Dom.	100%	100%	(Gonzales et al., 2011)
H7N1	A/turkey/Italy/1067/99	Dom.	100%	60%	(Gonzales et al., 2011)
H7N1	A/turkey/Italy/1067/99	Dom.	100%	80%	(Gonzales et al., 2011)
H7N1	A/turkey/Italy/1067/99	Dom.	100%	100%	(Gonzales et al., 2011)
H7N2	A/Chicken/NJ/118878/5/01	Dom.	100%	40%	(Lee et al., 2004)
H7N2	A/chicken/PA/3779-2/97	Dom.	93%	89%	(Lu et al., 2003)
H7N2	A/chicken/PA/3779-2/97	Dom.	100%	88%	(Lu et al., 2003)
H7N2	A/Turkey/VA/55/02	Dom.	100%	20%	(Lee and Suarez, 2004)
H7N7	A/Chicken/Netherlands/06022003/06	Dom.	83%	83%	(Post et al., 2013)
H7N7	A/Chicken/Netherlands/2006	Dom.	93%	63%	(Gonzales et al., 2012)
H7N1	A/T. tadorna/Belgium/3441-P3/09	WB	100%	83%	(Marche et al., 2012)
H7N2	A/Duck/Kr/A349/09	WB	100%	100%	(Kim et al., 2012)
H7N2	A/Duck/Kr/A349/09	WB	100%	75%	(Kim et al., 2012)
H7N6	A/Duck/Kr/A117/10	WB	25%	0%	(Kim et al., 2012)
H7N6	A/Duck/Kr/A117/10	WB	0%	0%	(Kim et al., 2012)
H7N7	A/B. canadensis/Belgium/13000-9-2/10	WB	67%	25%	(Marche et al., 2012)
H7N7	A/Duck/Kr/A75/10	WB	75%	0%	(Kim et al., 2012)
H7N7	A/Duck/Kr/A75/10	WB	0%	0%	(Kim et al., 2012)
H7N7	A/Duck/Kr/A76/10	WB	25%	0%	(Kim et al., 2012)
H7N7	A/Duck/Kr/A76/10	WB	0%	0%	(Kim et al., 2012)
H7N7	A/Magpie/Kr/07	WB	90%	57%	(Kim et al., 2010)
H7N7	A/Magpie/Kr/07	WB	66%	66%	(Kim et al., 2010)

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Summary

Avian influenza viruses (AIVs) are a diverse group of viruses that are a major global threat to the poultry industry. To date, 16 HA and 9 NA subtypes of avian influenza have been observed in birds. Most of these subtypes cause no or only mild disease in poultry and are therefore classified as low pathogenic avian influenza viruses (LPAIVs). However, as a result of mutations, LPAIVs belonging to the H5 or H7 subtypes (LPNAIVs) may evolve into a highly pathogenic avian influenza virus (HPAIV) when they are circulating in poultry. Since these HPAIVs cause severe sickness and mortality in poultry and are able to spread rapidly, such an outbreak can wreak havoc among poultry holdings in a large region.

In order to prevent the emergence of a HPAIV, it is important that LPNAI outbreaks in poultry are detected as soon as possible and that control measures can be applied before the virus has the opportunity to become widespread. In order to achieve this, active surveillance programs directed towards an early detection of LPNAIV in poultry holdings are established. It is believed that focusing the current active surveillance programs on those sectors of the poultry industry that are more susceptible to a LPNAIV infection, would lead to an earlier detection of LPNAIV. To this end, it is necessary that those factors that determine whether a poultry holding is at increased risk for infection with LPNAIV are identified. In this thesis, transmission experiments were designed to identify and study some putative risk factors for LPNAIV infection in poultry holdings.

In a first series of transmission experiments (**Chapter 3**), the transmission of three LPNAIVs between SPF chickens was studied. A H5N2 LPAIV, which had been isolated from chickens and a H7N1 LPAIV which had also been isolated from chickens, were found to be highly infectious and transmissible to SPF chickens. In contrary, a H5N3 LPAIV that had been isolated from wild ducks proved to be low infectious to SPF chickens and was not transmitted between them.

It was additionally examined whether keeping chickens in cage or barn housing could influence the transmission of LPNAIV. To achieve this, differences in virus transmission were studied between SPF chickens housed on a grid (to simulate housing in enriched cages) and SPF chickens housed on a floor covered with wood shavings (to simulate floor-based housing). The obtained results suggest that the transmission of LPNAIVs may be slightly enhanced by the accumulation of fecal matter as it occurs in floor-based housing systems. However, no large impact was observed. In order to decide whether this factor should be regarded as a risk factor and be included in the active LPNAI surveillance programs, further investigation should be conducted. This could be realized by conducting additional

transmission experiments or by conducting an epidemiological study looking for a relationship between LPNAI outbreaks and the type of housing system.

A second set of transmission experiments (**Chapter 4**) was dedicated to the investigation of the transmission of LPNAIVs between ducks and chickens. In these experiments, the transmission of the duck originated H5N3 LPAIV that was also used in chapter 3 was investigated between pekin ducks and SPF chickens. Despite this virus was previously found to be low infectious to SPF chickens, it was found to be efficiently transmitted from pekin ducks to SPF chickens, suggesting that this virus can be efficiently introduced in chicken farms if contact with wild waterfowl is possible. Additionally, from experiments with a special setting, it was suggested that drinking water that is fecally contaminated by visiting waterfowl may be one of the most important fomites by which LPNAIVs are introduced to poultry holdings. From these results, it can be suggested that the risk of a wild bird originated LPNAIV becoming established in poultry is only realistic if the virus itself has a high infectious potential to poultry. Indeed, our results suggest that, despite the fact that they can be relatively easily introduced, a LPNAIV with a low infectious potential to chickens has a significant risk of dying out spontaneously when it is introduced in an all-chicken population. On the other hand, in mixed poultry farms where contact between domestic waterfowl and gallinaceous poultry is possible, these low infectious LPNAIVs may be of a bigger problem; It is possible that such a LPNAIV can circulate among the holding's waterfowl and can then be repeatedly passed on to the chickens. This would theoretically enable the virus to adapt to chickens, which means that these mixed poultry holdings can act as a bridge for the virus to cross the species barrier from its natural host to gallinaceous poultry.

In a third study (**Chapter 5**); it was evaluated if transmission of the H5N2 and H7N1 LPAIVs used in chapter 3 still occurs when a more natural infection pressure is used. Indeed, it is often assumed that the inoculation of animals, as carried out in standard transmission experiments, may lead to a higher infectivity and thus to an artificially enhanced transmission. Extended transmission experiments in which susceptible SPF chickens were exposed to naturally infected SPF chickens were carried out. For the H5N2 LPAIV, it was observed that the reproduction ratio was similar to the one obtained in chapter 3. This suggests a similar infectivity for naturally infected SPF chickens as for inoculated SPF chickens, at least for this virus. Unfortunately, for the H7N1 LPAIV, no conclusions could be drawn; naturally infected

SPF chickens could not be obtained, most likely due to problems in the design of the experiment.

In a final study (**Chapter 6**), the multispecies NP-ELISA kit that was used throughout the thesis for the determination of infection was compared with other commercially available multispecies NP-ELISA kits. A selection of chicken and duck sera from the transmission experiments (chapter 3 and 4) were analyzed and results were compared. As these kits are also widely used in the active surveillance of LPNAIV in wild waterfowl, some additional field sera from wild geese, swans and ducks were included. The results suggest that the currently available commercial multispecies NP-ELISA kits perform equivalent for the analysis of chicken sera, and that they are most probably more sensitive than the current standard, the HI test. For wild bird sera however, a high degree of inconsistency between the different kits was observed. This indicates that, whilst the currently available commercial multispecies NP-ELISA kits are reliable for the active LPNAI surveillance in chickens, they can give unreliable results for wild bird sera. Hence, either these kits need to be better calibrated for the use in these species, or more reliable alternatives need to be investigated.

Samenvatting

Aviaire Influenza Virussen zijn een diverse groep van virussen die wereldwijd een belangrijke bedreiging vormen voor de pluimvee industrie. Tot op heden zijn 16 HA en 9 NA antigene subtypes van aviaire influenza beschreven bij vogels. De meesten van deze subtypes veroorzaken geen of slechts milde ziektesymptomen bij pluimvee en worden daarom geclassificeerd als laag pathogene aviaire influenza virussen (LPAIVs). Echter, wanneer LPAIVs die behoren tot de H5 of H7 subtypes (LPNAIVs) circuleren in pluimvee, kunnen zij als gevolg van een reeks mutaties ontaarden in een hoog pathogene variant (HPAIV) dat ernstige ziektesymptomen en een hoge mortaliteit veroorzaakt. Zulke HPAIVs kunnen zich snel verspreiden in pluimvee, en kunnen zo een ware economische ravage aanrichten in pluimveehouderijen in een grote regio.

Om dit te voorkomen dienen uitbraken van LPNAIVs zo spoedig mogelijk gedetecteerd en gecontroleerd te worden, vooraleer ze zich op grote schaal kunnen verspreiden in de pluimvee-industrie. Daarom worden actieve bewakingsprogramma's gericht op een snelle detectie van LPNAIVs in pluimveehouderijen geïnstalleerd. Om de effectiviteit van deze bewakingsprogramma's te optimaliseren is het belangrijk dat zij zich concentreren op die sectoren van de pluimvee-industrie die het gevoeligst zijn voor een LPNAIV-infectie. Hiervoor is het nodig dat de factoren die bepalen of een pluimveehouderij een verhoogd risico loopt op besmetting met LPNAIV geïdentificeerd worden. In het kader van deze thesis werden transmissie experimenten opgesteld met als doel het identificeren en bestuderen van enkele vermeende risicofactoren voor LPNAIV infectie in pluimveehouderijen.

In een eerste reeks transmissieproeven (**hoofdstuk 3**), werd de overdracht van drie LPNAIVs tussen SPF kippen bestudeerd. Een H5N2 LPAIV en een H7N1 LPAIV die waren geïsoleerd bij kippen, werden beide hoog infectieus bevonden voor SPF kippen en werden eveneens beide goed overgedragen van geïnoculeerde naar contactgevoelige SPF kippen. Een H5N3 LPAIV dat geïsoleerd werd bij wilde eenden bleek echter laag infectieus voor SPF kippen en werd niet overgedragen tussen geïnoculeerde en contactgevoelige SPF kippen. Bijkomstig werd onderzocht of het houden van kippen volgens een kooihuisvesting of volgens een scharrelhuisvesting een invloed heeft op de transmissie van deze drie LPNAIVs. Hiervoor werd het verschil in virusoverdracht bestudeerd tussen enerzijds SPF kippen gehuisvest op een roosteren vloer (ter nabootsing van huisvesting in verrijkte kooien) en anderzijds SPF kippen gehuisvest op een vloer bedekt met houtkrullen (ter nabootsing van scharrelhuisvesting). De bekomen resultaten suggereren dat de transmissie van LPNAIVs, door accumulatie van fecaal materiaal, mogelijks licht verhoogd is bij scharrelhuisvesting.

Het verschil was echter klein. Daarom, om concreet te kunnen besluiten of dit bij het opstellen van actieve bewakingsprogramma's moet beschouwd worden als risicofactor, zou verder onderzoek moeten worden uitgevoerd. Dit zou kunnen gebeuren via bijkomende transmissieproeven of als een epidemiologisch onderzoek dat een verband zoekt tussen LPNAI uitbraken in pluimvee en het type huisvesting.

In een tweede reeks transmissieproeven (**hoofdstuk 4**) werd dieper ingegaan op de transmissie van LPNAIVs tussen eenden en kippen. In deze proeven werd de transmissie van het, van wilde vogels afkomstige, H5N3 LPAIV van pekin eenden naar SPF kippen onderzocht. Ondanks het feit dat dit virus eerder niet overdraagbaar leek te zijn tussen kippen (hoofdstuk 3), toonden de resultaten van deze interspecies transmissieproeven aan dat dit virus wél goed kan worden overgedragen van pekin eenden naar SPF kippen. In een daaropvolgende speciale proefopstelling werd bijkomstig aangetoond dat fecaal gecontamineerd drinkwater hiervoor een van de belangrijkste vectoren is. Deze resultaten doen veronderstellen dat het risico dat een LPNAIV dat afkomstig is van wilde watervogels zich, na introductie in een pluimveehouderij met galliform pluimvee, ook werkelijk gaat circuleren enkel reëel is indien het virus zelf beschikt over een hoog infectieus potentieel voor kippen. Inderdaad, onze resultaten wijzen er immers op dat een LPNAIV met een laag infectieus potentieel voor kippen relatief makkelijk kan worden binnengebracht in kippenhouderijen indien contact met wilde vogels mogelijk is, maar dat het virus daarna veel kans loopt om spontaan uit te sterven. In gemengde pluimveehouderijen waar contact tussen gedomesticeerde watervogels en galliform pluimvee mogelijk is, vormen deze laag infectieuze LPNAIVs mogelijks wél een probleem. Het is immers mogelijk dat zulk een LPNAIV kan circuleren onder de gedomesticeerde watervogels van het bedrijf en zo herhaaldelijk kan worden doorgegeven aan de kippen. Dit zou er theoretisch gezien voor kunnen zorgen dat het LPNAIV in kwestie zich kan aanpassen aan kippen, waardoor zulke gemengde bedrijven voor het virus kunnen fungeren als 'brug' tussen de natuurlijke wilde gastheer en galliform pluimvee.

In een derde studie (**hoofdstuk 5**) werd geëvalueerd of de H5N2 en H7N1 LPAIVs die in hoofdstuk 3 gebruikt waren in vergelijkbare mate worden overgedragen tussen kippen indien een meer natuurlijke infectiedruk gegenereerd wordt. Inderdaad, het wordt vaak verondersteld dat de inoculatie van proefdieren, zoals gedaan wordt in standaard transmissie experimenten, zorgt voor een hogere infectiviteit van de dieren en zo zorgt voor een artificiële versterking

van de virustransmissie. Verlengde transmissie experimenten waarin contactgevoelige SPF kippen worden blootgesteld aan natuurlijk geïnfecteerde SPF kippen werden daarom uitgevoerd. Voor het H5N2 LPAIV werd vastgesteld dat de reproductie ratio gelijkaardig was aan datgene wat eerder bekomen was, in hoofdstuk 3. Hierdoor kan verondersteld worden dat de infectiviteit van natuurlijk geïnfecteerde SPF kippen gelijkaardig is aan die van geïnoculeerde SPF kippen, althans voor dit virus. Spijtig genoeg konden geen conclusies gemaakt worden voor de verlengde transmissie experimenten met het H7N1 LPAIV; natuurlijk geïnfecteerde SPF kippen konden hier niet worden bekomen, naar alle waarschijnlijkheid door een probleem in het ontwerp van het experiment.

In een laatste studie (**hoofdstuk 6**), werd de multispecies NP-ELISA kit die doorheen de thesis werd gebruikt voor het vaststellen van infectie vergeleken met andere, commercieel verkrijgbare multispecies NP-ELISA kits. Een selectie kippen- en eendensera die bekomen waren uit de transmissieproeven in de hoofdstukken 3 en 4 werden geanalyseerd en de resultaten werden vergeleken. Aangezien deze kits ook veelvuldig gebruikt worden bij de actieve bewaking van LPNAIV in wilde watervogels, werden bijkomstig een aantal sera die bekomen werden van wilde ganzen, zwanen en eenden geanalyseerd. De resultaten toonden aan dat de huidige commercieel verkrijgbare multispecies NP-ELISA kits over het algemeen gelijkaardig presteren wat betreft het analyseren van kippensera, en dat de meesten onder hen waarschijnlijk ook gevoeliger zijn dan de huidige standaard, de HI test. Voor de sera van wilde vogels echter, werd een hoge graad van inconsequentie tussen de verschillende NP-ELISA kits vastgesteld. Dit wijst erop dat de momenteel commercieel verkrijgbare multispecies NP-ELISA kits wel betrouwbaar zijn voor de actieve LPNAI bewaking in kippen, maar dat zij onbetrouwbare resultaten kunnen geven voor de analyse van sera afkomstig van watervogels. Daarom zouden ofwel deze kits beter gekalibreerd moeten worden voor deze diersoorten, of zouden betrouwbaardere alternatieven onderzocht moeten worden.

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Curriculum Vitae

Gerwin Claes werd geboren op 25 maart 1983 te Wilrijk. Na zijn middelbare studies (Wetenschappen-Wiskunde) aan het Sint-Ursula instituut te Onze-Lieve-Vrouw Waver startte hij in 2001 met de studie diergeneeskunde aan de universiteit van Antwerpen en later de universiteit van Gent. Hij behaalde in 2007 met onderscheiding het diploma van Dierenarts. Na enige tijd als praktiserend dierenarts gewerkt te hebben in voornamelijk de rundvee sector besloot hij in het onderzoek te stappen.

Als onderzoeker begon hij in januari 2010 aan het project Flutree, gefinancierd door CO FOD volksgezondheid, veiligheid van de voedselketen en leefmilieu en een joint venture tussen het CODA-CERVA en het KBIN-IRSNB. Na een succesvolle afronding van dit twee jaar durende project, werd een aanvraag voor een nieuw onderzoeksproject ingediend. Dit eveneens twee jaar durende project, Flutrans, werd gefinancierd door BELSPO en kon verder bouwen op de eerder bekomen resultaten. De resultaten van deze onderzoeksprojecten zijn gebundeld in deze doctoraatsthesis. Gerwin Claes is auteur of mede-auteur van meerdere wetenschappelijke publicaties en lichtte zijn onderzoeksresultaten toe op verschillende nationale en internationale congressen.

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Tijdens het werken aan dit doctoraat is er werkelijk een volledig nieuwe wereld voor mij opengegaan. Het onderzoek naar de transmissie van laag pathogene aviaire influenza heeft mij enorm gefascineerd. Echter, met enkel fascinatie kom je er niet. Toen ik begon aan dit doctoraat, wist ik bitter weinig over het uitvoeren van wetenschappelijk onderzoek, het verrichten van labowerk en over het onderwerp zelf. Gelukkig heb ik vier jaar lang steevast kunnen rekenen op de hulp van enkele mensen, die ik hiervoor zou willen bedanken.

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