Influenza A Viruses in Migratory Birds: Ecology, evolution and the wild-domestic interface

Josanne Hinke Verhagen

The research presented in this thesis was carried out at the Department of Viroscience of the Erasmus MC, Rotterdam, the Netherlands within the post-graduate school Molecular Medicine.

The research was financially supported by the Dutch Ministry of Economic Affairs, the EU DG Sanco monitoring program, EU Framework six program NewFluBird (044490), European Research Council project FLUPLAN (250136), NIAID/NIH contracts HHSN266200700010C and HHSN272201400008C, and Horizon 2020 project COMPARE.

Cover design by Josanne Verhagen
Photos by Jörgen Caris (p220), David della Rossa (p14, 98, 238, 266), Michelle Wille (p116), Josanne Verhagen (p6, 36, 62, 150, 160, 214, 248, 276, 280)
Printed by Ipskamp printing

This thesis should be cited as: Verhagen JH (2016) Influenza A Viruses in Migratory Birds: Ecology, evolution and the wild-domestic interface. PhD thesis. Erasmus University Rotterdam, Rotterdam, the Netherlands.

ISBN: 978-94-028-0294-8

Influenza A Viruses in Migratory Birds: Ecology, evolution and the wild-domestic interface

Griepvirussen in trekvogels:
ecologie, evolutie en de koppeling
tussen wilde en gedomesticeerde gastheren

Proefschrift

Ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de
rector magnificus

Prof.dr. H.A.P. Pols

en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op

woensdag 5 oktober 2016 om 9.30 uur

door

Josanne Hinke Verhagen
geboren te Gouderak

Erasmus University Rotterdam

Promotiecommissie

Promotoren	Prof.dr. R.A.M. Fouchier Prof.dr. T. Kuiken
Overige leden	Prof.dr. A.D.M.E. Osterhaus Prof.dr. J. Waldenström Prof.dr. A. Dhondt

Contents

Voorwoord		7
CHAPTER 1	General introduction	15
CHAPTER 2	Influenza in migratory birds	
2.1	Migratory birds reinforce local circulation of avian influenza viruses	37
2.2	Influenza A virus evolution and spatio-temporal dynamics in Eurasian wild birds: A phylogenetic and phylogeographic study of whole-genome sequence data	63
2.3	Epidemiology of influenza A virus among black-headed gulls, the Netherlands, 2006–2010	99
2.4	Long-term effect of serial infections with H13 and H16 low pathogenic avian influenza viruses in black-headed gulls	117
CHAPTER 3	Influenza at the wild-domestic interface	
3.1	Avian influenza A virus in wild birds in highly urbanized areas	151
3.2	Discordant detection of avian influenza virus subtypes in time and space between poultry and wild birds; Towards improvement of surveillance programs	161
3.3	How a virus travels the world: Wild birds may spread the H5N8 virus	215
3.4	Wild bird surveillance around outbreaks of highly pathogenic avian influenza A(H5N8) virus in the Netherlands, 2014, within the context of global flyways	221
CHAPTER 4	Summarizing discussion	239
CHAPTER 5	References	249
CHAPTER 6	Summary (English and Dutch)	267
CHAPTER 7	Author's affiliations	277
CHAPTER 8	About the author	281

Voorwoord

Een koude ochtend in het najaar van 2002 in een loods op een industrieterrein in Groningen. Een slager, de dierenarts van het dolfinarium, pathologen, parasitologen, virologen, een cateraar en duizend zeehonden kadavers komen samen. Het plan, onderzoeken wat de oorzaak is van de enorme sterfte onder de zeehonden in de Waddenzee. Ik was er bij als 3e jaars diergeneeskunde student. Het werd een onvergetelijke week, een geweldige combinatie van heel interessant werk en een groep vol gedreven types. Met werkdagen die om 7 uur begonnen en feestjes die om 3 uur eindigden vormde het een overweldigende kennismaking met de afdeling Viroscience van het Erasmus MC.

Wat een mazzel om werk te mogen doen waarbij je vragen mag stellen, veel mag lezen, kritisch mag zijn, analyseren, schrijven, en dat in een omgeving met veel mogelijkheden en omringd door super intelligente harde werkers, waaronder drie van de grootste influenza onderzoekers in de wereld: Ab, Ron en Thijs.

Ab, jouw buitengewone bevlogenheid, visie, en zorg voor je team bewonder ik diep. Het is ongelofelijk wat jij mogelijk hebt gemaakt, waaronder een bruisende onderzoeksafdeling Viroscience aan het Erasmus MC, een unieke werksfeer en meer dan duizend publicaties op jouw naam. Jouw rol voor de afdeling was om het allemaal draaiende te houden, en mogelijk te maken dat ik, en vele anderen, zich hoofdzakelijk met onderzoek bezig konden houden, en dat heb je met heel veel enthousiasme gedaan.

Ron, wat heb ik een geluk gehad met jou als promotor. Ik kan ik me geen betere promotor wensen. Je staat altijd klaar, teksten zijn snel terug, je hebt altijd weer een of ander geniaal commentaar en bent lekker direct. Ik bewonder je scherpheid, strategische visie en je originaliteit. Het is superfijn met je samenwerken. Dank voor het vertrouwen dat je mij hebt gegeven. Dank ook voor het bijspijkeren van m'n gezegden kennis, ik weet nu wat het is om boter op je hoofd te hebben, of met een vork te schrijven. En voor dat je me af en toe hebt laten zwemmen, dat was soms zwaar en traag, maar ook een uitdaging en nooit saai.

Thijs, jouw enorme enthousiasme voor onderzoek is aanstekelijk, en de aandacht die je hebt voor alle natuur om je heen is voor mij uniek. Dankzij jou ben ik in contact gekomen met Ted in Saskatoon, waar een avontuur als (veld) onderzoeker begon op een van de mooiste plekken die je kan verzinnen. En dan bedoel ik natuurlijk Doré Lake in Canada; om de intensiteit en ontwikkeling van een vogelbroedkolonie van dichtbij mee te maken is zoiets prachtigs. Dank voor dat ik van jou heb mogen leren en voor het bijdragen aan meer structuur in m'n werk.

Dear Ted and Catherine—the founders of my interest in research on infectious diseases in wild animal populations and in particular birds—in the summer of 2007 you gave me the opportunity to work in the prairies and boreal forest of beautiful Saskatchewan, and it was then when I experienced for the first time how incredibly interesting and fun it is to work with wild animal populations. Thank you!

Lieve Oanh, ongelofelijk hoeveel 10.000en vogelmonsters je hebt verwerkt de afgelopen jaren. Jij bent een keiharde werker, gemotiveerd, enthousiast en sociaal. En ik vind het tof hoe we door de jaren heen naar elkaar toe zijn gegroeid en ben trots op waar we nu staan. Ik wens je veel geluk met Richard en jullie kleine Kaj.

Judith, Manon, Martin en Susan, ik wil jullie bedanken voor het (mogelijk maken van het) testen van de vele, vele 'vogelpoepjes'.

Beste Ger, jij bent de essentiële schakel tussen de kooikers, de ganzenflappers, en alle andere vogelvangers en ornithologen, en het lab. Het is geweldig hoe jij je inzet voor de wetenschap, hoe je dat doet met gemak, en altijd een mooi sterk verhaal klaar hebt. Ik kom graag nog eens langs bij jullie aan het water!

Ik weet niet wat het precies is wat het zo ongelofelijk leuk maakt om met vogelvangers en ornithologen samen te werken. Misschien is het dat ze voor hun plezier voor zonsopgang de kou in gaan, dat ze voor hun plezier uren in een—op het eerste gezicht—leeg weiland kunnen staan of zitten, ze er meestal weinig om lijken te geven als ze ondergescheten worden, dat ze vaak omhoog kijken, dat een vogelaar zijn geen bevlieging lijkt te zijn, dat ze een vanzelfsprekend respect voor de natuur hebben, of is het vooral dat ze zo'n scherp oog hebben en veel meer zien dan wat een willekeurig andere persoon zou zien. Ik zag meer door jullie en ben met heel veel plezier met jullie op pad geweest. Een speciaal dank gaat uit naar de eendenkooikers waarvan een aantal al sinds het begin van het wilde vogel vogelgriep surveillance programma in 1998 bijdragen aan het onderzoek: Bert Pellegrom, Hans Zantinge, Teunis de Vaal, Jan Berkouwer, Jan en Lilian Slijkerman, Arie Keijzer en Henk ten Klooster. Ook de ganzenvangers en onderzoekers van Alterra hebben een heel grote bijdrage geleverd aan het onderzoek en heb ik met veel plezier mee samengewerkt, waaronder Engbert van Oort, Dick Jonkers, Kees Polderdijk, David Kleijn en Bart Ebbinge. Gerard Müskens, jou wil ik in het bijzonder bedanken vanwege de zeer prettige samenwerking. Hoe druk je het ook had, je probeerde altijd tijd te maken voor de ganzen, en geregeld was je 's nachts nog aan het werk om de vogel data te mailen.

Frank, ik bewonder je gedrevenheid en plezier voor het werken met de meeuwen en andere vogels. Het was steeds een feest om mee te gaan het veld in. Het water op, of de stad in met je bus gevuld met delicatessen voor de meeuwen. Geweldig vind ik het te zien dat je geen moment onbenut laat om een meeuw te vangen of een ring af te lezen. Op de boot naar Texel dient de lunch als lokvoer en in de haven bij een bord fish & chips zijn de laatste frieten voor de meeuwen. Ik hoop je in de toekomst af en toe te kunnen blijven assisteren in het veld. Ook wil ik je geweldige team van vangers en bemonsteraars bedanken waaronder Leon Kelder, Rob Voesten en Jeroen Nagtegaal. En de andere collega's van Sovon: Roy en Menno. Ik wil jullie bedanken voor de fijne samenwerking en discussies. Heel erg leuk om jullie grootschalige (broed)vogel monitorings programma's in te zetten om vogelgriep gerelateerde vragen te beantwoorden.

En Jan van de Winden en Sjoerd Dirksen, onze gesprekken vond ik heel interessant en ik had graag meer met jullie samen gewerkt, helaas liet de tijd in het laatste deel van m'n promotietraject dat niet meer toe. Désiré Karelse en Fons Mandigers, dank voor jullie initiatief en energie voor het maken van een boek over het ringen en onderzoek in eendenkooien en de mogelijkheid hieraan bij te dragen.

Jacintha! Wat was het leuk om met jou samen te werken, je bent een energieke, enthousiaste harde werker. En leuk om samen te schrijven: jij ('s nachts) in Australie en ik overdag in Nederland, super efficiënt. In het begin spraken we een andere taal, jij met een achtergrond in ecologie en ik met een achtergrond in diergeneeskunde, maar in de loop van de tijd is onze taal zich meer gaan mengen. Ik heb veel van je geleerd en met plezier kijk ik terug! Dank je wel voor de discussies en gezelligheid.

Bethany, to me you have been the perfect PhD student: the super intelligent, independent, hard worker. We didn't overlap that much in time, but with awe I looked at and in your thesis every now and then, hoping that I ever could make something that would look a bit like that.

Een virus uitbraak heeft ook goede kanten. Henk en Bart, de uitbraak van het H5N8 virus in 2014 maakte dat we meer intensief gingen samen werken. En dat vond ik erg leuk en leerzaam. De uitdaging om met verschillende achtergronden tot een standpunt of artikel te komen is prachtig.

Guus en Armin, ik wil jullie hartelijk bedanken voor de interessante discussies, en ook voor jullie geduld, want wat heeft het lang geduurd voordat dat manuscript werd ingestuurd.

En dan uiteraard alle mensen van de afdeling Viroscience, en in het bijzonder m'n kamergenoten. Theo, jij bent een gouden analist en een super collega. Een wandelende encyclopedie en betrokken bij de mensen om je heen (of gewoon rete nieuwsgierig?). En je ontbijt met cola en chips blijft hilarisch. Pascal, bedankt voor het vele werk dat je hebt verzet voor het vogelproject. Ik ben benieuwd naar of je ooit nog gaat promoveren want volgens mij ben je slim en eigenwijs genoeg. Ramona, Bernike, Mathilde, Monique, Björn, Stefan: bedankt voor het dagelijks delen van de lol en frustraties. Martin, Sacha, Bernadette, Kim, kleine Sander, Bri, Sander, Ben, Miranda, Carolien, Gerry, Tiny, Rogier, Eefje, Nella, Rory, Arwen, Stella: bedankt voor de gezelligheid en discussies. Joost en Guus, dank voor het lachen en het delen van jullie inzichten op het gebied van immunologie. Stefan, dank voor het bijspringen in het vogelproject in de winter van de H5N8 uitbraak.

Geert, je bent onmisbaar. Je bent echt heel goed met het hanteren van de meeuwen, het was superfijn en leerzaam met je samen te werken. Angela en Tanja, dank voor jullie goede zorgen en het vele werk dat jullie verricht hebben om het werken met de meeuwen mogelijk te maken.

Vincent, dank voor de overdracht van dit unieke vogelproject waar ik de afgelopen jaren aan heb mogen werken, en dank voor de discussies door de jaren heen.

Marjolein, ik vind het geweldig dat jij het vogelproject voortzet: het is zo'n prachtig netwerk en er liggen nog zoveel vragen om te beantwoorden. Ik vond, en vind, het geweldig om met je samen te werken. Ik wens je veel plezier en geluk! En we gaan gauw weer koffie drinken.

David, dank voor wat je me allemaal geleerd hebt, door jou is statistiek een stuk leuker. En Debby, Lonneke, Lineke, Jurre, Peter, Marco, Judith, Leslie, Byron, Stefanie en Do, bedankt voor de goede tijd en voor wat ik allemaal van jullie heb mogen leren. Marion, bedankt voor het runnen van de afdeling en ik hoop dat onder jouw leiding het vogelproject kan blijven voortbestaan. Robert, dank voor het faciliteren van de afdeling inclusief wekelijkse levering van gebakken bananen met pindasaus. Lieve dames van het secretariaat, Simone, Maria, Loubna, Anouk, Carola en Sabine, dank voor jullie gezelligheid en hulp.

Besides Erasmus MC colleagues and other Dutch colleagues, a whole network of international colleagues helped me improving my work. Nicola, I have a lot of respect for what you initiated and coordinate in Georgia: setting up a wild bird avian flu surveillance network, together with Zura and Ann and many others. Thank you so much for everything. Ursula, thank you for all the care and hard work you put into the gull project and the discussions we have had. Neus, Michelle and Jonas, I have great memories of visiting you in Kalmar and of our discussions through skype or in Rotterdam. One day a wader story will be finished. Camille, Jonathan, Nicola (H) and Dave, it has been very inspiring talking with you at the CEIRS meetings. Diane and Eric, thank you for the nice collaboration within CEIRS.

David della Rossa, Michelle Wille and Jörgen Caris, thank you for allowing me to use your wonderful pictures in this thesis.

Lieve Lilian, fijn dat je altijd in de buurt bent, zowel te lopen of te fietsen als met je hart. Carolien, Bianca, Marja, Wietske en Liset, te gek dat we elkaar nog steeds zien en spreken, dank voor de vele, vele fijne momenten, al 20 jaar! Dhoya, jij bent mijn grote inspirator. Hoe open en spontaan jij in de wereld staat vind ik prachtig, iedere keer weer. Marien, jouw pretogen, bakken energie en spel met taal zijn om nooit genoeg van te krijgen. Lieve Maaike, geweldig om met jou tijd door te brengen, altijd gezellig en superfijn ervaringen uit te wisselen over onderzoek. Rob, you are one of the most craziest, sweetest and funniest persons I know. Hope to see you, Jen & little Nathan, soon! Franco, it’s always great talking with you, as if we saw each other last Tuesday. Lieve Lidewij, dank voor de vragen, inzichten en voor het delen van werkelijk alles. Lieve (oud-) Groningers, Eva, Marc, Anne, Jabik, Marije, wat geweldig dat ik jullie ken, het leven is zo veel mooier met jullie. Lieve Utrechters (proefschrift-is-eindelijk-af-zul-je-bedoelen) en Groesbekers: te vaak 'moest ik nog even wat doen', maar als werk aan de kant stond was het altijd goed bier drinken, sjoelen of kamperen met jullie. And during the last but long bits and pieces of bringing this thesis together, Lina and Knut, thanks for the good times, conversations and support.

Lieve Bert en Ria, Paul en Femke. Dank voor jullie grote betrokkenheid, de open gesprekken, inzichten en voor dat ik me altijd welkom voel bij jullie.

Lieve familie. Dieuwer, Dinja, Melle, Froukje, Paul, Max, Marijn, Jef, Erika, Lotte, Arian en Mees. Dank voor de fijne momenten—met zijn tweeën of met zijn allen—en voor dat jullie allemaal van die prachtige, talentvolle mensen zijn.

Lieve pap en mam, jullie creativiteit en zorgzaamheid zijn onuitputtelijk. Dank voor de vrijheid die jullie mij altijd hebben gegeven. En pap, jouw impulsiviteit gecombineerd met je creativiteit blijft verwonderen en maakt me altijd aan het lachen. Lieve mam, ik bewonder jouw kracht, openheid en inventiviteit in hoe je om gaat met tegenslag. Ik kom altijd thuis bij jullie.

Dank lieve Joost, voor je geduld en liefde. Voor het altijd meedenken, de ruimte die je me geeft, je rust, het lachen, je enthousiasme, je actie, je eindeloze steun.

CHAPTER 1
Josanne H. Verhagen, Ron A.M. Fouchier
Vincent J. Munster

General introduction

In part based on: Ecology of Avian Viruses. In: Studies in Viral Ecology: Animal Host Systems: Volume 2
First edition. Christon J. Hurst, editor. Hoboken, New Jersey. John Wiley & Sons, Inc. (2011), 365-394

Few virus hosts are as mobile as birds. This mobility enables them to access a wide variety of environments and habitats. Birds are found throughout the world and on all continents, including remote places such as the world's oceans, the arctic tundras and Antarctica. One of the most important features of birds as potential vectors for emerging infectious diseases is the seasonal migration performed by many bird species, which potentially allows effective dispersal of pathogens over vast geographical areas and even between continents. The spread of the highly pathogenic avian influenza (HPAI) H5N1 virus from Asia to Europe and Africa and the spread of a virulent form of West Nile virus (WNV) across North America have revealed links between migratory birds and animal and human health. The transmission of these viruses and their geographical spread is dependent on the tight connection between the ecology of the migrating host and the ecology of the pathogen. The ecology of avian viruses within the host is determined by viral characteristics such as host cell receptor use, tissue tropism, replication efficiency and the capacity to evade the host's immune

system; and by host characteristics, such as species, diversity and distribution of virus-specific receptors, host cell transcription and translation machinery and the capacity of the immune system to recognize and fight the viral infection. In addition, the ecology of avian viruses depends largely on the behavior of the host species, such as diet and foraging behavior, habitat use, migratory patterns and behavior, population size and density, group size and frequency of aggregation (1) and on biotic and abiotic factors outside the host affecting viral environmental persistence (2).

The ecology of most avian viruses has been studied to a very limited extent, with the exception of classic poultry diseases, such as Newcastle disease virus and especially avian influenza virus that have been studied extensively in domestic and wild birds. The introductions of HPAI H5N1 virus in wild birds and its subsequent spread throughout Asia, the Middle East, Africa and Europe has put a focus on the role of wild birds in the geographical spread of HPAI H5N1 virus. Large-scale surveillance programs are ongoing to determine a potential role of wild birds in the spread of these H5N1 viruses and genetically closely related H5 viruses, and to serve as sentinel systems for introductions into new geographical regions (3-11). The unprecedented scale and coverage of these surveillance programs has made avian influenza virus the most intensively studied of all wildlife diseases in general.

Influenza A virus

Influenza A viruses are probably best known for their ability to cause pandemics and subsequent annual epidemics in humans, with the 1918 H1N1 Spanish influenza and the 2009 H1N1 swine origin pandemic as prime examples. In addition, outbreaks of HPAI virus, such as the HPAI H5N1 outbreaks, recently gained a high profile in both the scientific community and the general public. Less well known is the fact that influenza A viruses circulating in wild birds are the progenitors, either directly or indirectly, of all pandemic and HPAI viruses. Besides being prevalent in humans, influenza A viruses have been isolated from many other species including pigs, horses, mink, dogs, cats, marine mammals, bats and a wide range of domestic birds (12-14). However, wild migratory birds are the original virus reservoir of most influenza A viruses in nature (Figure 1).

Influenza A virus is an enveloped RNA virus, belonging to the family of *Orthomyxoviridae*. The influenza A virus particle is pleomorphic, with a diameter of approximately 120 nm. The viral envelope is derived from the host cell membrane. Influenza A viruses are classified on the basis of the viral surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), which mediate cell entry and release of virus particles, respectively. In wild birds, influenza A viruses representing 16 distinct

types of HA and 9 of NA have been found, which can be found in numerous combinations (also called subtypes, e.g. H5N1) (16, 17). In addition, influenza A viruses of the subtype H17N10 and H18N11 have been isolated from fruit bats exclusively (13, 14).

The influenza A virus genome consists of eight segments of negative sense, single-stranded RNA. The eight gene segments of influenza A virus encode 11 different proteins (Figure 2) (18). The virus proteins are important for binding and fusion with the host cell, virus transcription, virus replication, intracellular transport, virus assembly and structure, virus release from the host cell and evasion of the host immune response. The segmented nature of the influenza A virus genome enables evolution by a process known as genetic reassortment, that is, the mixing of gene segments from two or more influenza A viruses (12).

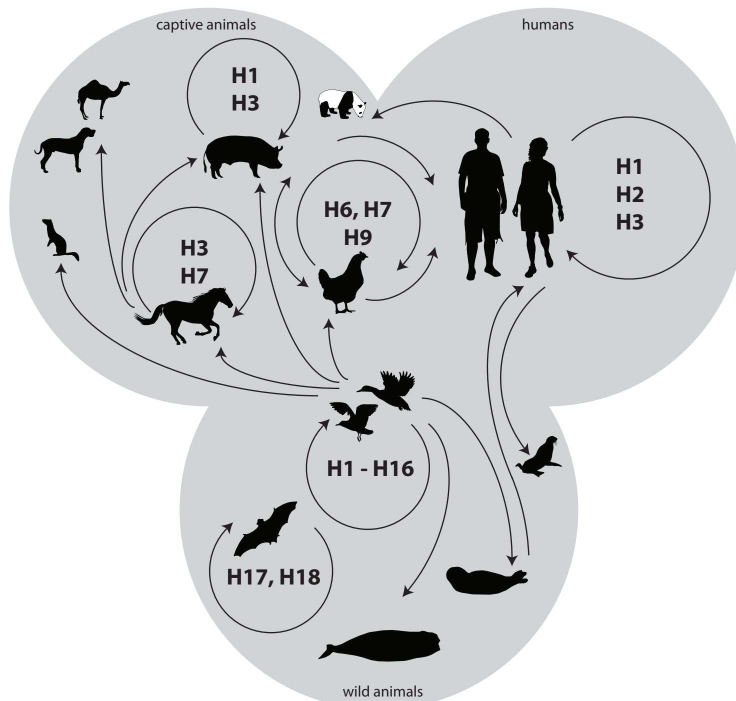


Figure 1. Wild migratory birds are the original virus reservoir of most influenza A viruses in nature. Figure adapted from Short *et al.* 2015 (15), DOI: 10.1016/j.onehlt.2015.03.001 (CC BY 4.0).

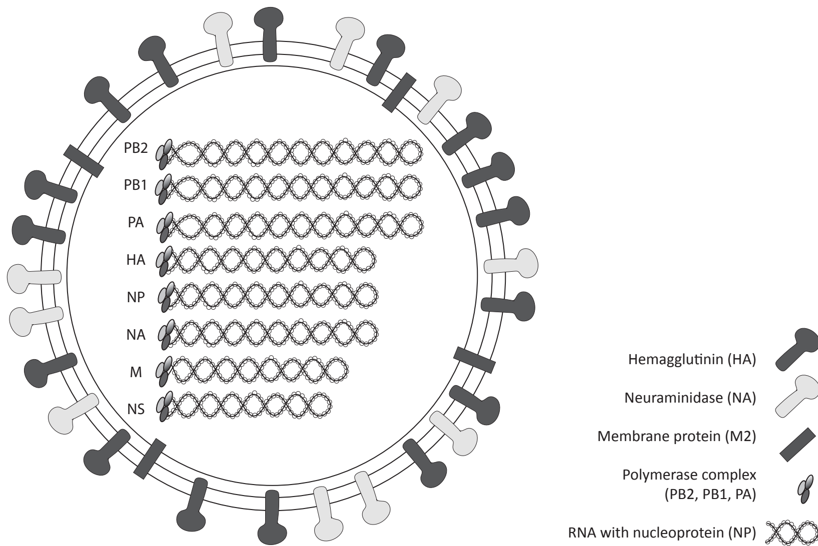


Figure 2. Influenza A virus particle

Pathogenic traits of influenza A viruses vary between different host-pathogen combinations and have shown to be partly determined by the HA protein. The HA protein of influenza A viruses is initially synthesized as a single polypeptide precursor (HA_0) which is cleaved into HA_1 and HA_2 subunits by host cell proteases. Influenza A viruses of subtypes H5 and H7, but not of other HA subtypes, may become highly pathogenic after introduction into poultry and cause HPAI outbreaks. The switch from a low pathogenic avian influenza (LPAI) virus phenotype—commonly circulating in wild birds—to the HPAI virus phenotype is achieved by the introduction of basic amino acid residues into the HA_0 cleavage site, which facilitates systemic virus replication and a mortality of up to 100% in poultry (19, 20). HPAI virus isolates have been obtained primarily from commercially raised poultry.

Avian influenza virus host species

Avian influenza viruses have been detected in at least 105 of ~10,000 free-living bird species (21, 22). Although many wild bird species may occasionally harbor avian influenza viruses, birds of wetlands and aquatic environments such as those belonging to the orders of Anseriformes (mainly ducks, geese and swans) and Charadriiformes (mainly gulls, terns and waders) appear to be central in the maintenance of avian influenza viruses (21). An overview of the prevalence of influenza A virus in wild birds is presented in table 1.

Avian influenza virus in ducks

Dabbling ducks of the *Anas* genus, with mallards (*Anas platyrhynchos*) by large the most extensively studied species (24-32), have been found to be infected with avian influenza viruses more frequently than other duck species, including diving and sea ducks (21, 26, 33). In addition, all avian influenza HA and NA subtypes, with the exception of H13 to H16, circulate in wild ducks and the largest diversity of HA/NA subtype combinations has been detected in ducks (Figure 3).

The virus prevalence in mallards in temperate climates varies in a seasonally predictable way, from low prevalence (<1%) during spring and early summer to high prevalence (up to 30%) during autumn migration and early winter (Figure 4) (12, 25, 26, 34). The peak in prevalence during fall migration is believed to be related to the large numbers of young, immunologically naïve birds of that breeding season that aggregate prior to and during their southbound migration (12).

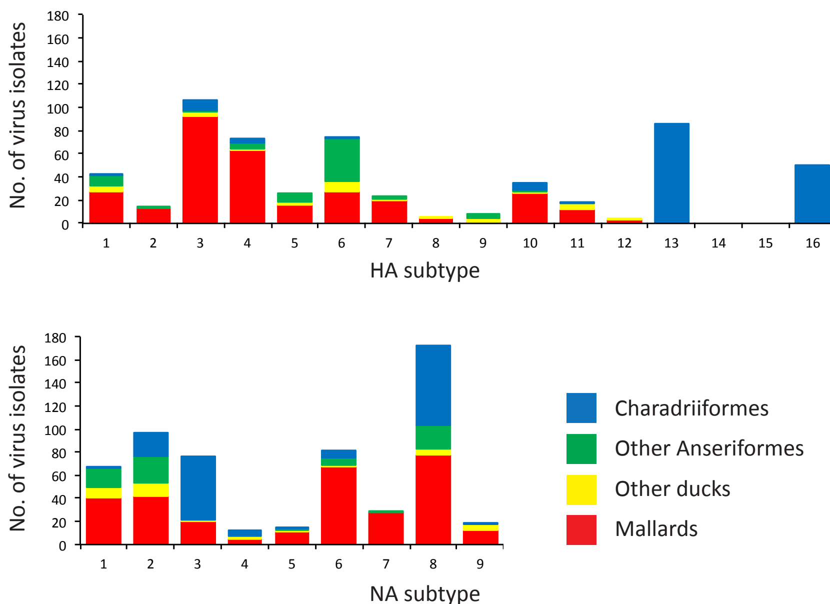


Figure 3. Distribution of hemagglutinin and neuraminidase subtypes in influenza A virus isolates obtained from wild birds sampled in the Netherlands from 1998 to 2011. Original data from Erasmus MC, Rotterdam, the Netherlands. Figure adapted from Van Dijk *et al.* 2013 (23), book chapter in 'Blauwgoed, helen en halven: 100 jaar ringwerk in eendenkooien'. With permission from editors Karelse & Mandigers.

Table 1. Influenza A virus prevalence per bird species sampled in the Netherlands from 1998 to 2011. Original data from Erasmus MC, Rotterdam, the Netherlands. Table modified from Van Dijk et al. 2013 (23), book chapter in 'Blauwgoed, helen en halven: 100 jaar ringwerk in eendenkooien'. With permission from editors Karelse & Mandigers.

Order	Family	Group	Species	No. samples tested	No. samples virus positive	Virus prevalence (%)
Anseriformes	Anatidae	Ducks	10 species	59866	3717	6.2
			Mallard (*Anas platyrhynchos*)	45449	3016	6.6
			Eurasian wigeon (*Anas penelope*)	9405	455	4.8
			Common teal (*Anas crecca*)	1585	107	6.8
			Gadwall (*Anas strepera*)	1143	69	6.0
			Egyptian goose (*Alopochen aegyptiaca*)	938	6	0.6
			Northern shoveler (*Anas clypeata*)	576	36	6.3
			Northern pintail (*Anas acuta*)	543	18	3.3
			Eider duck (*Somateria mollissima*)	99	6	6.1
			Tufted duck (*Aythya fuligula*)	65	2	3.1
			Common shelduck (*Tadorna tadorna*)	63	2	3.2
		Geese	7 species	20707	742	3.6
			Greater white-fronted goose (*Anser albifrons*)	13073	601	4.6
			Barnacle goose (*Branta leucopsis*)	2499	68	2.7
			Greylag goose (*Anser anser*)	2132	21	1.0
			Bean goose (*Anser fabalis*)	1510	35	2.3
			Brent goose (*Branta bernicla*)	1005	8	0.8
			Canadian goose (*Branta canadensis*)	257	3	1.2
			Pink-footed goose (*Anser brachyrhyngus*)	231	6	2.6
		Swans	3 species	2655	25	0.9
			Mute swan (*Cygnus olor*)	2434	4	0.2
			Bewick's swan (*Cygnus bewickii*)	208	20	9.6
			Black swan (*Cygnus atratus*)	13	1	7.7

Table 1 continued

Order	Family	Group	Species	No. samples tested	No. samples virus positive	Virus prevalence (%)
Charadriiformes	Laridae	Gulls	5 species	16168	425	2.6
			Black-headed gull (*Chroicocephalus ridibundus*)	10810	408	3.8
			Common gull (*Larus canus*)	2107	3	0.1
			Lesser black-backed gull (*Larus fuscus*)	1967	1	0.1
			Herring gull (*Larus argentatus*)	1259	12	1.0
			Great black-backed gull (*Larus marinus*)	25	1	4.0
	Scolopacidae	Waders	4 species	2036	26	1.3
			Ruddy turnstone (*Arenaria interpres*)	723	19	2.6
			Dunlin (*Calidris alpina*)	628	4	0.6
			Ruff (*Calidris pugnax*)	609	1	0.2
			Common redshank (*Tringa totanus*)	76	2	2.6
Gruiformes	Rallidae	Rails	2 species	950	2	0.2
			Common coot (*Fulica atra*)	641	1	0.2
			Common moorhen (*Gallinula chloropus*)	309	1	0.3
Passeriformes	Emberizidae	Buntings	1 species	136	1	0.7
			Reed bunting (*Emberiza schoeniclus*)	136	1	0.7
	Muscicapidae	Flycatchers	1 species	19	1	5.3
			European Pied Flycatcher (*Ficedula hypoleuca*)	19	1	5.3
Total				102537	4939	4.8

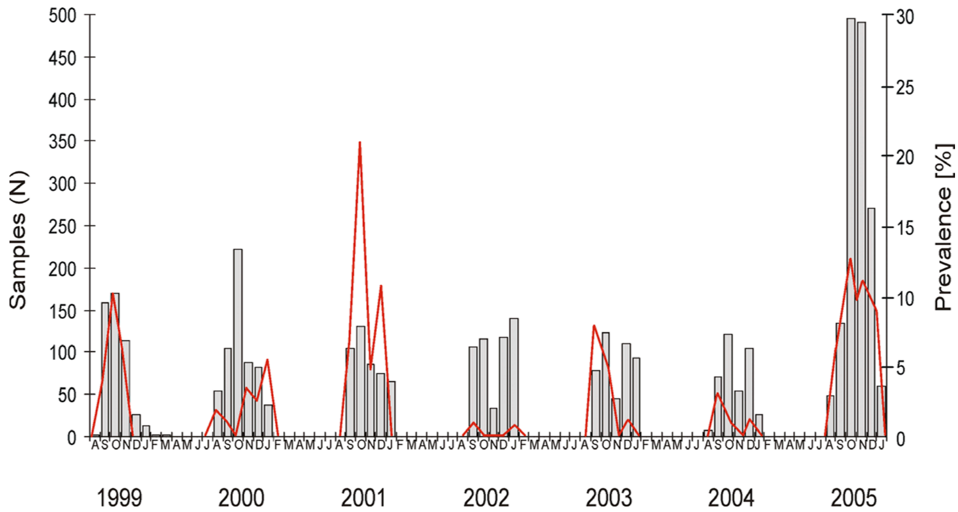


Figure 4. Annual influenza A virus prevalence in mallards during fall migration in the Netherlands from 1999 to 2005. Grey bars indicate sample size (left y-axis) and red line indicates virus prevalence (right y-axis). Figure from Munster *et al.* 2007, DOI: 10.1371/journal.ppat.0030061 (CC BY 4.0).

The differences in virus prevalence between ecological guilds of ducks are likely related in part to their behavior. Dabbling ducks feed mainly on the water surface allowing effective fecal-oral transmission, while diving ducks forage at deeper depths and more often in marine habitats. Dabbling ducks display a propensity for migration and the switching of breeding grounds between years, in part due to mate choice (35). This behavior could provide an opportunity for influenza A viruses to be transmitted between different host subpopulations.

Avian influenza virus in gulls and terns

Birds of the Laridae family, with gull species by large the most intensively studied, have been found to host most frequently avian influenza viruses of the H13 and H16 subtype (16, 26, 36-38). Both HA subtypes are rarely found in other bird species. The spatial and temporal patterns of avian influenza virus infections in gulls have been studied to a limited extent. Influenza viruses can be detected in a small proportion of gulls, with high virus prevalence reported in late summer and early fall in populations in North America, Europe and Russia (16, 39-41). Most gull species breed in dense colonies, potentially creating good opportunities for virus spread. Breeding in dense colonies contrasts with dabbling ducks that do not breed in dense colonies (35), and outbreaks are likely to be more easily initiated when birds congregate in large numbers during molt, migration, or wintering.

Avian influenza virus in other wild bird species

As opposed to the endemicity of avian influenza viruses in dabbling ducks, the avian influenza virus prevalence in other Anseriformes species suggests that avian influenza virus infections behave epidemically in those species (26, 42, 43). In greater white-fronted geese (*Anser albifrons albifrons*) in the Netherlands, the absence of avian influenza virus upon arrival on their wintering grounds is explained by the introduction of the virus after their arrival on their wintering grounds likely through spillover from other reservoir species, such as the ubiquitous mallards (42).

Avian influenza virus prevalence in wader species in the Charadriidae and Scolopacidae families—known for their extremely long-distance migrations—suggests that avian influenza virus infections behave epidemically in those species. Peak prevalence of influenza virus (~14%) in waders (especially ruddy turnstones, *Arenaria interpres*) was observed during mass spring migration in Delaware Bay on the east coast of the USA (27). During spring migration, over a million waders refuel in Delaware Bay on horseshoe crab eggs (*Linudus polyphemus*) to finish migration to their breeding grounds in the Arctic (44). In the Delaware Bay area, a unique combination of ecological factors facilitates efficient influenza A virus circulation and transmission. Surveillance activities performed at other geographical locations, such as Africa, Europe, Alaska and Australia, only identified very limited circulation of influenza A viruses in the respective wader populations (9, 26, 38, 45, 46). Locations comparable to Delaware Bay with respect to virus-host ecology have so far not been identified elsewhere in the world.

Avian influenza viruses have been found in numerous other bird species (21), but it is unclear whether avian influenza virus is endemic in these species or whether the virus is a transient pathogen. Bird species in which avian influenza viruses are endemic share the same habitat at least part of the year with other species in which influenza viruses are frequently detected including geese, swans, rails, quails, petrels, cormorants and, to a lesser extent, passerine species (22, 47). In these and other bird species, influenza A virus prevalence seems to be lower than in dabbling ducks, but studies that sample during the full annual cycle are limited, and it is possible that peak prevalence has been missed because of its seasonal nature or location. In addition, avian influenza virus surveillance has typically shown considerable bias towards species that are easily caught or are present in accessible areas at high concentrations. Therefore, the current status of our knowledge may only partly reflect the true ecology of avian influenza viruses with respect to host reservoir species.

Transmission of avian influenza viruses

The circulation of avian influenza viruses within wild bird host populations relies on the effective transmission of the virus between infected and susceptible hosts and populations. Susceptibility to infection with wild bird origin LPAI viruses of different subtypes may vary between wild bird species (48, 49). Avian influenza A viruses generally infect cells lining the intestinal tract (50-54) and are transmitted via the fecal-oral route in dabbling ducks (1, 12, 29). In contrast to dabbling ducks that feed and defecate on the surface water—thereby allowing effective indirect fecal-oral transmission—geese and certain swan species graze in pastures and agricultural fields (1). A less efficient fecal-oral transmission in geese and swan species may explain the lower influenza A virus prevalence and diversity as observed in these species in influenza A virus surveillance studies. Consequently, transmission via the respiratory route may be relevant for bird species in which fecal-oral transmission would prove difficult, like greater white-fronted geese (42). Thus, differences in diet and foraging behavior could account for the differences in virus prevalence between bird families, species and populations, and may select for viruses that can switch from fecal-oral to respiratory transmission.

The duration of influenza A virus shedding varies by species (55, 56), age (57, 58), prior exposure to influenza virus (59-61) and virus strain (51, 52). For example, the infectious virus excretion lasts for one to two weeks in immunologically naïve hand-raised ducks (52, 59, 61) and for three to eight days in free-living mallards (62). It is unknown if and to which extent LPAI virus excretion is affected by the annual life cycle of the migratory bird. The transient infection in combination with the relatively short shedding time suggests that the spatial dynamics of influenza A viruses are mainly explained by circulation within bird flocks or by relay transmission between staging areas where the birds congregate.

Migratory birds can disperse pathogens, particularly those that do not significantly affect the birds' health status and consequently interfere with migration, either as biological or mechanical carriers (e.g. influenza A virus on feathers due to contaminated waters) (63, 64). Within the large continents and along the major flyways (Figure 5), migration connects many bird populations in time and space, either at common breeding areas, during migration at stopover sites, or at shared non-breeding areas. However, these major flyways are simplifications, and there are numerous exceptions where individuals or populations behave differently from the commons patterns (e.g. frequency and duration of refueling at stopover sites along migration). Stopover and wintering sites may be important for transmission of viruses between wild and captive birds and between different species. It is important to realize that the transmission of

the viruses and their geographical spread is dependent on the ecology of the migrating hosts.

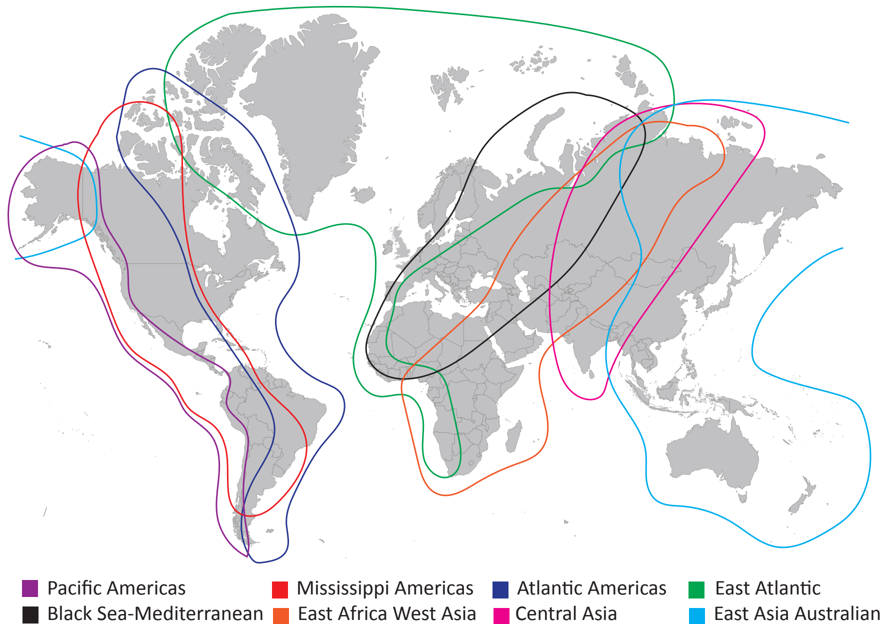


Figure 5. Major flyways of migratory waterbirds

Immunity to avian influenza viruses in birds

Birds have developed several lines of defense to prevent or limit virus invasion and replication. Physical and chemical barriers (skin, tears, mucus, cilia, stomach acid, gut bacteria) form the first line of defense against infection (65). Upon infection, the innate immune response provides immediate defense against infection. Infecting influenza viruses are being recognized by pattern recognition receptors, such as Toll-like receptors (TLRs) and retinoic acid-inducible gene-I (RIG-I)-like receptors (66, 67). The recognition of influenza viruses leads to the secretion of cytokines (like interferon (IFN) type I and type II) and chemokines to attract and activate inflammatory cells and antigen presenting cells. Cytokines upregulate antigen presentation by infected cells. The innate immune response plays a crucial role in the induction and direction of the adaptive immune response, which collectively stop influenza A virus excretion after several days (52, 53, 56, 61).

The adaptive immune response is highly pathogen specific and is able to provide partial protection against reinfection with the same avian influenza virus in ducks (59-

61). The adaptive immune response includes production of influenza A virus-specific antibodies and cell-mediated responses, such as T-lymphocyte-mediated responses (68). Consecutive or simultaneous infections with different subtypes of influenza A viruses are common in dabbling ducks, suggesting that only partial immunity is induced by infection of the birds with an influenza A virus (62). Avian influenza virus-specific antibodies target the virus proteins (e.g. HA, NA, NP) and exist in multiple isotypes (i.e. IgA, IgM and IgY). Antibodies of the IgY isotype are the equivalent to IgG in mammals and the main class of antibodies in blood. Antibodies of the IgA isotype are the main class of antibodies in mucosal excretions (e.g. tears, excretions respiratory tract and gastro-intestinal tract). The dose and location of virus invasion shapes the resultant immune response (69). Despite several studies on LPAI virus immunology in birds (43, 69, 70), little is known about the degree and duration of the protective effect of previous LPAI virus exposure(s).

Most studies in wild birds on the interaction between LPAI viruses and the host immune system are based on the detection of influenza A virus-specific antibodies in blood (e.g. (70)). It has been generally accepted that HA-specific antibodies against a certain LPAI virus strain afford protection against reinfection with the same strain, so called homosubtypic immunity. Also, HA-specific antibodies may have a partial protective effect against reinfection with a different strain, so-called heterosubtypic immunity. The potential protective effect of HA-specific antibodies as detected in blood of wild birds upon LPAI virus infection has been largely based on immunization experiments in chicken and domestic turkey (71). In ducks, LPAI virus-specific antibodies are detected for a limited period of time, due to mechanisms like translocation of IgA (72-74) and truncation of IgY (75). In wild birds, the significance of influenza A virus-specific antibodies, or the protective effect of these antibodies as detected in blood, has been rarely evaluated by means of a LPAI virus challenge infection (52).

Impact of avian influenza virus infection on host ecology

Little is known about the effect of avian influenza A virus infection on the individual wild bird, the wild bird population as a whole and the ecosystems occupied. Extensive avian influenza virus surveillance studies have shown high LPAI virus prevalence in asymptomatic wild birds (42, 62, 76). Upon experimental infection of ducks, avian influenza viruses replicate in the epithelial cells of the intestine of birds and virus may be shed in high concentrations in the feces, without inducing apparent signs of disease (52, 54, 60, 61, 67). However, it is hard to extrapolate these data directly to the situation in free-living wild birds, where mild or subclinical infections may have significant ecological fitness consequences. For instance, late arrival on the breeding grounds has

a negative effect on the reproduction success probably as a result of the occupation of the best breeding sites, the decreased quality of food and a higher pathogen pressure. The virulence of LPAI virus has been assessed in a review on both experimental as well as natural infection studies in wild waterbirds and concluded that the only remaining evidence for virulence was that presence and intensity of LPAI virus infection was negatively associated with body weight (62, 77).

Avian influenza viruses will likely cause less severe infections in wild bird species that are regularly exposed to avian influenza viruses than in wild bird species that are less frequently exposed. Avian influenza virus infections could therefore have a larger behavioral impact on transiently infected species, such as swan species, and limited impact on endemically infected species such as dabbling ducks. Due to the scarceness of studies linking virus ecology to host ecology, it is currently not known how avian influenza virus infections affect the various wild bird species during their annual life cycle and consequently affects the reproduction success and survival of these wild bird species.

Persistence of avian influenza viruses

Large-scale surveillance studies have identified a predominant role for dabbling ducks in the circulation of avian influenza A viruses (12, 27, 32, 78, 79). Factors contributing to this role of dabbling duck populations as influenza A virus host species include population size and structure, migration phenology and mode of transmission. The importance of population size, age structure and herd-immunity on the epidemiology of infectious diseases has been investigated in detail for human pathogens like measles (80). Large populations are probably more capable of sustaining a large variety of different influenza A virus subtypes, as observed in dabbling ducks. The dabbling duck population is estimated to consist of 10 million birds in Europe alone, with the mallard being the most abundant species (~5 million in Europe and ~27 million worldwide) (81). The estimated yearly turnover rate of mallards in Northern Europe is roughly 1/3 (82). A large part of the population is therefore rejuvenated every year, potentially allowing simultaneous co-circulation of multiple genetic lineages and subtypes within one meta-population of potential hosts for influenza A virus. In contrast, the population estimates for the different goose species in Europe are significantly lower compared to the dabbling ducks with a total population size of ~1.2 million geese (81). Smaller population sizes would likely limit the perpetuation and maintenance of multiple influenza A virus subtypes and allow only a limited number of influenza A virus subtypes to co-circulate within these populations. The predominant avian influenza virus detected within geese in The Netherlands over the last decade was of the H6 subtype, with around 60% of all viruses

isolated from geese populations of this subtype (26, 42). The relative abundance of the detection of the H6 subtype within the geese populations does not correlate with the predominant subtypes detected within mallards (42). The global populations of *Laridae* (mainly gulls, terns and skimmers) species appears to be large enough to allow co-circulation of two distinct influenza A virus lineages of H13 and H16 influenza A viruses, although other avian influenza virus subtypes are also occasionally detected in terns and gulls (26, 27, 37).

In addition to population size, the migration phenology of dabbling ducks (like mallards) may allow for continuous circulation of LPAI viruses. For instance, mallard populations in Europe consist of birds breeding in northeast Europe (the Baltic states, Finland, Sweden, north-west Russia) that migrate southwards to Western and Central Europe to winter, and birds that breed in more temperate regions in Western Europe that winter locally or disperse (81). The mixture of both residents and migratory birds within a single species (e.g. mallard) may add to the persistence of LPAI viruses in dabbling ducks, in contrast to species that consist of resident or (long-distance) migratory birds exclusively. Migrants of most bird species in the Americas seldom use the same stopover sites on northward, spring migration as they do on southward, fall migration (83).

Avian influenza viruses can stay infectious for prolonged periods of time in surface water, potentially allowing temporal and spatial connectivity of different host sub-populations by their respective virus populations. Influenza A viruses can survive in cold, wet and dark places for several weeks to months (51, 84-87). Viruses have a limited survival when exposed to high temperatures, high salinity, high pH and ultraviolet light (84, 88, 89). Environmental survival may be important at times when contact rates are low (e.g. during breeding).

The yearly replenishment of the susceptible host pool (82), the ability of reinfection of the host and large host population sizes likely result in a critical community size of the host species large enough to allow endemicity and persistence of the genetically and phenotypically diverse avian influenza viruses. Thus, although it has been speculated that influenza A viruses may persist in abiotic reservoirs such as arctic lakes, the continuous prevalence in dabbling ducks in combination with the abundance of these species (24, 81), may be sufficient for year-round perpetuation of the virus in these species without a need for environmental persistence.

Evolutionary genetics of avian influenza viruses

The segmented nature of the influenza virus genome enables evolution by a process known as genetic reassortment, i.e. the mixing of genes from two or more influenza

viruses (12). Reassortment is one of the driving forces behind the variability of influenza viruses and contributes greatly to the phenotypic variability among these viruses. The three most recent human influenza pandemic viruses and the multitude of viral genotypes associated with the outbreaks of HPAI H5N1 viruses were the result of reassortment of gene segments. Few details of the capacity for reassortment of different lineages of influenza A viruses, the exact rate of reassortment in nature or the effects of reassortment on the virus population are currently known (90). A study of influenza A viruses obtained from ducks in Canada indicates that genetic "sublineages" do not persist, but frequently reassort with other viruses (91). In addition, analysis of the genome constellation (the set of eight gene segments as a whole) of five H4N6 influenza A viruses isolated from mallards at the same day and location revealed four different genome constellations (92). Influenza viruses of a particular subtype do therefore not necessarily have the same genetic make-up, even within a single day, location, or host species. Combined with the continuous co-circulation of several influenza A virus sub- and genotypes in a staging population of hosts, together with the replacement of viruses in the individual hosts, sets a scene where reassortment of co-infecting viruses is very likely to occur at a high rate (90, 92). This indicates that influenza A viruses do not circulate as "fixed" genome constellations but rather that the continuous reassortment leads to "transient" genome constellations.

Avian influenza viruses can be divided into two main phylogenetic lineages: the Eurasian and American lineage (12, 21, 78, 93). The major geographic segregation is observed between viruses isolated from bird species that utilize the migratory flyways of the America's and Eurasia/Africa/Australia, respectively. Apparently, this led to a long-term ecological and geographical separation of these bird populations and hence the viruses circulating within these hosts. This allopatric separation has resulted in a major phylogenetic split between the Eurasian and American genetic lineages of influenza A viruses. Despite this phylogenetic split, the separation of these virus populations is not absolute. The avifauna of North America and Eurasia are not completely separated; some ducks (e.g., Northern pintail, *Anas acuta*) and shorebirds cross the Bering Strait during migration or have breeding ranges that include both the Russian Far East and North-Western America (35). Indeed, influenza viruses carrying a mix of genes from the American and Eurasian lineages have been isolated, indicating that allopatric speciation is only partial and that exchange of gene segments occurs between the two virus populations (79, 94-100). Analyses of H6 avian influenza viruses suggest the introduction of the Eurasian H6 HA gene segment in North America on several occasions (101, 102). However, so far there has been no evidence for cross-hemisphere circulation of entire LPAI virus genomes but only introduction of single gene segments that reassorted

with other segments found in the new hemisphere. The partial geographic isolation of influenza virus hosts seems therefore sufficient to facilitate divergent evolution and continue the existence of separate gene pools.

Besides the influence of geographical separation on the evolutionary genetics of avian influenza A viruses, differences in host species affinity have also resulted in clearly distinguishable virus populations, like H13 and H16 subtypes in gulls and terns (16, 36). Gene segments of gull viruses are genetically distinct from those circulating in other wild birds, suggesting that they have been separated for a sufficient amount of time to allow genetic differentiation by sympatric speciation (16, 99). Gull influenza viruses do not readily infect ducks upon experimental inoculation (36, 49, 54, 103), providing a biological explanation for the limited detection of these viruses in other avian influenza host species, although a limited number of gull viruses has been isolated from ducks and vice versa (26, 27, 37).

LPAI and HPAI viruses in domestic birds

Influenza A viruses may infect virtually all species of domestic birds, depending mostly on their direct contact with wild birds and wild bird excretions or indirect contact via human activities. Exposure to avian influenza viruses is likely to vary by geographic location and surrounding habitat, by poultry farm type and management (e.g. indoor or outdoor, level of biosecurity).

In general, influenza A viruses originating from wild birds cause mild disease in domestic birds, referred to as LPAI. Clinical signs of LPAI virus infection in domestic birds range from no noticeable clinical signs to depression, mild respiratory disease, decreased growth and/or decreased egg production. Most LPAI poultry outbreaks have a limited duration and limited geographical scale, although large-scale and long-term outbreaks have been reported, for instance the outbreaks caused by viruses of the H9N2 subtype in the Eastern Hemisphere (19, 104). Avian influenza viruses are unlikely to be maintained in domestic bird populations housed for commercial purposes according to all-in-all-out procedures, but may be maintained in outdoor facilities that are in contact with wild birds. Studies on the year-round bird distribution and behavior in different habitats near poultry farms may increase the knowledge on potential risk species, but so far these studies are limited in time and space (105-107).

In contrast to most LPAI virus outbreaks, HPAI viruses have a devastating impact on chickens and turkeys, with mortality rates of ~ 100% (19). Since the early 90s of the last century, HPAI outbreaks have occurred and been detected frequently, caused by influenza viruses of subtype H5N1 in Asia, Russia, the Middle East, Europe, and Africa

(ongoing since 1997), H5N2 in Mexico (1994), Italy (1997), Texas (2004), South Africa (2004, 2011) and Taiwan (2012), H7N1 in Italy (1999), H7N3 in Australia (1994), Pakistan (1994), Chile (2002), Canada (2003) and Mexico (2012), H7N4 in Australia (1997), and H7N7 in the Netherlands (2003), North Korea (2005), England (2008), Spain (2009) and Australia (2012) (19, 108, 109). While most HPAI outbreaks have been controlled relatively quickly by preventive measures focused on eradication of the causative agent—such as “stamping out” procedures aiming at infected poultry flocks and pre-emptive culling aiming at preventing the spread of the virus—HPAI H5N1 virus has been circulating in poultry continuously since 1997.

Compared to all other HPAI virus outbreaks, the outbreaks of HPAI H5N1 virus is highly unusual in many regards, such as the spread of HPAI H5N1 virus throughout Asia and into Europe and Africa, the large number of countries affected, the loss of hundreds of millions of poultry (108), the transmission to humans and other mammals, the continuously changing genotypes and the spill-back of the virus into wild birds, leading to outbreaks and circulation of HPAI H5N1 virus in those birds. The ancestral HPAI H5N1 virus likely originated from a virus circulating in domestic geese in the Guangdong province of China in 1996 (A/Goose/Guandong/1/1996) (110). In 1997, the HPAI H5N1 virus was detected in chicken farms and the live bird markets of Hong Kong, and caused the first reported human cases of respiratory disease and fatality attributable directly to avian influenza virus (111). The H5N1 HPAI virus reappeared in 2002 when it caused an outbreak in resident waterfowl and various other bird species in two waterfowl parks in Hong Kong (112, 113). In 2003 the virus resurfaced again, and has devastated the poultry industry in large parts of Southeastern Asia since 2004. Analyses of the large-scale spread of HPAI H5N1 virus indicated that virus introductions were likely related to both human activities (i.e. trade of live poultry or poultry products) and wild bird movements (114-116).

HPAI virus and wild birds

It has been much debated whether wild birds have played—and play—an active role in the geographic spread of the HPAI H5N1 viruses. Some have argued that infected birds would be too severely affected to continue migration and would thus be unlikely to spread the HPAI H5N1 virus (117). However, it has been shown—in experimental settings—that the pathogenesis of the HPAI H5N1 virus infection and the susceptibility of wild bird species to this infection may vary considerably, depending on bird species and previous exposure to viruses of the same or other avian influenza virus subtypes. Recent experimental infections suggest that pre-exposure to LPAI viruses of homologous

or heterologous subtypes may result in partial immunity to HPAI H5N1 virus infection (60). Such pre-existing immunity might protect birds from developing severe disease upon infection but may still allow replication and thus shedding and spreading of the virus. Upon experimental HPAI H5N1 virus infection, some duck species proved to develop minor, if any, disease signs while still excreting the virus, predominantly from the respiratory tract, whereas other species developed a largely fatal infection that would not allow them to spread the virus efficiently over a considerable distance (118-121).

The outcome of HPAI H5N1 virus infections in wild bird species generally ranges from high morbidity and mortality (geese, swan and certain duck species) to minimal morbidity without mortality (ducks of the *Anas* species). In Europe, infected wild birds have been found in several countries that have not reported outbreaks in poultry (122, 123), suggesting that wild birds may have carried the virus to previously unaffected areas. Although swan deaths have been the first indicator for the presence of the HPAI H5N1 virus in several European countries, this does not necessarily imply a role as predominant vectors; they could merely have functioned as sentinel birds infected via other migrating bird species.

Before the unprecedented spread of HPAI H5N1 viruses, there was only one report on the outbreak of an HPAI virus in wild birds, in a colony of common terns (*Sterna hirundo*) in South Africa in 1961 (124) with no direct evidence for association with poultry. In 2002, HPAI H5N1 virus caused an outbreak among resident waterfowl in Hong Kong in which several wild bird species were found infected (112, 113, 125). In 2005, an HPAI H5N1 outbreak in wild migratory birds occurred in April–June at Lake Qinghai, China. This HPAI H5N1 virus outbreak in wild birds affected large numbers of birds such as bar-headed geese (*Anser indicus*), brown-headed gulls (*Larus brunnicephallus*), great black-headed gulls (*Larus ichthyaetus*), and great cormorants (*Phalacrocorax carbo*) (126, 127). After the HPAI H5N1 virus outbreak in wild birds, the virus rapidly spread westwards across Asia, Europe, Middle East and Africa. Affected wild birds have been reported in several countries, predominantly in mute swans (*Cygnus olor*) and whooper swans (*Cygnus cygnus*), although a wide range of other bird species have been infected as well (including coots, grebes, storks, herons, geese, diving ducks, mergansers, gulls, corvids and birds of prey) (21, 122, 128).

Despite intensive surveillance programs in both live and dead birds, HPAI H5N1 virus has predominantly been found in dead wild birds (122, 129). Only in limited cases was HPAI H5N1 virus detected in apparently healthy birds (130, 131). For instance, HPAI H5N2 virus has been isolated from feces of naturally infected spur-winged geese (*Plectropterus gambensis*) in Africa (132). Many national surveillance programs aimed at the early detection of HPAI H5N1 virus have therefore focused on collecting samples

from birds exhibiting morbidity or mortality. The intrinsic problem associated with establishing a clear idea of the prevalence of HPAI H5N1 virus in wild bird populations is the number of birds that have to be caught and sampled for this purpose. The more prevalent a virus is in the respective bird population, the fewer individuals need to be sampled to actually detect the virus. However, the number of birds that would need to be caught and sampled to detect viruses with a very low prevalence with a 95% probability of detection will rapidly become unfeasible, as may currently be the case with the lack of detection of HPAI H5N1 virus in wild bird populations (129, 133) (Figure 6).

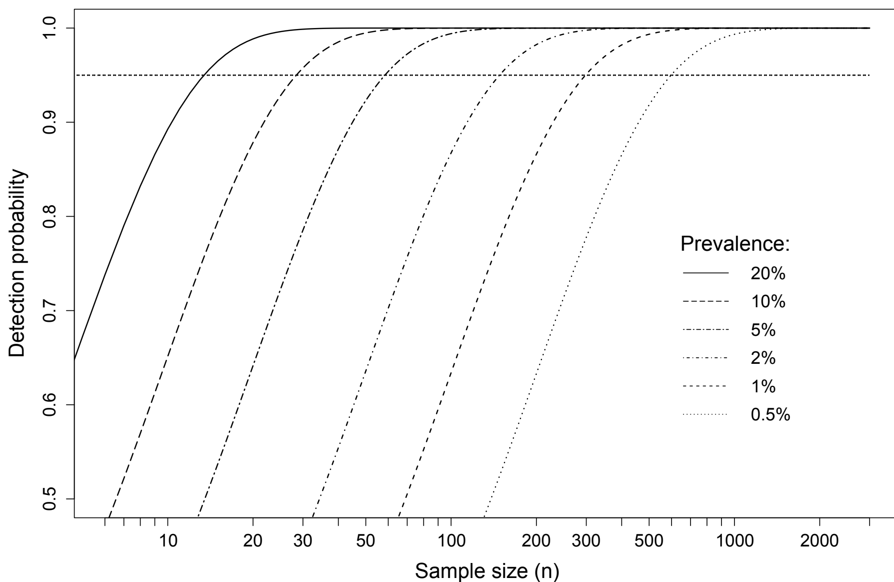


Figure 6. Sample size dependent on expected prevalence. The number of birds within a given population that would need to be sampled to have a 95% chance of detecting influenza A viruses. Figure from Hoye *et al.* 2010 (133), DOI: 10.3201/eid1612.100589 (CC BY 4.0).

This raises the question whether these infections have indeed become endemic in wild bird populations, or whether HPAI H5N1 virus is being re-introduced repeatedly by poultry or human activities. A recent study from China reported high prevalence of HPAI H5N1 virus, suggesting that HPAI H5N1 viruses are endemic in wild birds in China (10). Whether HPAI H5N1 viruses would eventually also cross the Atlantic or the Pacific Oceans to reach the Americas, remained a matter of speculation.

Thesis outline

Wild aquatic birds host a wide range of influenza A viruses that occasionally can be transmitted to other wild animals, domestic animals and humans. The first part of this thesis aims at improving our understanding of the ecology and evolution of avian influenza viruses in wild aquatic birds (chapter 2.1 – 2.4). The second part of this thesis aims at identifying risk factors associated with the wild bird and domestic interface (chapter 3.1 – 3.4).

The first part of the thesis contains studies designed to increase our knowledge on avian influenza virus ecology and evolution. Birds of the order *Anseriformes*, predominantly dabbling ducks like mallard, naturally host a high diversity of LPAI viruses. Wild birds with different migratory strategies are likely to differ in LPAI virus prevalence, however this has rarely been investigated within single species. The role of long-distance migrants, local migrants and residential birds in the introduction and infection dynamics of LPAI virus during a LPAI virus outbreak in mallards was investigated (chapter 2.1). Whereas wild birds can be categorized based on their migratory strategy, avian influenza viruses can be characterized and compared genetically to answer questions with respect to virus movement locally and globally. Based on whole-genome sequences of a wide variety of LPAI viruses isolated from wild birds during 15 years, we investigated the evolutionary, spatial and temporal dynamics of LPAI viruses in Eurasian wild birds (chapter 2.2). In contrast to LPAI viruses as detected in dabbling ducks, LPAI viruses of the H13 and H16 subtypes may have a more restricted host range limited to gulls. To better understand the epidemiology of these two HA subtypes; we choose the black-headed gull (*Chroicocephalus ridibundus*)—naturally infected with H13 and H16 viruses—as a model species for avian influenza viruses (chapter 2.3 and 2.4) to study LPAI virus epidemiology and immunity. For five consecutive years, black-headed gulls were sampled for virus- and antibody detection to describe avian influenza virus dynamics year round and investigate potential drivers of LPAI virus outbreaks in gulls (chapter 2.3). The degree and duration of protection in wild birds from previous LPAI virus infection, by the same or by a different subtype, is poorly understood. Hence, the long-term immune response and protective effect of one- or two re-infections with H13 and H16 LPAI virus—over a period of more than one year—was investigated in black-headed gulls (chapter 2.4).

The second part of the thesis contains studies related to risk assessment and prevention of influenza A virus transmission to domestic birds, and humans. While the vast majority of wild bird surveillance activities globally take place in rural areas, sampling in highly urbanized areas identified wild birds in cities as hosts for LPAI viruses

and thus connected to rural wild bird populations (chapter 3.1). To investigate which wild bird species may be important for the introduction of LPAI viruses into commercial poultry farms in the Netherlands, spatial, temporal and species variations of LPAI virus infection in wild birds were compared with LPAI viruses as detected on poultry farms based on data generated by large-scale surveillance programs (chapter 3.2). In response to the emergence of HPAI H5N8 virus in poultry and wild birds in Europe in winter 2014-2015, wild bird sampling activities in the Netherlands were intensified and resulted in the detection of HPAI H5N8 in feces from a long-distance migratory species, the Eurasian wigeon (*Anas penelope*) (chapter 3.3 and 3.4). Finally, the findings as presented in chapter 2 and 3 are evaluated in the summarizing discussion (chapter 4).

CHAPTER 2.1
Josanne H. Verhagen*, Jacintha G. B. van Dijk*, Oanh Vuong
Theo Bestebroer, Pascal Lexmond, Marcel Klaassen
Ron A. M. Fouchier

Migratory birds reinforce local circulation of avian influenza viruses

*Authors contributed equally to this study
PLoS ONE (2014), Volume 9, Issue 11: e112366. DOI:10.1371/journal.pone.0112366

Migratory and resident hosts have been hypothesized to fulfil distinct roles in infectious disease dynamics. However, the contribution of resident and migratory hosts to wildlife infectious disease epidemiology, including that of low pathogenic avian influenza virus (LPAIV) in wild birds, has largely remained unstudied. During an autumn H3 LPAIV epizootic in free- living mallards (*Anas platyrhynchos*) — a partially migratory species — we identified resident and migratory host populations using stable hydrogen isotope analysis of flight feathers. We investigated the role of migratory and resident hosts separately in the introduction and maintenance of H3 LPAIV during the epizootic. To test this we analysed (i) H3 virus kinship, (ii) temporal patterns in H3 virus prevalence and shedding and (iii) H3-specific antibody prevalence in relation to host migratory strategy. We demonstrate that the H3 LPAIV strain causing the epizootic most likely originated from a single introduction, followed by local clonal expansion. The H3 LPAIV strain was genetically unrelated to H3 LPAIV detected both before and after the epizootic at the study site. During the LPAIV epizootic, migratory

mallards were more often infected with H3 LPAIV than residents. Low titres of H3-specific antibodies were detected in only a few residents and migrants. Our results suggest that in this LPAIV epizootic, a single H3 virus was present in resident mallards prior to arrival of migratory mallards followed by a period of virus amplification, importantly associated with the influx of migratory mallards. Thus migrants are suggested to act as local amplifiers rather than the often suggested role as vectors importing novel strains from afar. Our study exemplifies that a multifaceted interdisciplinary approach offers promising opportunities to elucidate the role of migratory and resident hosts in infectious disease dynamics in wildlife.

INTRODUCTION

Migratory and resident (i.e. sedentary) hosts are thought to fulfil different, non-mutually exclusive, roles in infectious disease dynamics in wild animal populations, although empirical evidence is largely lacking. For one, migratory hosts may transport pathogens to new areas, resulting in the exposure and potential infection of new host species, thereby contributing to the global spread of infectious diseases (134). Resident hosts, immunologically naïve to these novel pathogens, may subsequently act as local amplifiers. For instance, the global spread of West Nile Virus (WNV) is considered to be greatly facilitated by migratory birds introducing the virus to other wildlife and humans in many parts of the world (135). Similarly, the introduction of Ebola virus into humans in the Democratic Republic of Congo, Africa, in 2007 coincided with massive annual fruit bat migration (136).

Additionally, migratory hosts may amplify pathogens upon arrival at a staging site, either because they are immunologically naïve to locally circulating pathogens (137) and/or as a consequence of reduced immunocompetence due to the trade-off between investment in immune defences and long-distance flight (134). Correspondingly, pathogen prevalence or the risk of disease outbreaks may locally be reduced when migratory hosts depart (134). Consistent with the role for migrants, residents in this scenario are suggested to act as reservoirs, permanently maintaining pathogens within their population and transmitting them to other hosts, including migrants (138, 139). Given these potentially distinct roles for migratory and resident hosts in the spatial and temporal spread of infectious diseases, it is important to differentiate between migratory and resident hosts when aiming to improve our understanding of the ecology, epidemiology, and persistence of diseases in wild animal populations.

Wild bird populations are considered the reservoir hosts of low pathogenic avian influenza A viruses (LPAIV). Predominantly birds from wetlands and aquatic

environments (orders *Anseriformes* and *Charadriiformes*) are infected with LPAIV (12), causing transient and mainly intestinal infections (54, 140), with no or limited signs of disease (77). LPAIV can be classified in subtypes based on antigenic and genetic variation of the viral surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). All subtypes that have been recognized to date, notably HA subtypes 1 through 16 (H1-H16) and NA subtypes 1 through 9 (N1-N9), have been found in wild birds (21). Recently, novel influenza viruses were identified in fruit bats that are distantly related to LPAIV (H17N10, H18N11), indicating that bats, alongside wild birds, harbour influenza viruses and might play a distinct role in the dynamics of this infectious disease (13, 14).

Despite a large number of studies on the ecology and epidemiology of LPAIV in wild birds, only few studies have focussed on the role of resident and migratory hosts in the dynamics of this infectious disease. Resident bird species likely facilitate LPAIV transmission, while migratory bird species harbour high LPAIV subtype diversity after arrival at the wintering grounds (141, 142). In most of these studies resident and migratory hosts belonged to different bird species, with presum- ably different LPAIV susceptibility. However, many bird species are composed of a mixture of resident and migratory individuals, so called partial migrants (143). Individuals that belong to the same species but use distinct migratory strategies, may differ in morphology and behaviour (e.g. body size, dominance; (144)), immune status and pathogen exposure. As a consequence, resident and migratory individuals of a single species may respond differentially to LPAIV infection and hence their contribution to local, and consequently global, LPAIV infection dynamics may differ. Hill et al. investigated the role of migratory and resident hosts of a single bird species in LPAIV infection dynamics. In their study, no differences were detected in LPAIV prevalence between migratory and resident host populations (145). However, migrants likely introduced LPAIV subtypes from their breeding areas to the wintering grounds and residents likely acted as LPAIV reservoirs facilitating year-round circulation of limited subtypes (145). A similar study in the same species conducted at a local scale instead of a macro-ecological scale, showed that susceptible migratory hosts were more frequently infected with LPAIV than residents, which had probably driven the epizootic in autumn (146). LPAIV epizootics in wild birds are likely to take place at local spatial and temporal scales, since LPAIV infections are generally short (i.e. up to a week; (62)), and most virus particles are shed within the first few days after infection (147). Yet, the precise role of migratory and resident hosts during local LPAIV epizootics in terms of virus introduction and reinforcement, including host immunity, has remained largely unstudied.

We build on the study of van Dijk et al. (146) to investigate the role of migratory and resident hosts of a single bird species during a local LPAIV epizootic. Throughout

an H3 LPAIV epizootic at the wintering grounds in autumn 2010, we sampled a partly migratory bird species, the mallard (*Anas platyrhynchos*), and connected host migratory strategy with (i) H3 virus kinship, (ii) H3 virus prevalence and shedding, and (iii) H3-specific antibody prevalence. H3 LPAIV is a dominant subtype in wild ducks in the northern hemisphere (26, 27). This study provides a detailed description of a monophyletic H3 LPAIV epizootic importantly associated with the influx of migratory mallards.

MATERIALS AND METHODS

Ethics statement

Capturing free-living mallards was approved by the Dutch Ministry of Economic Affairs based on the Flora and Fauna Act (permit number FF/75A/2009/067 and FF/75A/2010/011). Handling and sampling of free-living mallards was approved by the Animal Experiment Committee of the Erasmus MC (permit number 122-09-20 and 122-10-20) and the Royal Netherlands Academy of Arts and Sciences (KNAW) (permit number CL10.02). Free-living mallards were released into the wild after sampling. All efforts were made to minimize animal suffering throughout the studies.

Study species and site

Mallards are considered a key LPAIV host species, together with other dabbling duck species of the *Anas* genus, harbouring almost all LPAIV subtype combinations found in birds to date (21). Mallards are partially migratory, meaning that the population exists of both migratory and resident birds. Along the East Atlantic Flyway, mallards breeding in Scandinavia, the Baltic, and northwest Russia migrate to winter at more southern latitudes in autumn, congregating with the resident populations that breed in Western Europe, including the Netherlands (81).

During the 2010 LPAIV epizootic described here, free-living mallards were caught in swim-in traps of a duck decoy (148). The duck decoy was located near Oud Alblas (51u529380N, 4u439260E), situated in the province of Zuid-Holland in the Netherlands. This sampling site is part of the ongoing national wild bird avian influenza virus (AIV) surveillance program (dd 2014-09-20), executed by the department of Viroscience of Erasmus MC, where mallards, free-living and hunted in the near surrounding, were sampled for LPAIV from 2005 onwards.

Sampling

During the LPAIV epizootic (i.e. from August until December 2010) studied here, the duck decoy was visited, on average, seven times per month capturing approximately 11 birds per visit. Each captured mallard was marked using a metal ring with an unique code, aged (juvenile: <1 year, adult:>1 year) and sexed based on plumage characteristics (149). For virus detection, cloacal and oropharyngeal samples were collected using sterile cotton swabs as LPAIV may replicate in both the intestinal and respiratory tract of wild birds (150). Swabs were stored individually in virus transport medium (Hank's balanced salt solution with supplements (151)) at 4° C, and transported to the laboratory for analysis within seven days of collection. For detection of antibodies to AIV, blood samples (<1 ml, 2% of the circulating blood volume) were collected from the brachial vein, which were allowed to clot for approximately 6 h before centrifugation to separate serum from red blood cells (152). Serum samples were stored at -20° C until analysis. To determine a bird's migratory strategy using stable hydrogen isotope analysis, the tip (1–2 cm) of the first primary feather of the right wing was collected and stored in a sealed bag at room temperature. Of recaptured birds, both swabs and a blood sample were collected.

Migratory strategy

In the study of van Dijk et al. (146), the origin (and hence, migratory strategy) of mallards sampled during the 2010 LPAIV epizootic was determined using stable hydrogen isotope analysis in feathers. Stable isotope signatures in feathers reflect those of local food webs (153). During the period of growth (i.e. moult), local precipitation is incorporated into these feathers (154), causing the stable hydrogen isotope (δ^2H) ratio in feathers to be correlated with δ^2H of local precipitation (155). Across Europe, a gradient of δ^2H in feathers is found in mallards (156). Based on feather δ^2H and additional criteria, van Dijk et al. (146) classified mallards as resident, local migrant (i.e. short distance) and distant migrant (i.e. long distance). A resident bird had grown its feathers near the duck decoy (was captured during moult) and was recaptured multiple times either before or during the LPAIV epizootic. A local and distant migratory bird was seen and sampled once, i.e. only during the LPAIV epizootic and was not captured within one year before this epizootic. Based on feather δ^2H values of local (-103.5 to -72.6‰) and distant migrants (-164.5 to -103.7‰) and using a European feather δ^2H isoscape of mallards (156), local migrants originated roughly from central Europe and distant migrants roughly from north-eastern Europe. We used similar criteria to assess the migratory strategy of mallards caught during the H3 LPAIV epizootic. For 149 individual birds in this study we

were unable to assign them to either the resident or migratory population and these were excluded from analyses, except the genetic analysis.

For full details on the stable hydrogen isotope analysis, see van Dijk et al. (156). In short, feathers were cleaned and air-dried overnight. Feather samples were placed into silver capsules, stored in 96 well trays and shipped to the Colorado Plateau Stable Isotope Laboratory (Northern Arizona University, Flagstaff, USA). Stable hydrogen isotope analyses were performed on a Delta Plus XL isotope ratio mass spectrometer equipped with a 1400 C TC/ EA pyrolysis furnace. Feather δ^2H values are reported in units per mil (‰) relative to the Vienna Standard Mean Ocean Water- Standard Light Antarctic Precipitation (VSMOW-SLAP) standard scale.

Virus detection, isolation and characterization

As part of the national wild bird AIV surveillance program — including the 2010 LPAIV epizootic — LPAIV infection of free-living and hunted mallards was assessed using cloacal and oropharyngeal swab samples. RNA from these samples was isolated using the MagnaPure LC system with a MagnaPure LC total nucleic acid isolation kit (Roche Diagnostics, Almere, the Netherlands) and analysed using a real-time reverse transcriptase-PCR (RT-PCR) assay targeting the matrix gene. Matrix RT-PCR positive samples were used for the detection of H5 and H7 influenza A viruses using HA specific RT-PCR tests (151, 157). All matrix positive samples were used for virus isolation in embryonated chicken eggs and characterized as described previously (28).

Matrix RT-PCR positive samples collected during the 2010 LPAIV epizootic for which virus culture was not successful, were screened for the presence of H3 influenza A viruses using a H3 specific RT-PCR test (n = 126). Additionally, matrix RT-PCR positive samples collected half year prior to the LPAIV epizootic (November 2009-July 2010) were screened for the presence of H3 influenza A viruses to determine whether H3 LPAIV was detected in mallards prior to the epizootic (n = 20). Amplification and detection were performed on an ABI 7500 machine with the taqman Fast Virus 1 Step Master mix reagents (Applied Biosystems, Nieuwerkerk aan den IJssel, the Netherlands) and 5 μl of eluate in an end volume of 30 μl using 10 pmol oligonucleotides RF3226 (5’-GAACAACCGGTTCCAGATCAA-3’) and 40 pmol RF3227 (5’-TGGCAGGCCCACATAATGA-3’) and 10 pmol of the double-dye labelled probe RF3228 (5’-FAM-TCCTRTGGATTTCCTTTGCCATATCATGC-BHQ-3’). Primers and probe were designed with the software package Primer Express version 3.01 (Applied Biosystems, Nieuwerkerk aan den IJssel, the Netherlands), based on avian H3 nucleotide sequences obtained from GenBank (158).

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

Sequence analysis and phylogeny

To investigate H3 LPAIV diversity in time and space among resident and migratory mallards during the LPAIV epizootic, we performed a genetic analysis focussed on the HA segment, one of the two most variable gene segments of LPAIV. Nucleotide sequences of the HA gene segment were obtained from virus isolates that were previously characterized by hemagglutination inhibition (HI) assay as H3 LPAIV. The RT-PCR and sequencing of the HA segment was performed using HA specific primers (5'-GGATCTGCTGCTTGTCCTGT-3' and 5'-GRATAAGCATCTATTGGAC-3'), as described previously (159).

A total of 86 HA gene segments of 1576 nt in length were included in the genetic analysis. The genetic analysis comprised H3 nucleotide sequences obtained from (i) residents and migratory mallards during the 2010 LPAIV epizootic (n = 23), (ii) additional H3 LPAIV isolates from the national wild bird surveillance program of Erasmus MC (n = 35), and (iii) a BLAST analysis using public databases available as of 29 November 2013 (158, 160), from which only European virus sequences with a known isolation date were retrieved (n = 28). Duplicate and incomplete sequences were removed. Nucleotide sequences were aligned using the software MAFFT version 7 (161).

H3 nucleotide sequences were labelled based on sampling site, year of virus isolation, and host migratory strategy (i.e. resident, local migrant, distant migrant). During the 2010 LPAIV epizootic, H3 nucleotide sequences were obtained from 23 viruses, isolated from residents (n = 3), from local migrants (n = 13), from distant migrants (n = 2), and from birds of which the migratory strategy could not be assessed (n = 5). This was supplemented with 12 H3 nucleotide sequences obtained from viruses isolated from mallards sampled in the duck decoy in different years, notably in 2008 (n = 11) and 2011 (n = 1). There were 31 H3 nucleotide sequences from virus samples collected at other sampling locations in the Netherlands and elsewhere in Europe between 1999 and 2011. Of these virus samples, 18 originated from locations within the province of Zuid-Holland (5–30 km from the duck decoy), i.e. from Berkenwoude (n = 13) (51°57'00''N, 4°41'36''E), Lekkerkerk (n = 2) (51°53'41''N, 4°39'24''E), Oudeland van Strijen (n = 2) (51°46'56''N, 4°30'56''E) and Vlist (n = 1) (51°59'13''N, 4°45'56''E). Eleven viruses were isolated from birds in coastal regions in the Netherlands (i.e. 115–200 km from the duck decoy), i.e. Schiermonnikoog (n = 1) (53°28'41''N, 6°9'24''E), Vlieland (n =

1) (53°16’42’’N, 5°1’22’’E), Westerland (n = 8) (52°53’39’’N, 4°56’32’’E) and Wieringen (n = 1) (52°54’00’’, 4°58’11’’E). Outside the Netherlands, two H3 sequences were from viruses isolated in Hungary in 2009. The remaining 20 H3 nucleotide sequences originated from multiple locations throughout Europe (i.e. Belgium, Czech Republic, Germany, Iceland, Italy and Switzerland) and Russia.

A Maximum Likelihood (ML) phylogenetic tree was generated using the PhyML package version 3.1 using the GTR+I+G model of nucleotide substitution, performing a full heuristic search and subtree pruning and regrafting (SPR) searches. The best-fit model of nucleotide substitution was determined with jModelTest (162). Tree was visualized using the Figtree version 1.4.0 (163). Overall rates of evolutionary change (i.e. number of nucleotide substitutions per site per year) and time of circulation to the most recent common ancestor (TMRCA) in years was estimated using the BEAST program version 1.8.0 (164). To accommodate variation in the molecular evolutionary rate among lineages, the uncorrelated log-normal relaxed molecular clock was used. Isolation dates were used to calibrate the molecular clock. Three independent Bayesian Markov Chain Monte Carlo (MCMC) analyses were performed for 50 million states, with sampling every 2,000 states. Convergence and effective sample sizes of the estimate were checked with Tracer version 1.6 (165). Uncertainty in parameter estimates was reported as the 95% highest posterior density (HPD) (166). Nucleotide sequences are online available under the accession numbers as listed in Table S1 and S2.

Serology

To assess whether mallards had H3-specific antibodies during the 2010 LPAIV epizootic, all sera were first tested for the presence of AIV antibodies specific for the nucleoprotein (NP) using a multispecies blocking enzyme-linked immunosorbent assay (bELISA MultiS-Screen Avian Influenza Virus Antibody Test Kit; IDEXX Laboratories, Hoofddorp, the Netherlands), following manufacturer’s instructions. Each plate contained two positive and two negative controls. Samples were tested in duplicate. An infinite M200 plate reader (Tecan Group Ltd, Männedorf, Switzerland) was used to measure the absorbance (i.e. OD-value) at 620 nm. Samples were considered positive for the presence of NP antibodies when signal-to-noise ratios (i.e. mean OD-value of the sample divided by the mean OD-value of the negative control) were <0.5. NP antibody positive serum samples were subsequently tested for the presence of H3-specific antibodies using the HI assay according to standard procedures (167). Briefly, sera were pretreated overnight at 37°C with receptor destroying enzyme (*Vibrio cholerae* neuraminidase) and incubated at 56°C for 1 h. Two-fold serial dilutions of the antisera, starting at a 1:10 dilution, were

mixed with 4 hemagglutinating units of A/Mallard/Netherlands/ 10/2010 (H3N8) in 25 ml and were incubated at 37°C for 30 min. Subsequently, 25 ml 1% turkey erythrocytes was added and the mixture was incubated at 4°C for 1 h. Hemagglutination inhibition patterns were read and the HI titre was expressed as the reciprocal value of the highest dilution of the serum that completely inhibited agglutination of turkey erythrocytes.

Statistics

Birds were considered LPAIV positive when either cloacal or oropharyngeal swabs were positive. To exclude samples of birds that had been sampled twice within the same infectious period during the 2010 LPAIV epizootic, we used an interval of at least 30 days between the day that a bird tested LPAIV positive and the next sampling day. Mallards may shed virus up to 18 days (147).

During the LPAIV epizootic, 709 cloacal and oropharyngeal swabs were collected from 472 mallards of which 129 individuals were recaptured. Of these swabs, 84 tested positive for H3 LPAIV, 35 tested LPAIV positive but H3 negative (i.e. matrix-positive H3- negative), and 583 swabs tested LPAIV negative. Of 7 matrix- positive swabs we were unable to determine H3-positivity. To test H3 virus prevalence and shedding, we included H3-positive and H3-negative swabs (i.e. matrix-negative and matrix-positive). Swabs from birds of which the migratory strategy could not be assessed (n = 269) or with undefined age and sex (n = 13) were excluded. The exclusion of birds of which the migratory strategy could not be assessed did not affect the temporal pattern of H3 LPAIV prevalence. In total we included 420 cloacal and oropharyngeal swabs from 305 individual birds, of which 55 birds were sampled more than once (Table S3).

During the LPAIV epizootic, 428 serum samples were collected from 364 mallards of which 52 individuals were recaptured. Of these serum samples, 9 tested positive for H3-specific antibodies, 98 tested positive for AIV antibodies but negative for H3-specific antibodies (i.e. NP-positive H3-negative), and 321 sera tested negative for AIV antibodies. To investigate H3-specific antibody prevalence, we included H3-specific antibody positive and H3- specific antibody negative sera (i.e. NP-negative and NP-positive). Sera from birds of which the migratory strategy could not be assessed (n = 96) or with undefined age and sex (n = 5) were excluded. Thus in total we included 320 sera samples from 281 individual birds, of which 30 birds were sampled more than once (Table S3).

A generalized linear mixed model (GLMM) was used in the analysis of H3 virus prevalence, with migratory strategy (i.e. resident, local migrant, distant migrant), age, sex and month as fixed factors, all two-way interactions with migratory strategy, and individual bird as random factor. The interactions between migratory strategy and age,

migratory strategy and sex, and migratory strategy and month were tested to assess whether H3 virus prevalence differed per age class, sex and month for the three categories of migratory strategy. The fixed factors age and sex were merely included in the models to conduct the interactions. A general linear model (GLM) was used to test for differences in prevalence of H3-specific antibodies, with migratory strategy and month as fixed factors. Linear models (LMs) were used to determine differences in the degree of virus shedding of H3 LPAIV-particles based on viral RNA from the cloaca and the oropharynx (i.e. C_T value) with migratory strategy and month as fixed factors. A Tukey's post hoc test was performed to detect differences in H3 LPAIV prevalence between the three categories of migratory strategy and months. All analyses were conducted using R 2.14.1 (168). Package lme4 was used to fit the GLMM (169) and multcomp to perform a Tukey's post hoc test (170).

RESULTS

Virus prevalence

Each year, from 2005 until 2011, LPAIV prevalence in mallards peaked between the end of summer (August) and the beginning of winter (December), with some exceptions in March 2009 and June 2011 (Figure 1A). Detection of the various HA subtypes varied per year, with most virus isolates found in autumn, notably H2 to H8, H10, and H12. H3 LPAIV was isolated from mallards every year, except in 2007 and 2009, and was the dominant HA subtype in 2006, 2008 and 2010 (Figure 1B).

During the 2010 LPAIV epizootic, mallards were infected with H3 LPAIV (84 of 709, 12%) and with other LPAIV subtypes, namely H4, H6 and H10 (35 of 709, 5%; Figure 1B). The H3 LPAIV epizootic started on the 12th of August 2010 (Figure 2A) and H3 virus prevalence differed between months (Table 1). H3 virus prevalence increased in September, peaked in October, and decreased in November and December (Figure 2A and 2C). Shortly before the 2010 LPAIV epizootic, a single mallard of unknown origin was infected with H3 LPAIV on the 10th of February 2010, followed by a period of five months where no H3 infections were detected among 536 mallards sampled.

Local and distant migrants were more often infected with H3 LPAIV (37 of 113, 33% and 22 of 98, 22% respectively) than residents (20 of 209, 10%; Figure 2C, Table 1). The peak month of the H3 LPAIV epizootic differed between the three mallard populations (Table 1): in local migrants H3 LPAIV infection peaked in September, whereas in residents and distant migrants infection peaked in October (Figure 2C). At the start of the H3 LPAIV epizootic (12th of August), three residents and one local migrant were infected with H3 LPAIV, with their populations constituting respectively 88% and 12% of the sampled

mallard population. Two weeks later (26th of August), the first distant migrant infected with H3 LPAIV was detected (44% of the sampled mallard population). In September and October, most mallards infected with H3 LPAIV were local migrants (respectively 12 of 22 and 15 of 35 total H3 LPAIV positives), while local migrants comprised respectively 24% and 40% of the sampled mallard population. In October, 11 residents and nine distant migrants were infected with H3 LPAIV, the latter constituting only 17% of the sampled mallard population. In November, only nine local and five distant migrants were infected with H3 LPAIV (comprising respectively 29% and 25% of the sampled mallard population). The last month of the H3 LPAIV epizootic, only one distant migrant and two residents were infected with H3 LPAIV, although distant migrants and residents constituted respectively 43% and 32% of the sampled mallard population.

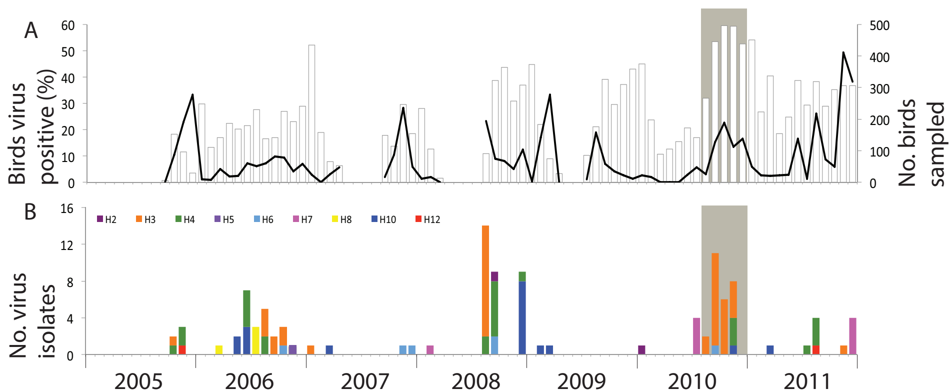


Figure 1. Prevalence and subtype diversity of low pathogenic avian influenza viruses (LPAIV) in mallards sampled at Oud Alblas, the Netherlands, 2005–2011. The grey-shaded area indicates the H3 LPAIV epizootic from August until December 2010. (A) Number of free-living and hunted birds sampled (bars, right Y-axis) and percentage of birds tested virus positive based on M RT-PCR (line, left Y-axis). (B) Number of virus isolates per HA subtype: H2 (purple), H3 (orange), H4 (green), H5 (light purple), H6 (light blue), H7 (pink), H8 (yellow), H10 (dark blue) and H12 (red).

Virus shedding

H3 virus shedding from the cloaca and oropharynx did not differ between the three mallard populations ($F_{2,10}$ = 1.051, P = 0.385 and $F_{2,63}$ = 0.025, P = 0.976, respectively). Nor were there any differences in the monthly amount of H3 virus shed from the cloaca and oropharynx during the H3 LPAIV epizootic ($F_{3,10}$ = 1.945, P = 0.186 and $F_{4,63}$ = 1.124, P = 0.353, respectively).

Table 1. Linear model test results of the analysis of H3 low pathogenic avian influenza virus (LPAIV) prevalence during the LPAIV epizootic in 2010. Besides migratory strategy, age, sex, month and two-way interactions were included. Significant values ($P < 0.05$) are shown in bold.

	H3 virus prevalence	
Variable	X^2	p-value
Age	0.144	0.705
Sex	0.659	0.417
Month	44.928	**<0.001**
Migratory strategy	23.681	**<0.001**
Migratory strategy * Age	0.777	0.678
Migratory strategy * Sex	0.558	0.757
Migratory strategy * Month	21.510	**0.006**

Antibody prevalence

During the 2010 LPAIV epizootic, NP-specific LPAIV antibody prevalence increased from September onwards to 60% in December (Figure S1). During the H3 LPAIV epizootic, the proportion of local and distant migrants with H3-specific antibodies (3 of 106, 3% and 4 of 96, 4% respectively) was similar to that in residents (2 of 118, 2%; X^2 = 0.543, P = 0.762; Figure 3). There were no differences in H3-specific antibodies between months (X^2 = 6.996, P = 0.136). During the H3 LPAIV epizootic, H3-specific antibodies were detected on four sampling dates. On the 5th of August, before the start of the H3 LPAIV epizootic, one distant migrant had H3-specific antibodies (while distant migrants constituted 14% of the sampled mallard population). During the H3 LPAIV epizootic, the first resident with H3-specific antibodies was sampled on the 21st of September, with 9% of the sampled mallard population comprised of residents. After the peak of the H3 LPAIV epizootic (1st of November), two local migrants, one distant migrant and one resident had antibodies specific for H3 LPAIV. That day, local migrants constituted the largest proportion of the sampled mallard population (71%). At the end of the epizootic (21st of December), only migrants (local migrant: 1, distant migrant: 2) had specific antibodies against H3 LPAIV (constituting 38% and 44% of the sampled mallard population, respectively).

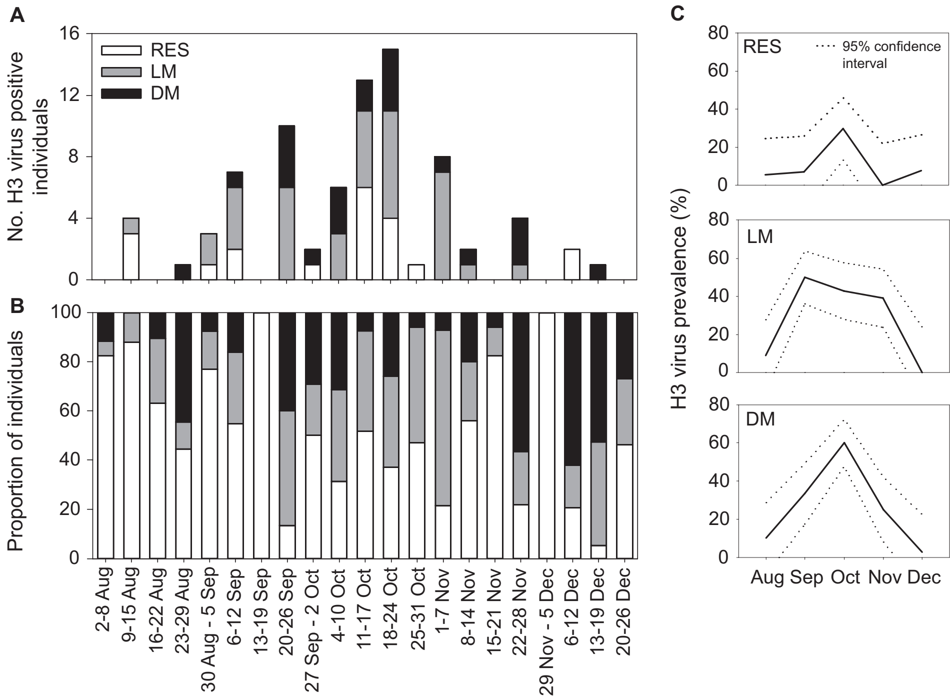


Figure 2. Prevalence of H3 low pathogenic avian influenza viruses (LPAIV) in residents, local and distant migratory mallards during the H3 LPAIV epizootic in 2010. For residents (RES), local migrants (LM) and distant migrants (DM) the (A) number of H3 virus positive individuals per week, (B) proportion of individuals sampled per week, and (C) H3 virus prevalence (±95% CI) per month are depicted.

Virus kinship

The HA gene sequences of the H3 LPAIV strains isolated from free-living mallards during the H3 LPAIV epizootic were monophyletic, suggesting the outbreak resulted from a single virus introduction. Although migratory mallards kept arriving at the study site during the H3 LPAIV epizootic, the genetic analysis indicates that no other H3 LPAIVs were introduced. The estimated time to the most recent common ancestor of the H3 LPAIV strains of the epizootic was spring 2009 (TMRCA 12 May 2009, LHPD95% 1 July 2008, UHPD95% 18 November 2009). The H3 LPAIV strain detected in a single mallard at our study site prior to the H3 LPAIV epizootic (10th of February 2010) differed from the H3 LPAIV strains of the epizootic (HA could only be sequenced partially and is not shown

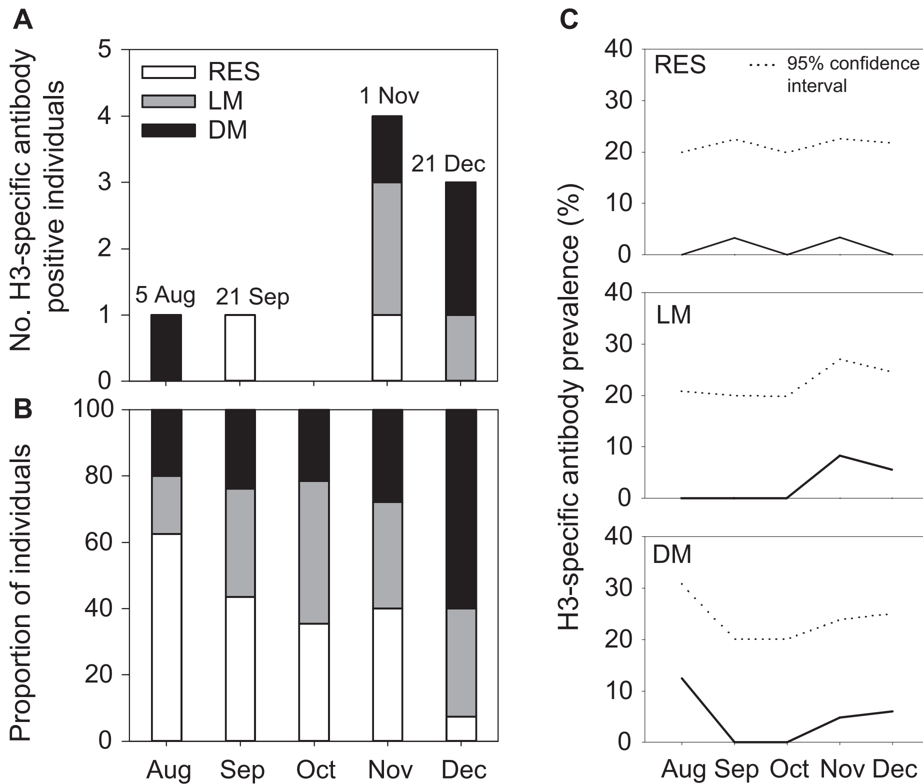


Figure 3. Prevalence of avian influenza H3-specific antibodies in residents, local and distant migratory mallards during the H3 low pathogenic avian influenza virus (LPAIV) epizootic in 2010. For residents (RES), local migrants (LM) and distant migrants (DM) the (A) number of H3-specific antibody positive individuals, (B) proportion of individuals sampled, and (C) H3-specific antibody prevalence (±95% CI) per month are depicted.

in the tree), and was therefore unlikely to have seeded the outbreak. Furthermore, the H3 LPAIV strains isolated during the H3 LPAIV epizootic were not closely related to isolates obtained from mallards at our study site in autumn 2008 (sequence identity 0.958–0.967), or November 2011 (sequence identity 0.954–0.957; Figure 4). However, the H3 LPAIV strains isolated from the H3 LPAIV epizootic were genetically closely related to H3 isolates from mallards at two sampling sites 8 to 12 km away from the study site one year later, in autumn 2011 (i.e. locations Berkenwoude and Vlist; Figure 4).

H3 LPAIV strains isolated from the resident, local and distant migratory population belonged to the same cluster with little variation in nucleotide sequences (sequence identity 0.995–1; detail of Figure 4). No consistent substitutions were detected in the nucleotide sequences that correlated with the migratory strategy of birds. Evolutionary divergence of the HA of H3 LPAIV was 2.5^{e-3} nucleotide substitutions per site per year, which is lower than reported by Hill et al. (18): 1.38 $(\pm 0.40)^{e-2}$.

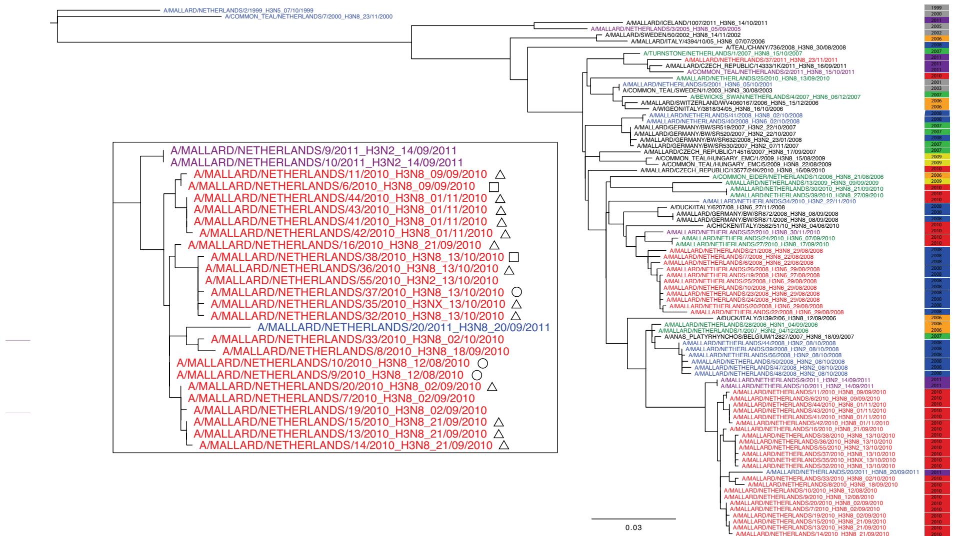


Figure 4. Phylogenetic analysis of HA gene of H3 low pathogenic avian influenza virus (LPAIV) isolated during the H3 LPAIV epizootic in 2010. The Maximum Likelihood (ML) tree contains samples of wild birds collected at various locations in and outside the Netherlands from 1999 until 2011. Each sampling location within the Netherlands is grouped by colour: Oud Alblas (red); Berkenwoude (blue); Lekkerkerk aan de IJssel, Oudeland van Strijen and Vlist (purple); Schiermonnikoog, Vlieland, Westerland and Wieringen (green). Locations are closely situated to the study site (i.e. duck decoy near Oud Alblas), except the locations shown in green, which are located at the coast. Year of virus isolation is listed next to isolate and grouped by colour. Detail of ML tree contains samples of the H3 LPAIV epizootic described in this study and migratory strategy of mallards: residents (RES; circle), local migrants (LM; triangle) and distant migrants (DM; square).

DISCUSSION

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

H3 LPAIV isolations from residents, local and distant migrants belonged to the same genetic cluster (Figure 4). However, we cannot fully exclude the possibility that

novel introductions of H3 LPAIV, or other LPAIV HA subtypes, by migratory birds occurred that were subsequently outcompeted by the dominant epizootic H3 LPAIV strain and thus remained undetected during our monitoring (i.e. competitive exclusion principle; (171)). For instance, another H3 LPAIV epizootic in the area (i.e. Berkenwoude in 2008) resulted from multiple virus introductions. The H3 LPAIV that induced the 2010 epizootic was closely related to H3 LPAIV strains isolated in the near surrounding one year after the epizootic (i.e. Berkenwoude and Vlist in 2011). This suggests that after the epizootic H3 LPAIV may have overwin- tered and had been maintained locally. H3 virus prevalence in migratory mallards was higher (especially in distant migrants) and more prolonged (especially in local migrants) than in resident individuals. This finding corresponds with the results of van Dijk et al. (146) who found a three-fold increase in overall (i.e. non LPAIV-subtype specific) virus prevalence in migratory mallards. However, during the peak of the H3 LPAIV epizootic many residents were also infected with H3 LPAIV, which may be a consequence of the local amplification and increased viral deposition in the environment (i.e. water and sediment) at the study site. The local amplification may thus be a self-reinforcing process.

At the start of the H3 LPAIV epizootic, almost exclusively resident birds were infected with H3 LPAIV. However, it is not surprising that the majority of H3 LPAIV infections were found in residents, since the sampled mallard population consisted mainly out of resident birds (88%). What is remarkable though is that one week after detection of the first H3 LPAIV infections, no migrants were infected while a large proportion of the sampled mallard population consisted of migrants (~40%). Either migratory birds were not, or to a lesser extent, susceptible to H3 LPAIV infection, or contact rates and the amount of H3 virus particles in the surface water were still too low to infect arriving migrants. Interestingly, the peak of virus infection in October in the resident population was mainly induced by recaptured resident birds (i.e. captured multiple times) (Figure S2). H3 virus prevalence in primary residents (i.e. captured for the first time) remained relatively low and increased in December. Potentially recaptured residents were trap-prone and had a higher probability of being exposed—and consequently becoming infected—than primary residents. In addition, in October the population of recaptured residents sampled was three-times higher than the population of primary residents sampled, increasing the probability of virus detection in recaptured residents.

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

Hypothetically, this individual could have been infected with H3 LPAIV when transiting through southern Sweden (i.e. feather hydrogen stable isotope -129.2‰ suggest it originated from southern Scandinavia, Baltic States or Russia; (156)), introducing this virus to the wintering grounds. H3 LPAIV is detected frequently in mallards sampled in southern Sweden in early autumn (172). Although our genetic analysis does not support this theory, it should be noted that only few H3 LPAIV originating from Sweden or other northern European countries were available and were included in the genetic analysis.

Several local and distant migrants had H3-specific antibodies after the peak of the H3 LPAIV epizootic. Since these birds were captured once during the H3 LPAIV epizootic, we cannot exclude that an H3 LPAIV infection outside the study site triggered this antibody response (i.e. genetically different H3 LPAIV were isolated at other locations in the Netherlands). Resident mallards with H3-specific antibodies most likely have been infected by the H3 LPAIV of the epizootic. Only 20% (1 of 5) of residents that had been infected with H3 LPAIV during the epizootic had H3- specific antibodies when recaptured (i.e. recaptured within 31 days since longevity of detectable HA specific antibodies is short; (173)). As result of H3 LPAIV infection an H3 specific antibody titre may have been generated, yet not detected due to antibody dynamics and timing of sampling, and/or sensitivity of the HI assay.

In conclusion, by combining virology, serology and phylogeny analyses with stable isotopes we demonstrate that a local H3 LPAIV epizootic in mallards was likely induced by a single virus introduction into susceptible residents, followed by a period of local virus amplification that was associated with the influx of migratory mallards. In addition to the study of Hill et al. (145), who showed long-distance movement of LPAIV genes by migrating mallards on a macro-ecological scale, we showed an association between local amplification of H3 LPAIV and the arrival of migratory mallards at the wintering grounds at a much smaller ecological scale. We suggest an additional role for migrating mallards as local amplifiers, based on the difference in H3 LPAIV prevalence between resident and migratory mallards upon arrival at the wintering grounds. This study exemplifies the difficulty of elucidating the role of migratory and resident hosts in infectious disease dynamics in wildlife, but provides encouraging indications that the here presented multifaceted approach may open a window on these processes.

ACKNOWLEDGEMENTS

We thank Teunis de Vaal for catching mallards in the duck decoy at Oud Alblas and assisting with sampling the birds. Peter de Vries, Audrey van Mastrigt and Lennart Zwart are also thanked for their help in the field. We thank Ger van der Water, Judith Guldemeester and Kim Westgeest for logistical and technical assistance, and Richard

Doucett and Melanie Caron of the Colorado Plateau Stable Isotope Laboratory for performing the stable hydrogen isotope analysis. The sequences of the H3 LPAIV used in this study are available from GenBank (158) and GISAID EpiFlu Database (160) and listed in Table S1 and S2. This is publication 5677 of the NIOO-KNAW. We thank David Stallknecht and an anonymous reviewer for comments on earlier versions of this paper.

SUPPORTING INFORMATION

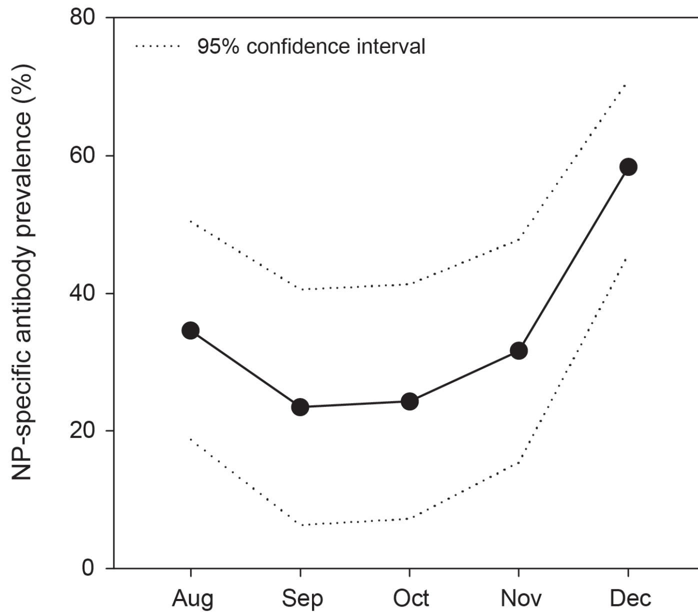


Figure S1. Prevalence of avian influenza-specific anti-bodies in free-living mallards during H3 epizootic. This figure shows prevalence of avian influenza virus nucleoprotein (NP)-specific antibodies in mallards (*Anas platyrhynchos*) during the H3 low pathogenic avian influenza virus epizootic in 2010.

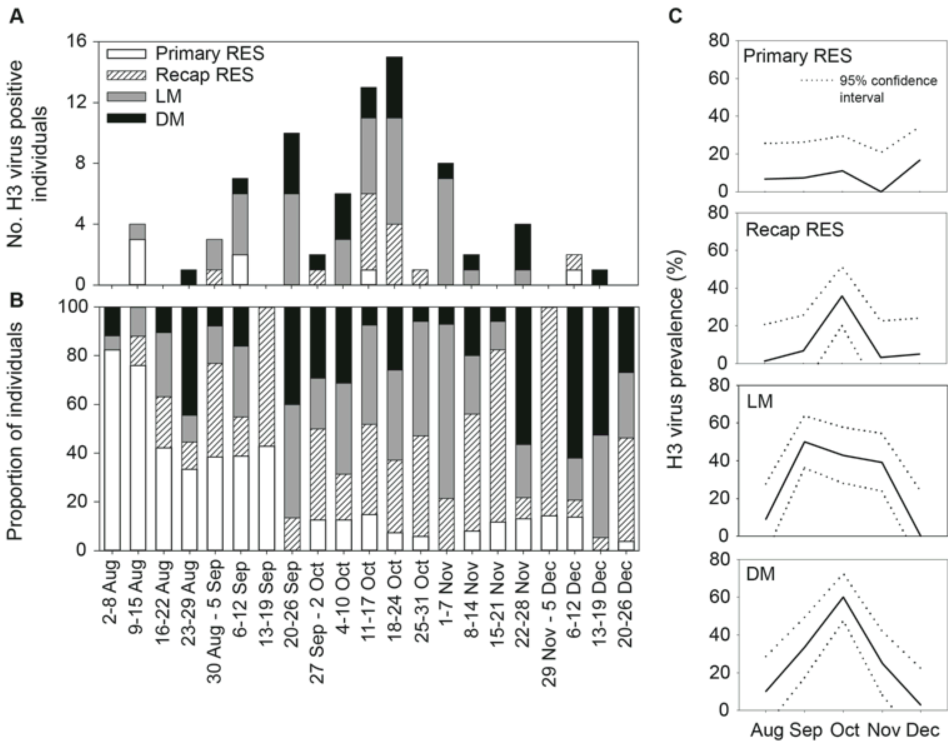


Figure S2. H3 low pathogenic avian influenza virus (LPAIV) prevalence in residents, local and distant migratory mallards during the H3 epizootic in 2010. For residents that were first captured (primary RES), recaptured residents (recap RES), local migrants (LM) and distant migrants (DM) the (A) number of H3 virus positive individuals per week, (B) proportion of individuals sampled per week, and (C) H3 virus prevalence (± 95% CI) per month are depicted.

Table S1. List of virus strain names and accession numbers of H3 influenza A viruses included in this study as listed in online databases Influenza Research Database (IRD) (http://www.fludb.org) and GISAID EpiFlu (http://platform.gisiad.org).

Virus strain name	Accession no.
A/Bewick's Swan/Netherlands/4/2007(H3N6)	IRDAccession_1099728
A/Common Teal/Hungary-EMC/1/2009(H3N8)	IRDAccession_1099753
A/Common Teal/Hungary-EMC/5/2009(H3N8)	IRDAccession_1099754
A/Mallard/Netherlands/52/2010(H3N8)	IRDAccession_1099755
A/Common Teal/Netherlands/2/2011(H3N8)	IRDAccession_1099756
A/Mallard/Netherlands/6/2008(H3N6)	IRDAccession_1099759
A/Mallard/Netherlands/7/2008(H3N8)	IRDAccession_1099760
A/Mallard/Netherlands/10/2008(H3N6)	IRDAccession_1099761
A/Mallard/Netherlands/56/2008(H3N2)	IRDAccession_1099762
A/Mallard/Netherlands/39/2008(H3N2)	IRDAccession_1099763
A/Mallard/Netherlands/40/2008(H3N6)	IRDAccession_1099764
A/Mallard/Netherlands/41/2008(H3N8)	IRDAccession_1099765
A/Mallard/Netherlands/44/2008(H3N2)	IRDAccession_1099767
A/Mallard/Netherlands/19/2010(H3N8)	IRDAccession_1099768
A/Mallard/Netherlands/7/2010(H3N8)	IRDAccession_1099769
A/Mallard/Netherlands/13/2010(H3N8)	IRDAccession_1099770
A/Mallard/Netherlands/14/2010(H3N8)	IRDAccession_1099771
A/Mallard/Netherlands/16/2010(H3N8)	IRDAccession_1099772
A/Mallard/Netherlands/13/2009(H3N3)	IRDAccession_1099773
A/Mallard/Netherlands/9/2010(H3N8)	IRDAccession_1099774
A/Mallard/Netherlands/27/2010(H3N8)	IRDAccession_1099775
A/Mallard/Netherlands/30/2010(H3N8)	IRDAccession_1099776
A/Mallard/Netherlands/39/2010(H3N8)	IRDAccession_1099777
A/Mallard/Netherlands/24/2010(H3N6)	IRDAccession_1099778
A/Mallard/Netherlands/25/2010(H3N8)	IRDAccession_1099779
A/Mallard/Netherlands/34/2010(H3N2)	IRDAccession_1099780
A/Mallard/Netherlands/8/2010(H3N8)	IRDAccession_1099781
A/Mallard/Netherlands/33/2010(H3N8)	IRDAccession_1099782
A/Mallard/Netherlands/9/2011(H3N2)	IRDAccession_1099783
A/Mallard/Netherlands/10/2011(H3N2)	IRDAccession_1099784
A/Mallard/Netherlands/20/2011(H3N8)	IRDAccession_1099785
A/Mallard/Netherlands/37/2011(H3N8)	IRDAccession_1099786
A/Mallard/Netherlands/19/2008(H3N6)	IRDAccession_1099787
A/Mallard/Netherlands/20/2008(H3N6)	IRDAccession_1099788
A/Mallard/Netherlands/21/2008(H3N8)	IRDAccession_1099789
A/Mallard/Netherlands/22/2008(H3N6)	IRDAccession_1099790
A/Mallard/Netherlands/23/2008(H3N6)	IRDAccession_1099791
A/Mallard/Netherlands/24/2008(H3N8)	IRDAccession_1099792
A/Mallard/Netherlands/25/2008(H3N6)	IRDAccession_1099793

Virus strain name	Accession no.
A/Mallard/Netherlands/26/2008(H3N6)	IRDAccession_1099794
A/Mallard/Netherlands/47/2008(H3N2)	IRDAccession_1099795
A/Mallard/Netherlands/48/2008(H3N2)	IRDAccession_1099796
A/Mallard/Netherlands/50/2008(H3N2)	IRDAccession_1099797
A/Mallard/Netherlands/20/2010(H3N8)	IRDAccession_1099798
A/Mallard/Netherlands/11/2010(H3N8)	IRDAccession_1099799
A/Mallard/Netherlands/6/2010(H3N8)	IRDAccession_1099800
A/Mallard/Netherlands/15/2010(H3N8)	IRDAccession_1099801
A/Mallard/Netherlands/35/2010(H3Nx)	IRDAccession_1099802
A/Mallard/Netherlands/32/2010(H3N8)	IRDAccession_1099803
A/Mallard/Netherlands/38/2010(H3N8)	IRDAccession_1099804
A/Mallard/Netherlands/36/2010(H3N8)	IRDAccession_1099805
A/Mallard/Netherlands/41/2010(H3N8)	IRDAccession_1099806
A/Mallard/Netherlands/42/2010(H3N8)	IRDAccession_1099807
A/Mallard/Netherlands/43/2010(H3N8)	IRDAccession_1099808
A/Mallard/Netherlands/44/2010(H3N8)	IRDAccession_1099809
A/Mallard/Netherlands/10/2010(H3N8)	IRDAccession_1099810
A/Mallard/Netherlands/37/2010(H3N8)	IRDAccession_1099811
A/Mallard/Netherlands/55/2010(H3N2)	IRDAccession_1099812
A/Anas_platyrhynchos/Belgium/12827/2007(H3N8)	EPI_ISL_26267
A/mallard/Germany-BW/SR872/2008(H3N8)	EPI_ISL_79643
A/mallard/Germany-BW/SR871/2008(H3N8)	EPI_ISL_79642
A/mallard/Germany-BW/SR632/2008(H3N2)	EPI_ISL_79640
A/mallard/Germany-BW/SR530/2007(H3N2)	EPI_ISL_79639
A/mallard/Germany-BW/SR520/2007(H3N2)	EPI_ISL_79638
A/mallard/Germany-BW/SR519/2007(H3N2)	EPI_ISL_79637
A/common_teal/Netherlands/7/2000(H3N8)	EPI_ISL_15008
A/mallard/Iceland/1007/2011(H3N6)	EPI_ISL_148200
A/mallard/Czech_Republic/14333-1K/2011(H3N8)	EPI_ISL_116136
A/mallard/Czech_Republic/14516/2007(H3N8)	EPI_ISL_63529
A/mallard/Sweden/50/2002(H3N8)	EPI_ISL_73381
A/mallard/Netherlands/5/2001(H3N6)	EPI_ISL_73371
A/mallard/Netherlands/2/1999(H3N5)	EPI_ISL_73370
A/common_teal/Sweden/1/2003(H3N3)	EPI_ISL_73363
A/mallard/Netherlands/1/2007(H3N2)	EPI_ISL_33850
A/mallard/Switzerland/WV4060167/2006(H3N5)	EPI_ISL_33832
A/turnstone/Netherlands/1/2007(H3N8)	EPI_ISL_30805
A/common_eider/Netherlands/1/2006(H3N8)	EPI_ISL_30804
A/mallard/Netherlands/3/2005(H3N8)	EPI_ISL_30793
A/teal/Chany/736/2008(H3N8)	EPI_ISL_97501
A/mallard/Czech_Republic/13577-24K/2010(H3N8)	EPI_ISL_89980
A/mallard/Netherlands/28/2006(H3N1)	EPI_ISL_84553
A/wigeon/Italy/3818-34/05(H3N8)	EPI_ISL_85911

Virus strain name	Accession no.
A/mallard/Italy/4394-10/05(H3N8)	EPI_ISL_85910
A/chicken/Italy/3582-51/10(H3N8)	EPI_ISL_85902
A/duck/Italy/3139-2/06(H3N8)	EPI_ISL_85901
A/duck/Italy/6207/08(H3N6)	EPI_ISL_85900

Table S2. Low pathogenic avian influenza virus sequence information. We gratefully acknowledge the authors, originating and submitting laboratories of the sequences from GISAID's EpiFluTM Database on which this research is based. All submitters of data may be contacted directly via the GISAID website. CODA-CERVA, Veterinary and Agrochemical Research Institute, Uccle, Belgium; FLI, Friedrich-Loeffler-Institut, Riems, Germany; IZSV, Istituto Zooprofilattico Sperimentale Delle Venezie, Venice, Italy.

Segment ID	Segment	Country	Collection date	Isolate name	Originating laboratory	Submitting laboratory	Authors
EPI_ISL_26267	HA	Belgium	2007-09-18	A/Anas_platyrhynchos/ Belgium/12827/2007(H3N8)	CODA-CERVA	CODA-CERVA	Thierry van den Berg
EPI_ISL_79643	HA	Germany	2008-09-08	A/mallard/Germany-BW/ SR872/2008(H3N8)	FLI	FLI	Elke Starick
EPI_ISL_79642	HA	Germany	2008-09-08	A/mallard/Germany-BW/ SR871/2008(H3N8)	FLI	FLI	Elke Starick
EPI_ISL_79640	HA	Germany	2008-01-23	A/mallard/Germany-BW/ SR632/2008(H3N2)	FLI	FLI	Elke Starick
EPI_ISL_79639	HA	Germany	2007-11-07	A/mallard/Germany-BW/ SR530/2007(H3N2)	FLI	FLI	Elke Starick
EPI_ISL_79638	HA	Germany	2007-10-22	A/mallard/Germany-BW/ SR520/2007(H3N2)	FLI	FLI	Elke Starick
EPI_ISL_79637	HA	Germany	2007-10-22	A/mallard/Germany-BW/ SR519/2007(H3N2)	FLI	FLI	Elke Starick
EPI_ISL_15008	HA	Netherlands	2000-11-23	A/common_teal/ Netherlands/7/2000(H3N8)			
EPI_ ISL_148200	HA	Iceland	2011-10-14	A/mallard/ Iceland/1007/2011(H3N6)			
EPI_ ISL_116136	HA	Czech Republic	2011-09-16	A/mallard/Czech_Republic/14333-1K/2011(H3N8)			
EPI_ISL_63529	HA	Czech Republic	2011-09-17	A/mallard/Czech_ Republic/14516/2007(H3N8)			
EPI_ISL_73381	HA	Sweden	2002-11-14	A/mallard/Sweden/50/2002(H3N8)			
EPI_ISL_73371	HA	Netherlands	2001-10-05	A/mallard/ Netherlands/5/2001(H3N6)			
EPI_ISL_73370	HA	Netherlands	1999-10-07	A/mallard/ Netherlands/2/1999(H3N5)			
EPI_ISL_73363	HA	Sweden	2003-08-30	A/common_teal/ Sweden/1/2003(H3N3)			
EPI_ISL_33850	HA	Netherlands	2006-12-04	A/mallard/ Netherlands/1/2007(H3N2)			
EPI_ISL_33832	HA	Switzerland	2006-12-15	A/mallard/Switzerland/ WV4060167/2006(H3N5)			
EPI_ISL_30805	HA	Netherlands	2007-10-15	A/turnstone/ Netherlands/1/2007(H3N8)			

Segment ID	Segment	Country	Collection date	Isolate name	Originating laboratory	Submitting laboratory	Authors
EPI_ISL_30804	HA	Netherlands	2006-08-05	A/common_eider/ Netherlands/1/2006(H3N8)			
EPI_ISL_30793	HA	Netherlands	2005-09-05	A/mallard/ Netherlands/3/2005(H3N8)			
EPI_ISL_97501	HA	Russia	2008-08-30	A/teal/Chany/736/2008(H3N8)			
EPI_ISL_89980	HA	Czech Republic	2010-09-16	A/mallard/Czech_Republic/13577-24K/2010(H3N8)			
EPI_ISL_84553	HA	Netherlands	2006-09-04	A/mallard/ Netherlands/28/2006(H3N1)			
EPI_ISL_85911	HA	Italy	2006-10-16	A/wigeon/Italy/3818-34/05(H3N8)	IZSV	IZSV	Isabella Monne
EPI_ISL_85910	HA	Italy	2006-07-07	A/mallard/Italy/4394-10/05(H3N8)	IZSV	IZSV	Isabella Monne
EPI_ISL_85902	HA	Italy	2010-06-04	A/chicken/Italy/3582-51/10(H3N8)	IZSV	IZSV	Isabella Monne
EPI_ISL_85901	HA	Italy	2006-09-12	A/duck/Italy/3139-2/06(H3N8)	IZSV	IZSV	Isabella Monne
EPI_ISL_85900	HA	Italy	2008-11-27	A/duck/Italy/6207/08(H3N6)	IZSV	IZSV	Isabella Monne

Table S3. Samples collected for virus and antibody detection from free-living mallards (Anas platyrhynchos) during the H3 low pathogenic avian influenza virus (LPAIV) epizootic in 2010. Samples were collected from resident birds that were first captured (primary), recaptured residents, local and distant migratory birds, and were specified by age (juvenile: <1 year, adult: >1 year) and sex.

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

CHAPTER 2.2

Nicola S. Lewis*, Josanne H. Verhagen, Zurab Javakhishvili, Colin A. Russell
Pascal Lexmond, Kim B. Westgeest, Theo M. Bestebroer, Rebecca A. Halpin
Xudong Lin, Amy Ransier, Nadia B. Fedorova, Timothy B. Stockwell
Neus Latorre-Margalef, Björn Olsen, Gavin Smith, Justin Bahl
David E. Wentworth, Jonas Waldenström, Ron A. M. Fouchier
Miranda de Graaf*

Influenza A virus evolution and spatio-temporal dynamics in Eurasian wild birds: A phylogenetic and phylogeographic study of whole-genome sequence data

*Authors contributed equally to this study
Journal of General Virology (2015), Volume 96, Issue 8: 2050–2060. DOI:10.1099/vir.0.000155

Low pathogenic avian influenza A viruses (IAVs) have a natural host reservoir in wild waterbirds and the potential to spread to other host species. Here, we investigated the evolutionary, spatial and temporal dynamics of avian IAVs in Eurasian wild birds. We used whole-genome sequences collected as part of an intensive long-term Eurasian wild bird surveillance study, and combined this genetic data with temporal and spatial information to explore the virus evolutionary dynamics. Frequent reassortment and co-circulating lineages were observed for all eight genomic RNA segments over time. There was no apparent species-specific effect on the diversity of the avian IAVs. There was a spatial and temporal relationship between the Eurasian sequences and significant viral migration of avian IAVs from West Eurasia towards Central Eurasia. The observed viral migration patterns differed between segments. Furthermore, we discuss the challenges faced when analysing these surveillance and sequence data, and the caveats to be borne in mind when drawing conclusions from the apparent results of such analyses.

INTRODUCTION

Low pathogenic avian influenza (LPAI) viruses have been isolated from more than 136 species of wild birds, most commonly from ducks, but also from other *Anseriformes* (geese and swans) and *Charadriiformes* (mainly gulls, waders and terns) (12, 19, 21). These bird groups have diverse annual life cycles and many are highly migratory, thereby potentially affecting spatial and temporal dynamics of avian influenza virus (AIV) at different geographical scales. Many species also frequent habitats where there is potential for direct or indirect contact with domestic birds (12), primarily ducks and geese, with the concurrent risk of cross-species transmission of AIVs into domestic animals. This incursion of virus from the wild bird reservoir may have several animal and human health implications, including the risk of emergence of highly pathogenic avian influenza (HPAI) viruses and threat to food security. It also provides a means by which AIV might be brought into closer proximity to humans (174). For Eurasia, waterbird migration can be broadly divided in five flyways: East Atlantic flyway, Black Sea–Mediterranean flyway, East Africa–West Asia flyway, Central Asia flyway and the East Asia Australian flyway. It should be noted that these flyways are oversimplifications and numerous exceptions exist (21, 26, 35, 175). Bird migration along the Central Asian flyway was reported to correlate with outbreaks of HPAI H5 and emphasized the need for bird surveillance (174). Despite widespread surveillance (176), there remain substantial unanswered questions about the spatial, temporal and ecological role of the host populations in defining the genetic structure of AIVs, and in inferring the role wild birds might play in trans-locating AIV from one geographical region to another. Such information is key for considering measures to reduce the risk of pathogen emergence from wildlife host reservoirs.

Previous work on identifying predictors of HPAI virus H5N1 occurrence have shown that human population size, duck density, rice cropping intensity, wild bird migration and poultry trade all contribute to virus prevalence and potential for detection (177, 178). Ideally, we would also want to use such spatial risk map approaches to better understand the ecology of LPAI viruses in wild birds, prior to any transmission to domestic birds. The challenges to such analyses are large as there are numerous host species with different ecological dynamics covering broad and far-reaching areas in short time frames and differences in intrinsic reservoir capacities. The prevalence of AIVs in their natural hosts depends on geographical location, seasonality, immune processes and species (21, 26, 179). The ecological drivers of these prevalence fluctuations and how they affect viral genetic diversity are less well-characterized (146, 150, 172). Previous studies to investigate patterns in the genetic diversity among wild bird AIVs have focused predominantly on North America, partly because of the existence of larger

longitudinal AIV surveillance datasets in wild birds. Studies on North American wild birds documented a high rate of genome reassortment (92), and a significant viral clustering by time and location of sampling (180). Other work suggested that ducks in Alberta were representative of the total AIV diversity in North American *Anseriformes* and, whilst there might be spatial segregation to a particular migratory flyway over short time frames, the long-term persistence of AIV was independent of bird flyways with migration between populations throughout North America (181). Extensive surveillance studies of AIV in ducks and shorebirds in North America have permitted analyses of reassortment rates, selection pressures and patterns of genetic diversity, but until recently there has only been limited whole-genome sequence data available for AIVs in Eurasia, Africa, South America and Oceania. AIVs found in Eurasian wild birds are predominantly genetically distinct from those of wild birds in the Americas (27, 92, 182), representing major geographical/continental lineages. Wild bird migratory flyways are different in Eurasia; thus patterns characterized for the Americas could differ substantially from those in Eurasia.

To explore the evolutionary and ecological dynamics of AIV in Eurasian wild birds, we used whole-genome sequences of AIVs isolated from several *Anseriformes* species sampled in West Eurasia along the East Atlantic flyway as part of an intensive wild bird surveillance study. These full-genome sequences were combined with genetic data of AIVs isolated throughout Eurasia. This large-scale study describes gene reassortment and viral migration within Eurasia in the light of wild bird migration and supports new directions in wild bird AIV surveillance.

RESULTS AND DISCUSSION

To study the spatio-temporal dynamics of AIVs in wild birds in Eurasia, more than 100 virus isolates collected from 1999 to 2007 were selected for full genomic sequencing of the coding regions. These virus isolates represented a diverse range of wild bird hosts, and included different subtypes and sampling locations predominantly within West Eurasia (Table 1). In addition, AIV full-genome sequences spanning NA1–NA9 and HA1–HA12 were retrieved from GenBank (158) (Table S1). To focus on evolution of LPAI viruses in wild birds, we excluded all sequences from domestic birds and all sequences related to poultry outbreaks, particularly HPAI H5N1, H7 and H9.

Although AIVs have been isolated from more than 136 species of birds, the role of each of these species in maintaining virus diversity and virus spread is unclear. Differences in AIV prevalence and in prevalence of haemagglutinin (HA) subtypes and HA/neuraminidase (NA) subtype combinations have been observed among wild bird species (26, 172). However, it is possible that for specific host species certain AIV subtypes are endemic, allowing for genetic evolution and diversification of the virus, whereas in other host species this AIV subtype is more likely to be a transient pathogen and does not become established. Here, the role of host species on influenza virus diversity was investigated using maximum likelihood (ML) trees coloured by the bird species group from which the virus was sampled (Figure 1; also see Figure S1 for ML trees of all segments with strain names and Table 1 for host categories). Overall, no clear species-specific patterns could be identified. The observed genetic diversity did not seem to originate from a particular host nor were there genetic lineages limited to a single species. Nevertheless, our sequence dataset was biased with respect to bird species as the majority of AIVs included in our study were isolated from dabbling ducks (Table 1). Dabbling ducks more frequently harbour AIVs and therefore they are a 'target species group' for surveillance (21). Due to the over-representation of dabbling ducks, we cannot exclude that the lack of species-specific patterns in the tree topology is an artefact. Most of the Eurasian shorebird sequences appeared to cluster together in the ML tree of the HA gene, suggesting a species-specific niche wider than the H13 and H16 niche, which has been reported previously for gulls in Eurasia and North America (27, 36, 183) (Figure S1). It should be noted that in our dataset most shorebird sequences were sampled in Oceania and were much older compared with the other Eurasian AIV sequences. Thus, our findings suggest that there is no strong species effect associated with virus diversity, similar to the results described previously for North American AIV (180).

Table 1. Number (n) of sequences per host species, country, year of isolation and subtype. (N = 211 complete genomes). *The number of newly submitted sequences is given within parentheses.

Species	N*	Species category	Country	N*	Year	N*	Subtypes	N*
Mallard	75 (57)	Dabbling duck	Netherlands	52 (51)	1956	2	H3N8	24 (4)
Duck	55	Dabbling duck	Australia	34	1963	1	H5N2	15 (4)
Red-necked stint	12	Shorebird	Sweden	32 (32)	1972	1	H4N6	14 (4)
Black duck	5	Dabbling duck	China	12	1973	1	H5N3	12 (1)
Common teal	5 (4)	Dabbling duck	Hong Kong	12	1975	3	H11N9	9 (5)
Gadwall	5 (1)	Dabbling duck	Russia	11	1976	1	H4N8	9 (1)
Gray teal	4	Dabbling duck	Italy	9	1977	3	H1N1	8 (1)
Eurasian wigeon	3 (3)	Dabbling duck	France	8	1978	9	H6N1	8 (5)
Northern shoveler	3 (2)	Dabbling duck	Japan	8	1979	8	H6N2	8 (5)
Shearwater	3	Shorebird	Mongolia	7	1980	7	H9N2	8 (4)

Species	N*	Species category	Country	N*	Year	N*	Subtypes	N*
Teal	3	Dabbling duck	Germany	6	1981	1	H7N7	7 (6)
Barheaded goose	2	Goose	Denmark	3	1982	1	H10N4	6 (2)
Bewick swan	2 (2)	Swan	Taiwan	3	1983	4	H4N2	5 (2)
Black-headed gull	2 (2)	Shorebird	UK	3	1984	3	H5N1	5
Common eider	2 (2)	Diving and other ducks	Czech Republic	2	1985	2	H7N1	5 (1)
Goose	2	Goose	New Zealand	2	1986	1	H8N4	5 (4)
Northern pintail	2 (1)	Dabbling duck	Portugal	2	1988	1	H12N3	4
Ruddy shelduck	2	Diving and other ducks	Belgium	1	1991	1	H2N2	4 (2)
Sharp-tailed sandpiper	2	Shorebird	Malaysia	1	1992	1	H2N9	4 (1)
Greater white-fronted goose	2 (2)	Goose	Slovenia	1	1992	1	H6N5	4 (1)
Dunlin	1 (1)	Shorebird	Spain	1	1994	1	H11N2	3 (2)
Eurasian coot	1	Shorebird	Ukraine	1	1998	1	H2N3	3 (3)
Fowl	1	Fowl			1999	12 (9)	H3N2	3 (1)
Garganey	1	Dabbling duck			2000	7 (4)	H3N6	3 (1)
Greylag goose	1 (1)	Goose			2001	5 (2)	H10N7	2 (2)
Gull	1	Shorebird			2002	21 (18)	H10N9	2 (1)
Herring gull	1 (1)	Shorebird			2003	10 (7)	H11N8	2 (2)
Mute swan	1 (1)	Swan			2004	14 (1)	H12N9	2
Pink-footed goose	1 (1)	Goose			2005	29 (15)	H4N3	2 (2)
Red-crested pochard	1	Diving and other ducks			2006	30 (18)	H6N8	2 (2)
Slaty-backed gull	1	Shorebird			2007	19 (9)	H7N2	2 (1)
Spot-billed duck	1	Dabbling duck			2008	6	H7N3	2 (1)
Swan	1	Swan			2009	4	H7N9	2 (1)
Tufted duck	1	Diving and other ducks					H10N1	1 (1)
Ruddy turnstone	1 (1)	Shorebird					H10N8	1 (1)
Wedge-tailed shearwater	1	Shorebird					H11N1	1 (1)
Whooper swan	1	Swan					H11N6	1
Barnacle goose	1 (1)	Goose					H1N4	1 (1)
Tern	1	Shorebird					H1N5	1 (1)
Whistling swan	1	Swan					H3N1	1 (1)
							H3N5	1 (1)
							H4N4	1
							H4N5	1 (1)
							H5N6	1
							H5N7	1
							H5N9	1 (1)
							H6N9	1
							H7N6	1
							H7N8	1 (1)
							H9N6	1
							H10N6	1 (1)

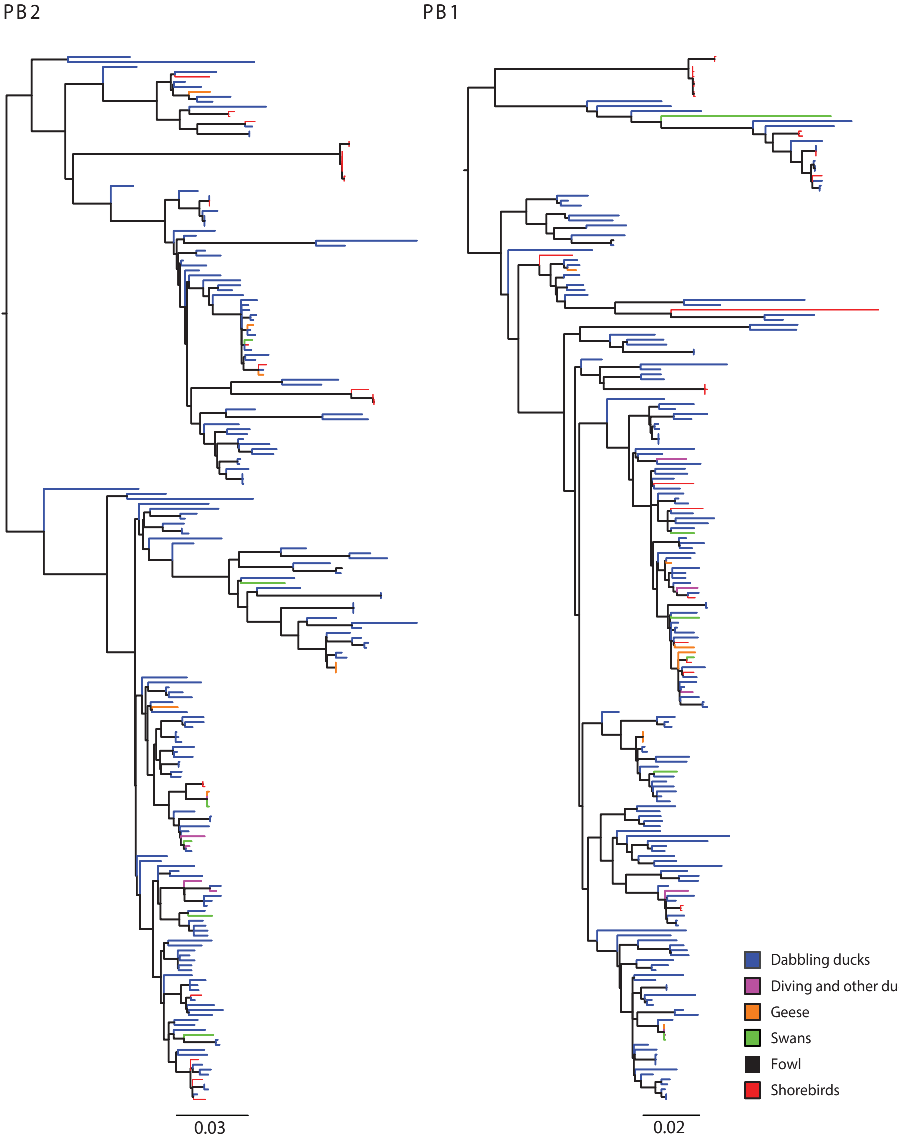


Figure 1. Maximum Likelihood (ML) trees for PB2 and PB1 displaying the genetic diversity of avian IAVs in Eurasian wild birds. The taxa colour indicates the bird species group from which the sample was isolated.

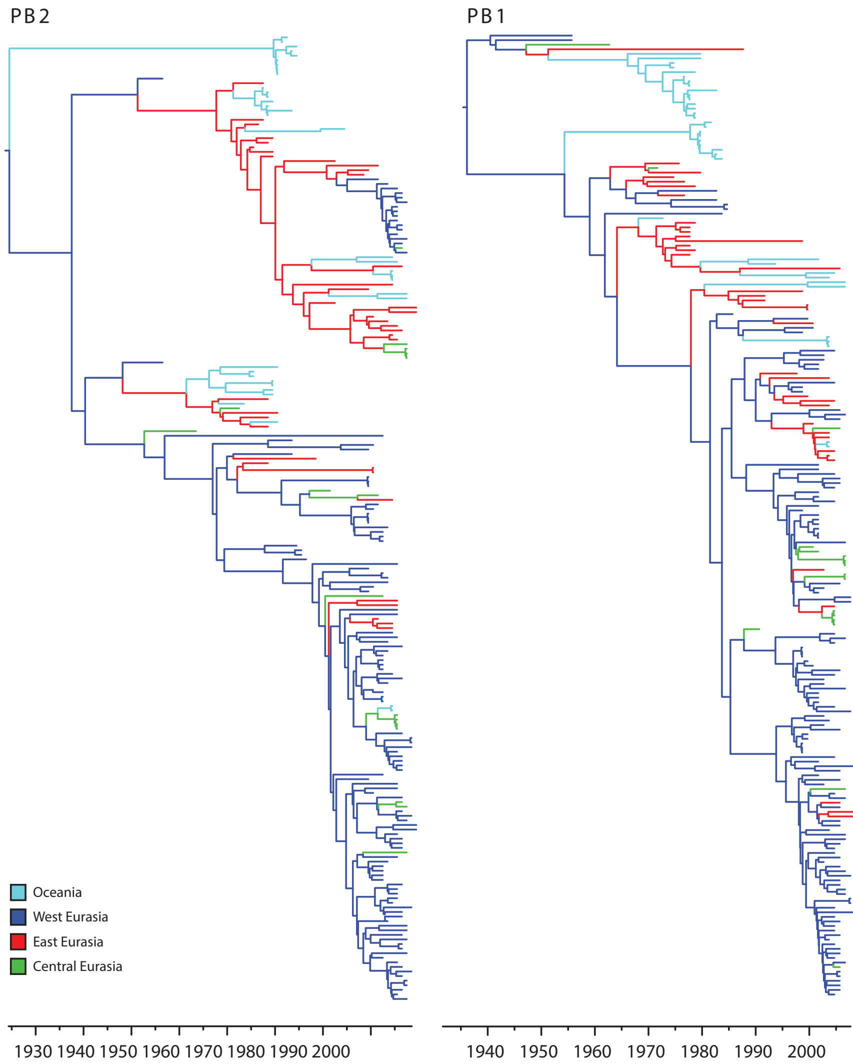


Figure 2. Maximum clade credibility (MCC) trees summarizing the results of the Bayesian phylogenetic inference of PB1 and PB2, and displaying the genetic diversity in different locations in Eurasia. The taxa colour indicates the regional location from where the sample was isolated. Year is indicated.

To investigate how the genetic diversity partitioned according to geographical location, ML trees were coloured by four discrete regions; West (i.e. West Eurasia), East (i.e. East Eurasia), Central (i.e. Central Eurasia) and Oceania (Figure S2). These four geographical regions also approximate migratory flyways: West Eurasia lies within the East Atlantic flyway, Central Eurasia lies within the Black Sea–Mediterranean and Central Asian flyway, and East Eurasia and Oceania represent the East Asian–Australasian flyways. Despite overlap in migratory flyways among these four regions, viruses sampled from one geographical region and from a particular time period were most closely related to other viruses sampled from the same geographical region and could be related to different migration patterns. To further investigate the spatial and temporal processes, BEAST was used to infer Bayesian phylogenetic trees in which all viruses were assigned to the four discrete regions (Figure 2 and S3).

The Bayesian analysis revealed that for all internal segments, except for NS, the most recent common ancestors (MRCAs) containing these segments circulated *72–108 years ago (Table 2, Figure 2; see Figure S3 for Bayesian trees of all segments with strain names). This recent ancestry is suggestive of hemispheric sweeps of all genetic diversity in fairly recent times, as suggested previously by others (184). The genetic diversity for the HA, NA and NS gene segments was maintained, corresponding with MRCAs much older than those of the other gene segments (PB2, PB1, PA, NP and M). However, the genetic diversity within each HA and NA subtype and NS allele was similar to that of the internal segments. For HA and NA, it was proposed that immunity in previously exposed bird populations allows the maintenance of multiple subtypes (184). It has also been described that NS alleles A and B differentially suppress innate immune responses (185), perhaps allowing for maintenance of both alleles. Despite generally short times to the MRCA for the internal segments, multiple lineages co-circulated within the same years at the same locations. In our dataset, there was a high sample density of West Eurasian AIVs isolated between 2002 and 2009. However, despite this high sampling density, the genetic diversity found in West Eurasia did not completely represent the genetic diversity of AIVs throughout Eurasia during that time period. For example, for PB1 there is a lineage containing AIVs isolated from East and Central Eurasia and Oceania of which the common ancestor to the most closely related AIV from West Eurasia circulated >20 years ago. Despite probable host population and ecological differences between Eurasia and North America (26), we found similar nucleotide substitution rates for Eurasian AIV strains compared with previous studies including both North American and Eurasian AIV sequences (186).

Table 2. Rates of nucleotide substitution and times to the MRCA

Gene segment	Mean nucleotide substitution rate (10^{-3} substitutions per site per year)	Time to MRCA (95 % higher posterior density interval) (years)
PB2	2.06 (1.80–2.32)	85 (66–111)
PB1	2.18 (1.94–2.44)	73 (64–81)
PA	1.99 (1.74–2.25)	78 (69–87)
HA	2.39 (1.91–2.88)	1003 (696–1340)
NP	1.78 (1.50–2.05)	109 (76–146)
NA	2.51 (1.99–3.08)	1294 (906–1673)
MP	1.29 (1.01–1.59)	92 (62–140)
NS	2.43 (1.70–3.18)	271 (147–428)

To test if more closely related viruses were more likely to share the same location than expected by chance alone (Parker et al., 2008), Bayesian trees were analysed for evidence of taxa association by location of sampling using the Bayesian Tip-significance (BaTS) package. When the entire period of sampling was analysed, we found strong clustering by location for all regions and all gene segments (data not shown). Although this indicated that there was a spatial component to the dataset and regional maintenance of a particular clade, it could also have been due to a bias in sampling during a particular time period. To reduce this bias, the same analysis was performed using time periods of 5 years (Table 3). Despite the shorter time period, there was significant clustering of viruses isolated from the same location. Ideally, 1 year would be most relevant to the annual life cycle – and thus annual migration – of the host; this dataset comprised insufficient data for statistical power to analyze just 1 year. This illustrates one of the confounders with these data. When one attempts to reduce potential sampling bias or inconsistent sampling effort throughout the region, and capture diversity on a timescale that is relevant to the host species, one likely reduces statistical power. See Table 1 for further details on sampling by species, time and sampling site.

The Bayesian analysis was also used for ancestral state reconstruction of geographical location and to estimate the rate of viral migration among the geographical regions (Figure 3, Table S2) (187). The highest rates of viral migration were observed from Eastern Eurasia to Oceania for PB2, Western to Eastern Eurasia for PB1, HA, NP, NA and M, and Eastern to Western Eurasia for PA and NS. Such a lack of consistent and directional spatial pattern among gene segments was also observed for North American strains (181). The inconsistent directionality observed here was likely due to differences

Table 3. Support for geographical clustering, based on BaTS testing (P values). Significant clustering of sequences from the four geographical regions was investigated by coding the regional location from which the virus was sampled onto the tips (taxa) of 900 posterior sampled trees, generating 100 null distributions, and testing the significance of the observed data. P ≤ 0.05 indicates significant geographical clustering, whilst P > 0.05 indicates that traits were randomly distributed across the phylogeny. Significant values are given in italics. Only datasets with at least three sequences were included.

		Gene segment							
Location	Period	PB2	PB1	PA	HA	NP	NA	MP	NS
Central Eurasia	2001-2005	*0.01*	*0.01*	*0.01*	*0.01*	*0.01*	*0.01*	*0.01*	*0.01*
Central Eurasia	2006-2010	*0.01*	*0.01*	0.06	*0.01*	*0.01*	*0.01*	*0.01*	*0.01*
East Eurasia	1976-1980	*0.11*	*0.01*	*0.01*	0.11	*0.01*	*0.01*	*0.01*	*0.02*
East Eurasia	1996-2000	0.06	*0.03*	*0.02*	*0.03*	*0.02*	*0.04*	*0.02*	*0.01*
East Eurasia	2001-2005	*0.05*	*0.01*	0.15	0.14	*0.01*	0.06	*0.01*	0.10
East Eurasia	2006-2010	*0.03*	*0.01*	1.00	*0.02*	0.06	1.00	1.00	1.00
Oceania	1971-1975	*0.02*	*0.02*	*0.02*	1.00	*0.01*	1.00	*0.01*	*0.01*
Oceania	1976-1980	*0.01*	*0.01*	*0.01*	*0.01*	*0.02*	*0.01*	*0.01*	*0.01*
Oceania	1981-1985	*0.01*	*0.01*	*0.01*	*0.02*	0.06	0.06	*0.02*	*0.01*
Oceania	2001-2005	*0.05*	*0.05*	*0.03*	*0.02*	*0.04*	*0.04*	*0.04*	0.06
West Eurasia	1981-1985	*0.01*	*0.01*	*0.05*	*0.01*	*0.05*	*0.02*	1.00	*0.01*
West Eurasia	1996-2000	*0.01*	*0.01*	*0.02*	*0.01*	*0.01*	*0.02*	*0.01*	*0.01*
West Eurasia	2001-2005	*0.02*	*0.01*	0.28	0.09	*0.01*	*0.01*	*0.01*	*0.01*

in sampling bias and the high rates of reassortment. For HA and NA, the different subtypes are highly divergent and ancestral state reconstruction will also include modelling on the long inter-subtype branches potentially influencing the results. It should also be noted that due to sparse sampling before 1999, migration events inferred for older viruses were much more susceptible to sample bias.

We assessed reassortment by rooting the Bayesian maximum clade credibility (MCC) nucleotide substitution trees by older Australian strains and making tanglegrams (Figure 4). Tanglegrams enable visualization of the locations of particular taxa within the PB2 tree and each of the trees of the other segments. In the absence of reassortment, the taxa should have a nearly horizontal linkage. The tanglegram patterns indicate that there was extensive reassortment, but without completely distorting clustering between sequences of the same geographical region. Viruses of a particular subtype do not necessarily have the same genetic makeup, even for a particular species, location or year. For NS, we observed co-circulation of the A and B alleles, and similar to HA and NA, these two alleles were not associated with separate lineages for other segments.

Differences in reassortment rates between the internal segments of AIVs belonging to different subtypes have been reported for Eurasian AIVs (188). In particular, internal segments belonging to subtypes H1–H4 reassort with a lower rate compared with H5 and H9. It should be noted that this dataset included poultry AIV and poultry-outbreak-related AIV sequences, likely influencing reassortment rates (188).

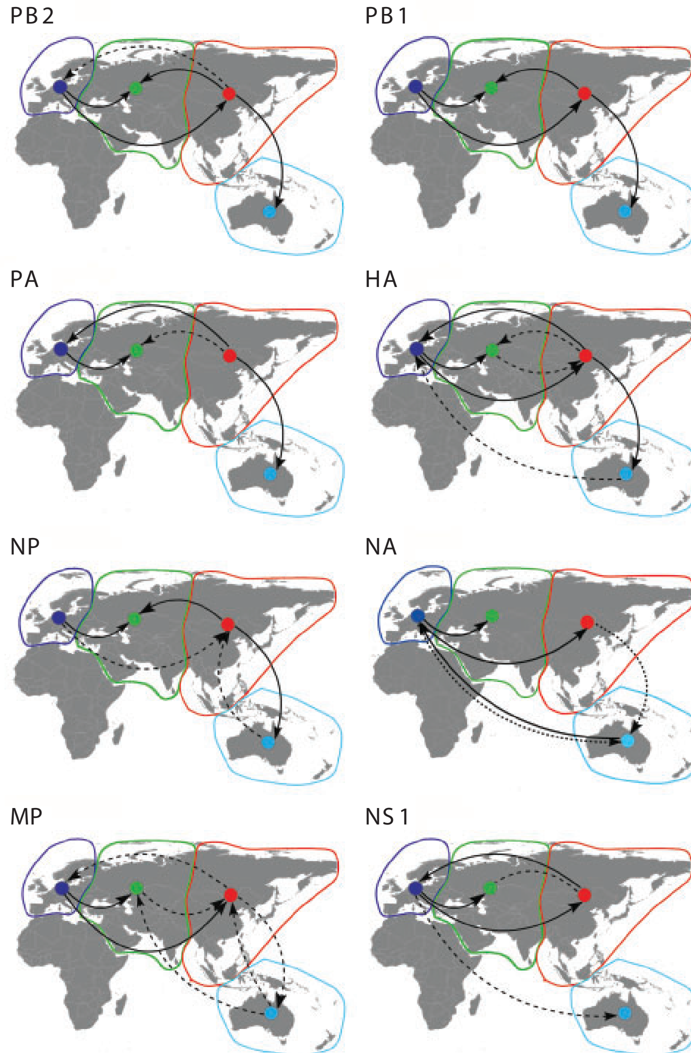


Figure 3. Patterns of viral migration among regions, visualized for each segment on a Eurasian map. A Bayes factor (BF) test was applied to assess the statistical support for viral migration between the discrete geographical states. Lines connecting discrete regions (cyan, Oceania; red, East Eurasia; green, Central Eurasia; blue, West Eurasia) indicate statistically supported ancestral state changes. Dotted lines, 8≤BF≤100 (supported); solid lines, BF>100 (strongly supported).

Overall genetic diversity of AIVs in Eurasian *Anseriformes* can be captured by the genetic diversity found in dabbling ducks. AIVs isolated from dabbling ducks in Alberta are a good representation of the genetic diversity of AIVs circulating in North America (181). In contrast, AIVs from West Eurasia, East Eurasia, Central Eurasia or Oceania do not represent the genetic diversity of the whole of Eurasia well. The genetic diversity of AIVs is shaped by many factors such as immunogenicity of the host, reassortment, migration patterns and life span of the hosts as well as virus durability in aquatic environments (189). The influence of heterosubtypic immunity is seen on the prevalence of both HA groups and on the level of HA clades in recaptured wild ducks (190). The incidence and prevalence of AIVs shows clear seasonal patterns due to host–pathogen interactions. The influx of immunologically naive juveniles in summer and the arrival of susceptible migrants in autumn as well as moult aggregations are also likely drivers of AIV infection dynamics in temperate Eurasian latitudes (146, 191). Whether these disease dynamics patterns can be generalized over multiple subpopulations in different latitudes within Eurasia remains to be investigated. In some North American flyways, resident birds can also act as reservoirs of virus diversity and although migratory birds introduce AIV in these wild bird populations, these viruses do not necessarily become the predominantly circulating viruses (145). Whilst this might be true at sites in Eurasia where resident and migratory bird populations overlap, in many areas there is likely less opportunity for resident maintenance. Therefore, virus diversity is more likely driven by migration.

Here, we map the long-term spatial-temporal dynamics of the whole-genome of AIV in Eurasia. Despite in-depth wild bird surveillance in Eurasia, it is clear from this study that to assess the implication of migration patterns on the genetic diversity of AIV in Eurasia future whole-genome sequencing should be directed towards increased numbers of samples within a short time frame in locations along the different flyways. Such high-resolution studies have been performed in North America and West Eurasia, and are currently being actively pursued in the rest of Eurasia. Incorporating of metadata such as host species, location and date of sampling, age, sex, and migratory status will illuminate future host-focused studies by including the impact of ecological factors like individual species diversity and life cycle on AIV genetic diversity.

METHODS

Dataset and genomic sequencing

Over a period of 15 years, 186 054 samples from 440 different bird species were analysed for the presence of AIVs. Positive isolates were subtyped and sequenced. In collaboration with the National Institutes of Health and the J. Craig Venter Institute,

~83 full or nearly full-genomes and 30 partial genomes of AIVs have been submitted to GenBank. The coding complete genomes of the influenza viruses were sequenced using a high-throughput next-generation sequencing pipeline at the J. Craig Venter Institute, which included the 454/Roche GS-FLX and the Illumina HiSeq 2000 platforms. Viral RNA was isolated using a ZR 96 Viral RNA kit (Zymo Research). The influenza A genomic RNA segments were simultaneously amplified from 3 ml purified RNA using a multisegment reverse transcription (M-RT)- PCR strategy (192, 193). The influenza M-RT-PCR amplicons were barcoded and amplified using an optimized SISPA (sequence-independent single primer amplification) protocol (194, 195). Subsequently, the SISPA amplicons were purified, pooled and size selected (~800 or ~200 bp), and the pools were used for both Roche 454 (Roche Diagnostics) and Illumina (Illumina) library construction. Samples were sequenced on the 454/Roche GS-FLX and Illumina HiSeq 2000 platforms.

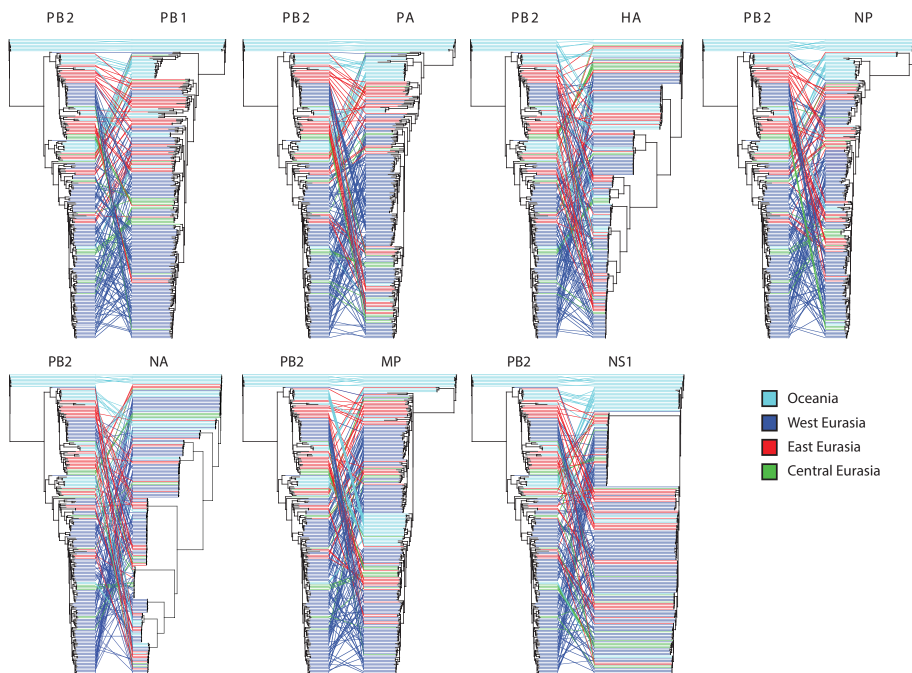


Figure 4. Tanglegrams constructed by rooting the MCC nucleotide substitution trees by older Australian strains. Corresponding taxa in the two trees are connected by a line. In the absence of reassortment one would expect to see a horizontal, or near horizontal, line connecting taxa between trees. The connecting lines are coloured by the region of sampling of the taxa. We show only the tree comparison for each segment with PB2 as the reference topology, but reassortment patterns were similar when other gene segments were used as the reference.

Libraries were prepared for sequencing on the 454/Roche GS-FLX platform using Titanium chemistry or for sequencing on the Illumina HiSeq 2000. The sequence reads were sorted by barcode, trimmed and searched by TBLASTX against custom nucleotide databases of full-length influenza A segments downloaded from GenBank to filter out both chimeric influenza sequences and non-influenza sequences amplified during the random hexamer-primed amplification. The reads were binned by segment and the 454/ Roche GS-FLX reads were de novo assembled using the clc_novo_assemble program (CLC Bio). The resulting contigs were searched against the corresponding custom full-length Influenza segment nucleotide database to find the closest reference sequence for each segment. Both 454/Roche GS-FLX and Illumina HiSeq 2000 reads were then mapped to the selected reference influenza A virus segments using the clc ref_assemble_long program (CLC Bio). At loci where both 454/Roche GS-FLX and Illumina HiSeq 2000 sequence data agreed on a variation (as compared with the reference sequence), the reference sequence was updated to reflect the difference. A final mapping of all next-generation sequences to the updated reference sequences was then performed. Any regions of the viral genomes that were poorly covered or ambiguous after next-generation sequencing were amplified and sequenced using the standard Sanger sequencing approach.

These viruses were isolated from different wild bird species, and included different subtypes and sampling locations within West Eurasia throughout the time period of the study. In addition, all full-genome sequences from AIV genomes containing NA1–NA9 and HA1–HA12 available from GenBank were retrieved. All sequences from domestic birds and all sequences related to poultry outbreaks, particularly HPAI H5N1, H7 and H9, were excluded. Our final datasets of matched genome sequences for PB2 (2266 nt), PB1 (2259 nt), PA (2142 nt), HA (1716 nt), NP (1482 nt), NA (1374 nt), MP (979 nt) and NS (838 nt) were aligned with BioEdit version 7.1 (a total of 211 complete genomes; see Table S1 for GenBank accession numbers).

ML analysis

Phylogenetic trees for each segment were reconstructed with PhyML version 3.0 (196), using the general time reversible (GTR) nucleotide substitution model with a pro- portion of invariant sites and a C distribution of among-site rate variation, all estimated from the data (determined by ModelTest as the appropriate nucleotide substitution model). GARLI version 0.96 (197) was run on the best tree from PhyML for 1 million generations to optimize tree topology and branch lengths.

Temporal phylogeny and relative genetic diversity

To identify potential errors in sequence data annotation that might have affected the clock estimation, we used the reconstructed ML nucleotide trees in Path-O-Gen version 1.3 (198) to generate linear regression plots of the years of sampling versus root-to-tip distance. We did not observe any anomalies in the eight segment datasets, which all exhibited a clock-like behaviour (199).

We estimated rates of evolutionary change (nucleotide substitutions per site per year) and times of circulation of the MRCA (years) with BEAST version 1.7.3 using time-stamped sequence data with a relaxed-clock Bayesian Markov chain Monte Carlo (MCMC) method (200-202). For all analyses, the uncorrelated log-normal relaxed molecular clock and a C site heterogeneity model with four C categories was used in combination with the GTR nucleotide substitution model. A normal rate prior with a mean of 0.0033 substitutions per site per year (SD50.0016) was used (203). These analyses were conducted with a Bayesian Skyline coalescent model, a random starting tree and a constant rate of migration. We performed at least three independent analyses of at least 100 million MCMC chains to ensure convergence and combined these analyses after removal of the burn-in of 10% using LogCombiner version 1.7.3. Finally, the MCMC chains were summarized to reconstruct the MCC trees using TreeAnnotator version 1.7.3. Trees were visualized and coloured with the FigTree version 1.4.0 (163).

Phylogeography

We grouped our country-level dataset into West Eurasia, Central Eurasia, East Eurasia and Oceania because of insufficient sampling density to reconstruct exact sampling location of ancestral viruses. Discrete state ancestral reconstruction of viral sampling locations and migration rates between geographical regions were estimated with an asymmetrical state transition model. Given the large number of states, a Bayesian stochastic search variable selection (BSSVS) was employed to reduce the number of parameters to those with significantly non-zero transition rates (187). From the BSSVS results, a Bayes factor (BF) test could be applied to assess the support for individual transitions between discrete geographical states. The BF was deemed statistically significant if the combined independent analyses resulted in a binary indicator w0.5 and a BFw6. Therefore, our minimal critical cut-offs for statistical support were 8jBFj100 indicating support and BFw100 indicating strong support (187, 203).

The migration routes that had the strongest support as indicated by the highest BF (187) were determined using SPREAD (204). In addition, significant clustering of

sequences from the four geographical regions was investigated by coding the regional location from which the virus was sampled onto the tips of 900 posterior sampled trees, generating 100 null distributions, and testing the significance of the observed data using BaTS package (205). Pv0.05 indicated significant clustering, whilst Pw0.05 indicated that traits were randomly distributed across the phylogeny.

Reassortment analyses

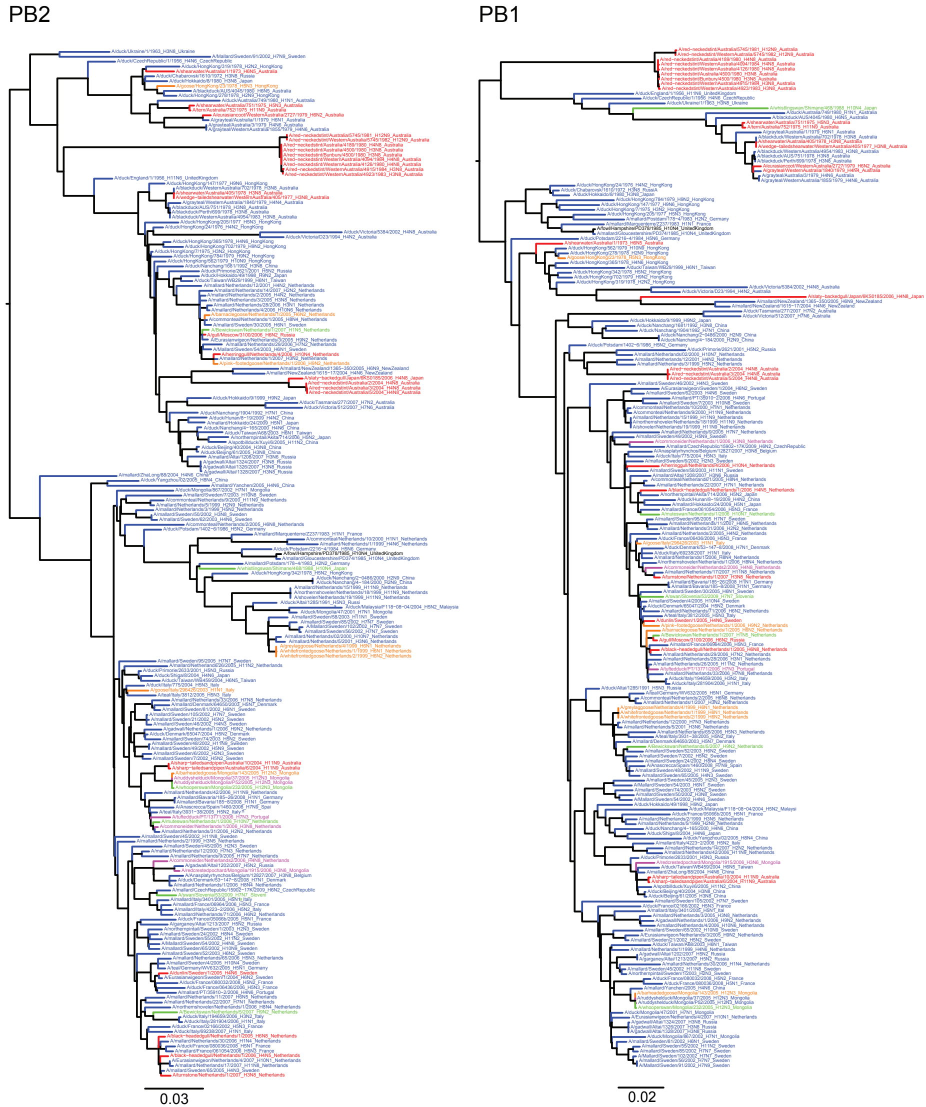
To visualize similarities and differences between the phylogenies, and investigate reassortment, tanglegrams were generated using the nucleotide substitution MCC trees generated by BEAST and TreeMap version 1.0 (206). These tanglegrams consisted of two rooted phylogenetic trees of which taxa that corresponded to each other in the two trees were connected. In the absence of reassortment, one would expect to see nearly horizontal linkage connecting one taxa to another.

ACKNOWLEDGEMENTS

We thank all the ornithologists and other collaborators for their continuous support. We thank V. Munster, E. Skepner, O. Vuong, C. Baas, J. Guldemeester, M. Schutten, G. van der Water, D. Smith and E. Bortz for technical support and stimulating discussions. This manuscript was prepared while D. E. W. was employed at the J. Craig Venter Institute. The opinions expressed in this article are the authors' own and do not reflect the view of the Centers for Disease Control, the Department of Health and Human Services or the US Government. This work was supported by the NIAID/NIH (HHSN266200700010C, HHSN272201400008C, HHSN272201400006C and HHSN272200900007C), a Wellcome Trust Fellowship Strategic Travel Award (WT089235MF), a DTRA FRCWMD Broad Agency Announcement (HDTRA1-09-14-FRCWMD GRANT11177182), by the EU Framework 6 Program NewFluBird (044490), by contracts with the Dutch Ministry of Economic Affairs and a NIAID/NIH CEIRS Travel Grant (HHSN266200700010C). The Swedish sampling and analysis was supported by the Swedish Research Councils VR and FORMAS.

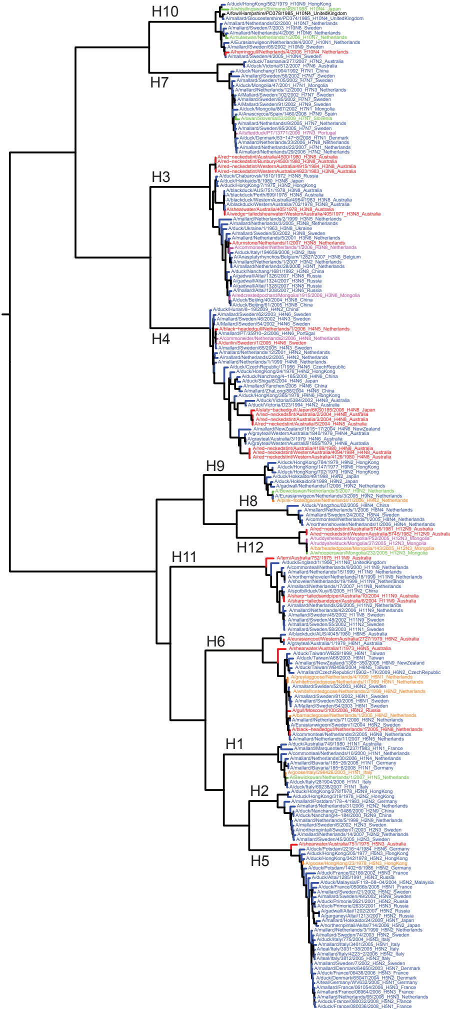
SUPPLEMENTARY MATERIAL

A



Figures S1. Maximum likelihood trees of (A) PB2 and PB2, (B) PA and HA, (C) NP and NA and (D) MP and NS, displaying the genetic diversity of avian influenza A viruses in Eurasian wild birds. The taxa color indicates the bird species group from which the sample was isolated: Dabbling ducks (blue), diving and other ducks (pink), geese (orange), swans (green), fowl (black), gulls and other shorebirds (red).

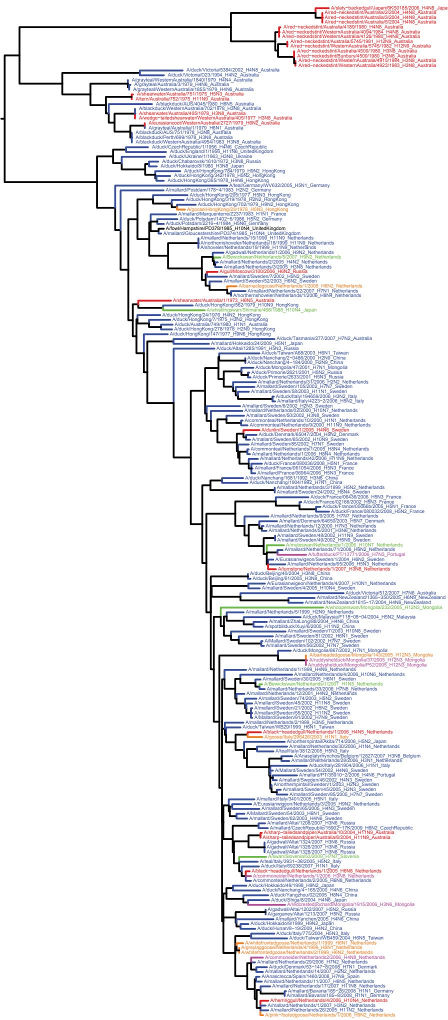
B

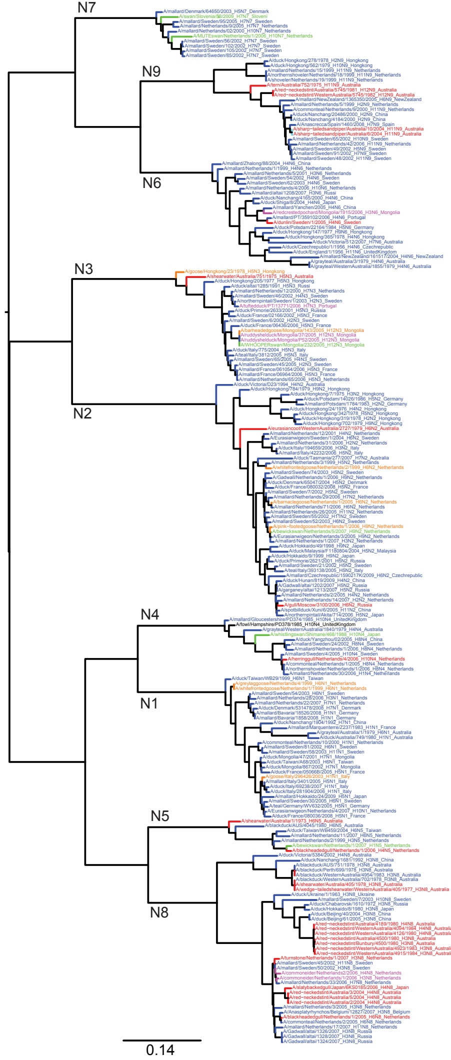


C

NP

NA

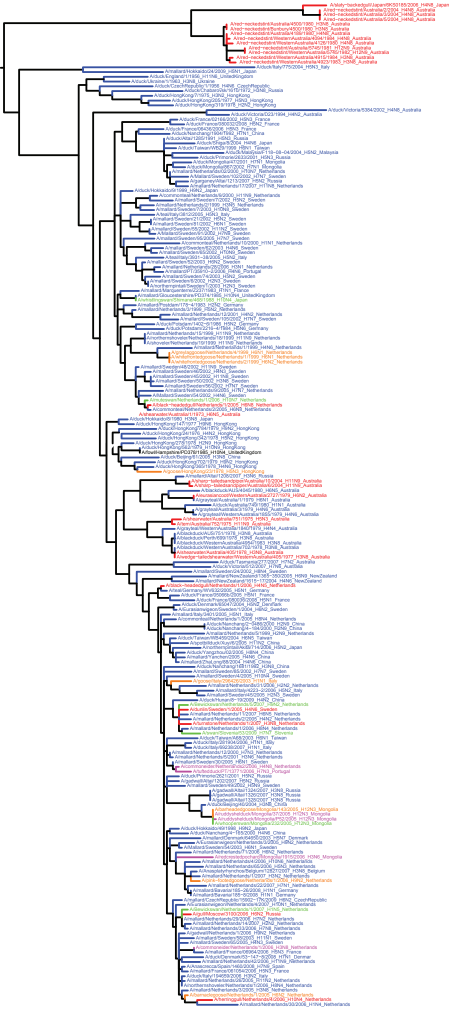




D

MP

NS



0.02

0.2

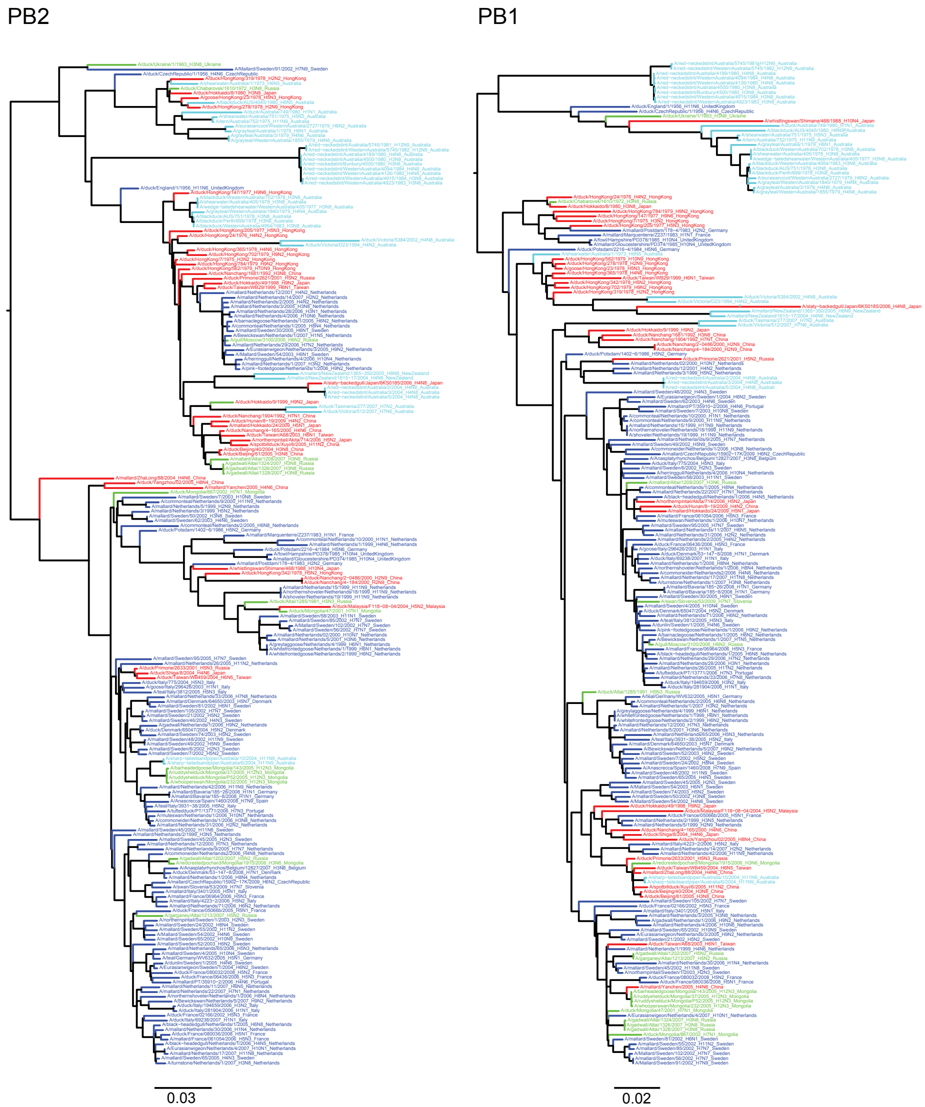
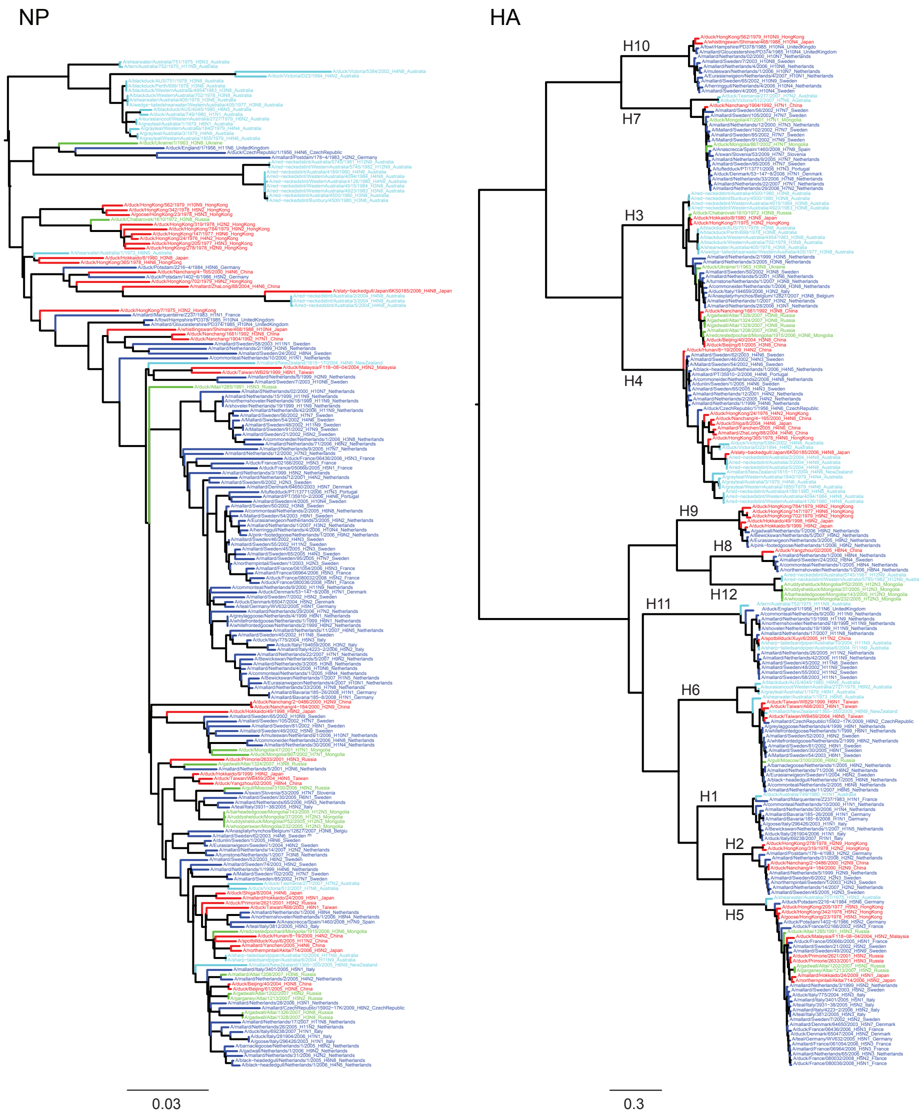
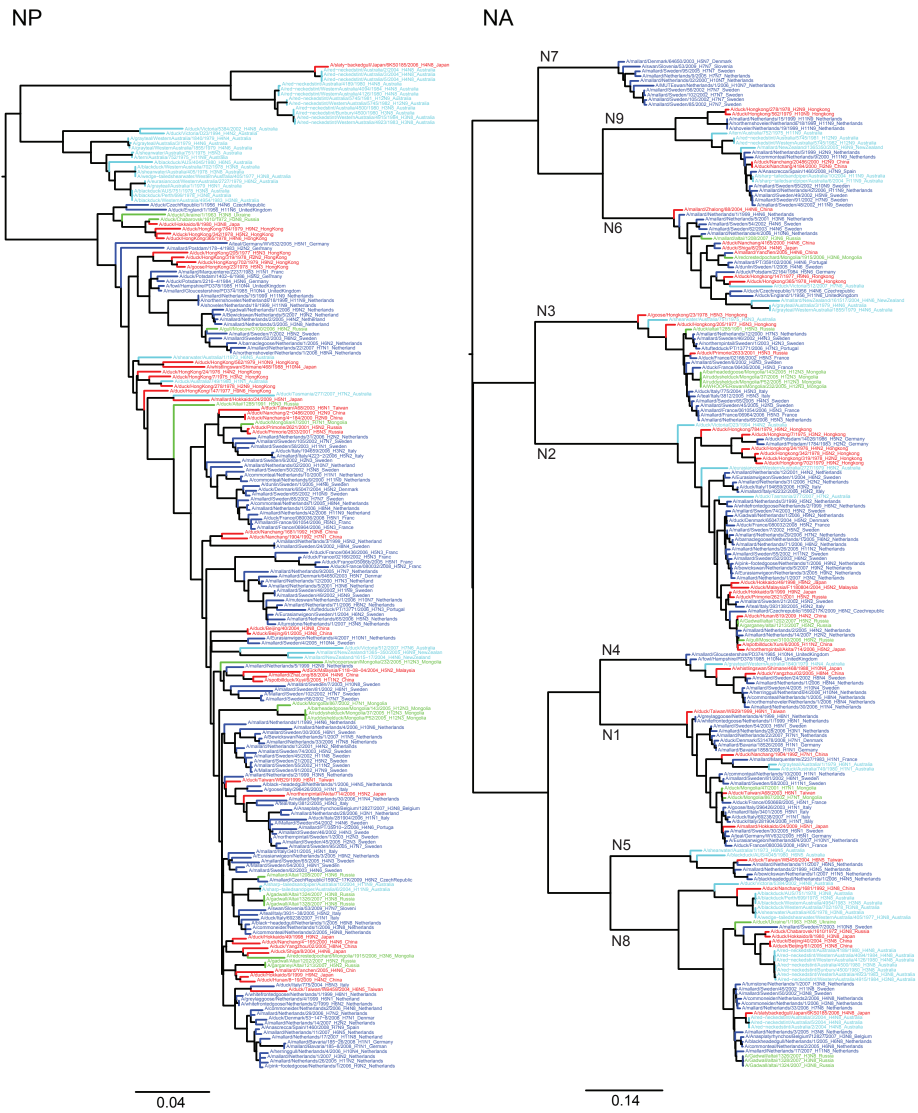


Figure S2. Maximum likelihood trees of (A) PB2 and PB2, (B) PA and HA, (C) NP and NA and (D) MP and NS, displaying the genetic diversity in different locations in Eurasia. The taxa color indicates the regional location from where the sample was isolated: Oceania (cyan), East Eurasia (red), Central Eurasia (green), West Eurasia (blue).

B




C
NP
NA
N7
N9
N6
N3
N2
N4
N1
N5
N8
0.04
0.14

D

MP NS

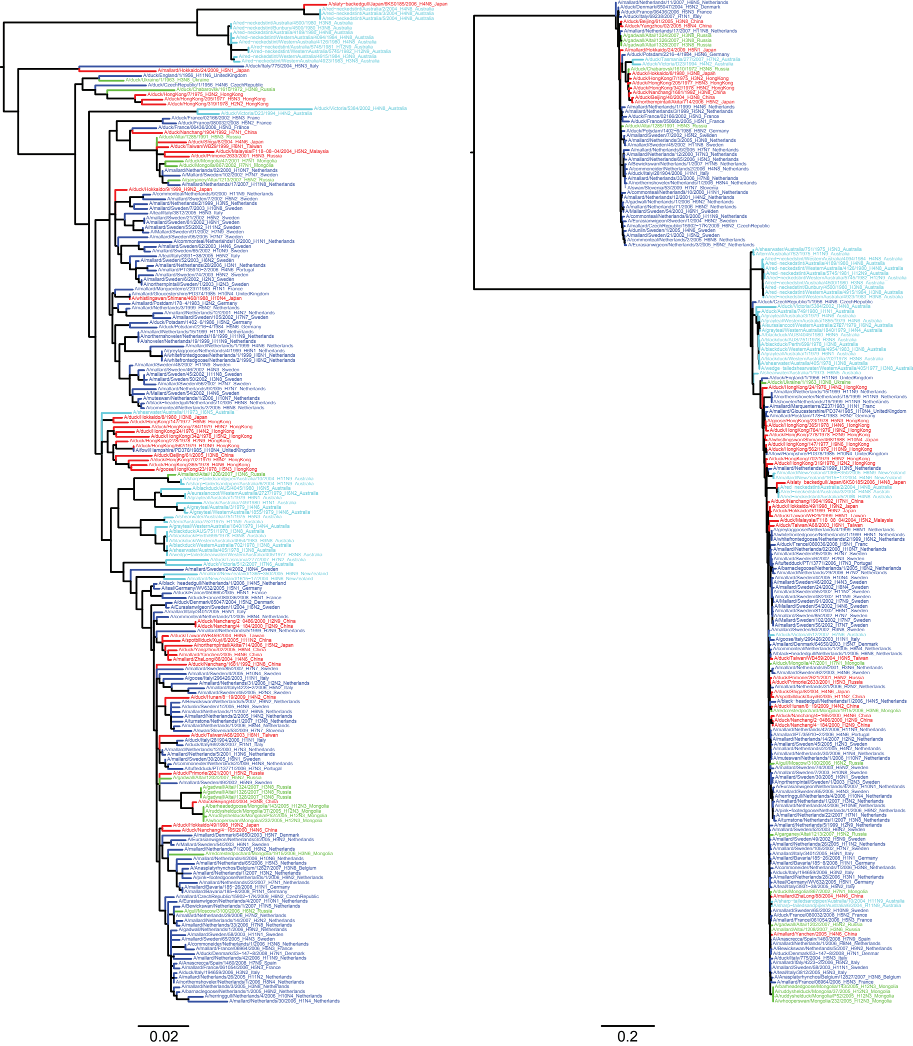
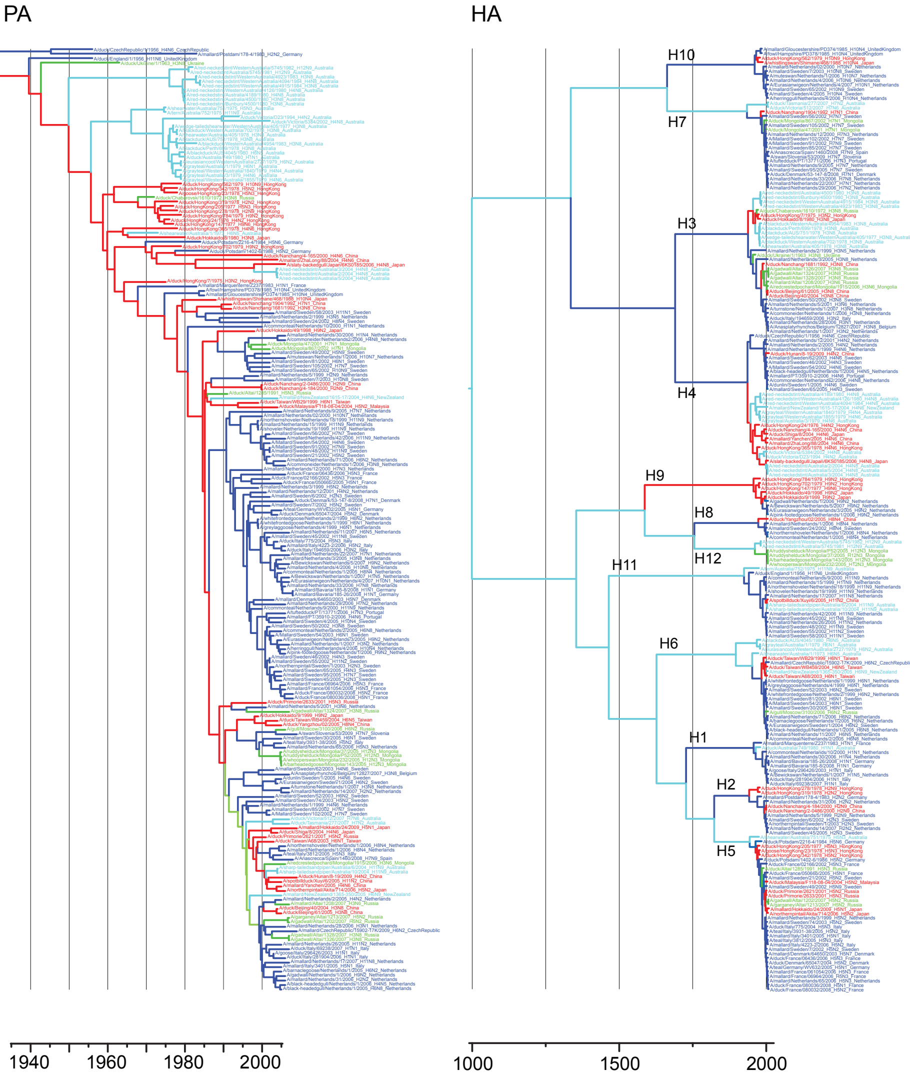
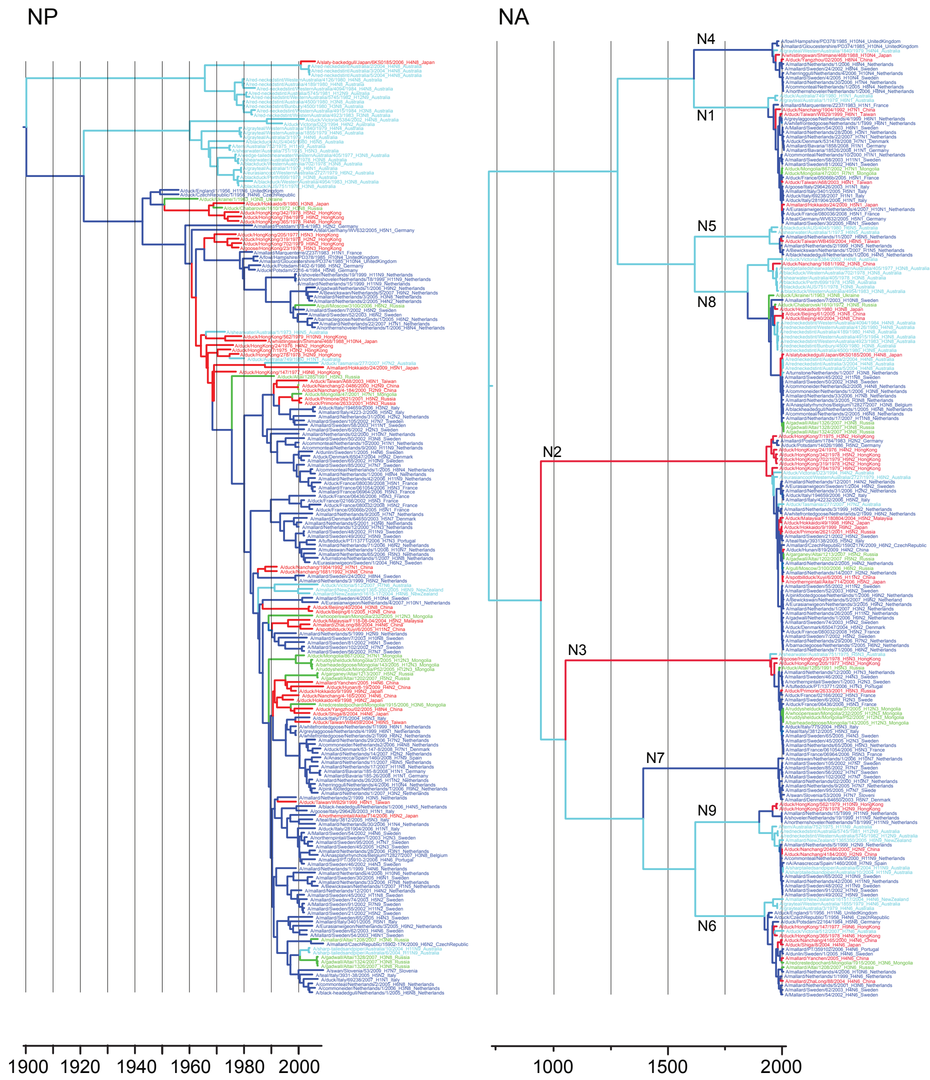


Figure S3. Maximum clade credibility (MCC) trees, summarizing the results of the Bayesian phylogenetic inference of (A) PB2 and PB2, (B) PA and HA, (C) NP and NA and (d) MP and NS, displaying the genetic diversity in different locations in Eurasia. The taxa color indicates the regional location from where the sample was isolated: Oceania (cyan), Central Eurasia (green), East Eurasia (red), West Eurasia (blue). The scale bar represents time in years.

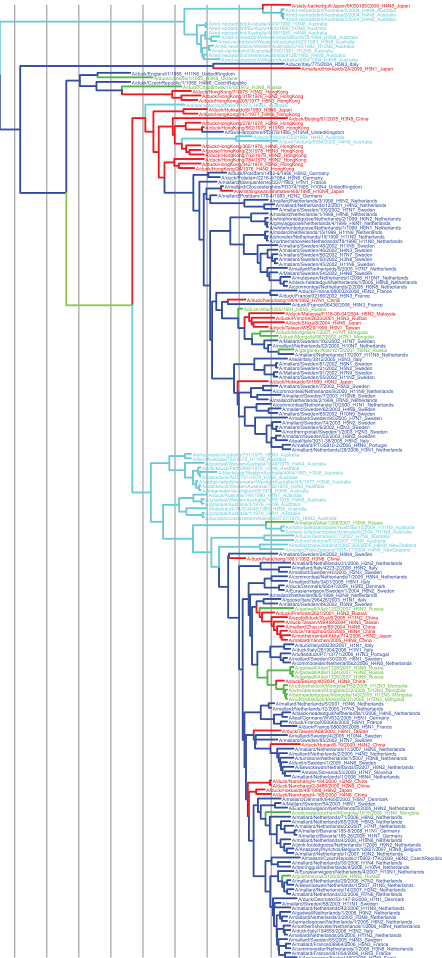
B



C



D



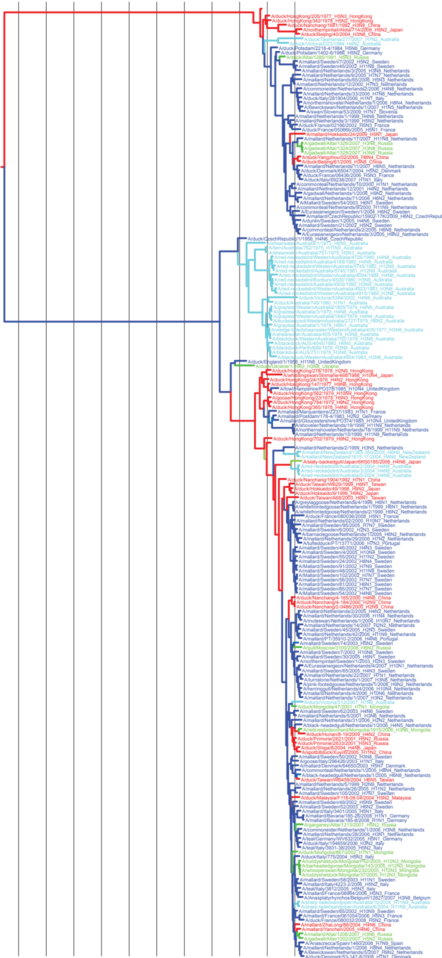


Table S1. Accession numbers

Virus Name	PB2	PB1	PA	HA	NP	NA	MP	NS
A/Anascrecca/Spain/1460/08	HQ244404	HQ244405	HQ244406	HQ244407	HQ244408	HQ244409	HQ244410	HQ244411
A/Anasplatyrhynchos/Belgium/12827/07	HM570065	HM570064	HM570063	HM570058	HM570061	HM570060	HM570059	HM570062
A/barheadedgoose/Mongolia/143/05	GQ907293	GQ907292	GQ907291	GQ907286	GQ907289	GQ907288	GQ907287	GQ907290
A/barnaclegoose/NL/1/05	CY041393	CY041392	CY041391	CY041386	CY041389	CY041388	CY041387	CY041390
A/Bewickswan/NL/1/07	CY076983	CY076982	CY076981	CY076976	CY076979	CY076978	CY076977	CY076980
A/Bewickswan/NL/5/07	CY041281	CY041280	CY041279	CY041274	CY041277	CY041276	CY041275	CY041278
A/blackduck/AUS/4045/80	CY005698	CY005697	CY005696	CY005691	CY005694	CY005693	CY005692	CY005695
A/blackduck/AUS/751/78	CY005659	CY005658	CY005657	CY006031	CY005655	CY005654	CY005653	CY005656
A/blackduck/Perth/699/78	CY028643	CY028642	CY028641	CY028636	CY028639	CY028638	CY028637	CY028640
A/blackduck/WesternAus/4954/83	CY028274	CY028273	CY028272	CY028267	CY028270	CY028269	CY028268	CY028271
A/blackduck/WesternAus/702/78	CY041313	CY041312	CY041311	CY041306	CY041309	CY041308	CY041307	CY041310
A/black-headedgull/NL/1/05	CY041385	CY041384	CY041383	CY041378	CY041381	CY041380	CY041379	CY041382
A/black-headedgull/NL/1/06	CY076999	CY076998	CY076997	CY076992	CY076995	CY076994	CY076993	CY076996
A/commoneider/NL/1/06	CY077007	CY077006	CY077005	CY077000	CY077003	CY077002	CY077001	CY077004
A/commoneider/NL2/06	CY041345	CY041344#	CY041343	CY041338	CY041341	CY041340	CY041339	CY041342
A/commonteal/NL/1/05	CY041265	CY041264	CY041263	CY041258	CY041261	CY041260	CY041259	CY041262
A/commonteal/NL/10/00	CY060175	CY060176	CY060177	CY060178	CY060179	CY060180	CY060181	CY060182
A/commonteal/NL/2/05	CY041377	CY041376	CY041375	CY041370	CY041373	CY041372	CY041371	CY041374
A/commonteal/NL/9/00	CY060187	CY060188	CY060189	CY060190	CY060191	CY060192	CY060193	CY060194
A/duck/Altai/1285/1991	GQ227557	GQ227558	GQ227554	GQ227551	GQ227553	GQ227552	GQ227555	GQ227556
A/duck/Aus/749/80	CY005690	CY005689	CY005688	CY014627	CY005687	CY005686	CY014628	CY014629
A/duck/Beijing/40/04	EU492488	EU492494	EU492500	EU492530	EU492512	EU492518	EU492524	EU492506
A/duck/Beijing/61/05	EU492492	EU492498	EU492504	EU492534	EU492516	EU492522	EU492528	EU492510
A/duck/Chabarovsk/1610/1972	CY014709	CY014708	CY014707	CY014702	CY014705	CY014704	CY014703	CY014706
A/duck/CzechRepublic/1/1956	CY045334	CY045333	CY045332	CY045327	CY045330	CY045329	CY045328	CY045331
A/duck/Denmark/53-147-8/08	GQ401162	GQ401163	GQ401164	GQ401157	GQ401159	GQ401158	GQ401160	GQ401161
A/duck/Denmark/65047/04	DQ251449	DQ251450	DQ251451	DQ251447	DQ251452	DQ251448	DQ251453	DQ251454
A/duck/England/1/1956	CY014686	CY014685	CY014684	CY014679	CY014682	CY014681	CY014680	CY014683
A/duck/France/02166/02	CY046117	CY046118	CY046119	AJ632268	CY046120	AJ849934	AM040281	CY046121
A/duck/France/05066b/05	CY046134	CY046135	CY046136	AJ971297	CY046137	AJ972921	AJ973609	CY046138
A/duck/France/06436/06	CY046147	CY046148	CY046149	CY046150	CY046151	CY046152	CY046153	CY046154
A/duck/France/080032/08	CY046171	CY046172	CY046173	CY046174	CY046175	CY046176	CY046177	CY046178
A/duck/France/080036/08	CY046179	CY046180	CY046181	CY046182	CY046183	CY046184	CY046185	CY046186
A/duck/Hokkaido/49/1998	AB473937	AB473938	AB473939	AB125928	AB473940	AB251944	AB473941	AB473942
A/duck/Hokkaido/8/80	AB274963	AB274964	AB274965	AB275283	AB275284	AB275285	AB275286	AB275287
A/duck/Hokkaido/9/99	AB262460	AB262461	AB262462	AB262463	AB262464	AB262465	AB262466	AB262467
A/duck/HongKong/147/77	CY005646	CY005645	CY005645	CY005639	CY005642	CY005641	CY005640	CY005643
A/duck/HongKong/205/77	CY005596	CY005595	CY005594	CY014615	CY005592	CY005591	CY005590	CY005593
A/duck/HongKong/24/1976	CY005631	CY005630	CY005629	CY006030	CY005627	CY005626	CY005625	CY005628
A/duck/HongKong/278/78	CY014614	CY005552	CY005551	CY005546	CY005549	CY005548	CY005547	CY005550
A/duck/HongKong/319/78	CY005545	CY005544	CY005543	CY005538	CY005541	CY005540	CY005539	CY005542

Virus Name	PB2	PB1	PA	HA	NP	NA	MP	NS
A/duck/HongKong/342/78	CY005582	CY005581	CY005580	CY005575	CY005578	CY005577	CY005576	CY005579
A/duck/HongKong/365/78	CY005574	CY005573	CY005572	CY006027	CY005570	CY005569	CY005568	CY005571
A/duck/HongKong/562/79	CY005652	CY005651	CY005650	CY014619	CY005648	CY014620	CY005647	CY005649
A/duck/HongKong/7/75	CY005559	CY005558	CY005557	CY006026	CY005555	CY005554	CY005553	CY005556
A/duck/HongKong/702/79	CY031256	CY031257	CY031258	CY031259	CY031260	CY031261	CY031262	CY031263
A/duck/HongKong/784/79	CY005638	CY005637	CY005636	CY005632	CY005634	CY014618	CY005633	CY005635
A/duck/Hunan/8-19/09	HQ285883	HQ285884	HQ285885	HQ285886	HQ285887	HQ285888	HQ285889	HQ285890
A/duck/Italy/194659/06	FJ432769	FJ432768	FJ432767	FJ432762	FJ432765	FJ432764	FJ432763	FJ432766
A/duck/Italy/281904/06	FJ432777	FJ432776	FJ432775	FJ432770	FJ432773	FJ432772	FJ432771	FJ432774
A/duck/Italy/69238/07	FJ432761	FJ432760	FJ432759	FJ432754	FJ432757	FJ432756	FJ432755	FJ432758
A/duck/Italy/775/04	CY024753	CY024752	CY024751	CY024746	CY024749	CY024748	CY024747	CY024750
A/duck/Malaysia/F118-08-04/04	EU249545	EU249546	EU249547	DQ104701	EU249548	DQ104703	EU249550	EU249549
A/duck/Mongolia/47/01	AB473548	AB268552	AB268553	AB268557	AB268554	AB302788	AB268555	AB268556
A/duck/Mongolia/867/02	AB473540	AB473541	AB473542	AB473543	AB473544	AB473545	AB473546	AB473547
A/duck/Nanchang/1681/1992	CY005475	CY005474	CY005473	CY006016	CY005471	CY005470	CY005469	CY005472
A/duck/Nanchang/1904/1992	CY005500	CY005499	CY005498	CY014612	CY005496	CY005495	CY005494	CY005497
A/duck/Nanchang/2-0486/00	CY005437	CY005436	CY005435	CY014608	CY005433	CY005432	CY005431	CY005434
A/duck/Nanchang/4-165/00	CY005493	CY005492	CY005491	CY006017	CY005489	CY005488	CY005487	CY005490
A/duck/Nanchang/4-184/00	CY005444	CY005443	CY005442	CY014609	CY005440	CY005439	CY005438	CY005441
A/duck/Potsdam/1402-6/1986	CY005783	CY005782	CY005781	CY014642	CY005779	CY005778	CY005777	CY005780
A/duck/Potsdam/2216-4/84	CY005776	CY005775	CY005774	CY006036	CY005772	CY005771	CY005770	CY005773
A/duck/Primorie/2621/01	GQ162793	GQ162792	GQ162791	GQ162786	GQ162790	GQ162788	GQ162787	GQ162789
A/duck/Primorie/2633/01	GQ227610	GQ227611	GQ227607	GQ227604	GQ227606	GQ227605	GQ227608	GQ227609
A/duck/Shiga/8/04	AB304144	AB304145	AB304146	AB304147	AB304148	AB304149	AB304150	AB304151
A/duck/Taiwan/A68/03	DQ376898	DQ376862	DQ376826	DQ376646	DQ376754	DQ376718	DQ376682	DQ376790
A/duck/Taiwan/WB29/99	DQ376879	DQ376843	DQ376807	DQ376627	DQ376735	DQ376699	DQ376663	DQ376771
A/duck/Taiwan/WB459/04	DQ376903	DQ376867	DQ376831	DQ376651	DQ376759	DQ376723	DQ376687	DQ376795
A/duck/Tasmania/277/07	CY033168	CY033167	CY033166	CY033161	CY033164	CY033163	CY033162	CY033165
A/duck/Ukraine/1/1963	CY005819	CY005818	CY005817	CY006038	CY005815	CY014648	CY005814	CY005816
A/duck/Victoria/512/07	CY061617	CY061616	CY061615	CY061610	CY061613	CY061612	CY061611	CY061614
A/duck/Victoria/5384/02	CY028258	CY028257	CY028256	CY028251	CY028254	CY028253	CY028252	CY028255
A/duck/Victoria/D23/1994	CY045254	CY045253	CY045252	CY045247	CY045250	CY045249	CY045248	CY045251
A/duck/Yangzhou/02/05	EF061121	EF061124	EF061120	EF061122	EF061123	EF061126	EF061125	EF061119
A/dunlin/Sweden/1/05	CY076991	CY076990	CY076989	CY076984	CY076987	CY076986	CY076985	CY076988
A/eurasiancoot/WesternAus/2727/79	CY028250	CY028249	CY028248	CY028243	CY028246	CY028245	CY028244	CY028247
A/Eurasianwigeon/NL/3/05	CY043863	CY043862	CY043861	CY043856	CY043859	CY043858	CY043857	CY043860
A/Eurasianwigeon/NL/4/07	CY077055	CY077054	CY077053	CY077048	CY077051	CY077050	CY077049	CY077052
A/Eurasianwigeon/Sweden/1/04	CY041369	CY041368	CY041367	CY041362	CY041365	CY041364	CY041363	CY041366
A/fowl/Hampshire/PD378/1985	GQ176113	GQ176114	GQ176115	GQ176120	GQ176117	GQ176118	GQ176119	GQ176116
A/gadwall/Altai/1202/07	CY049753	CY049754	CY049755	CY049756	CY049757	CY049758	CY049759	CY049760
A/gadwall/Altai/1324/07	CY049785	CY049786	CY049787	CY049788	CY049789	CY049790	CY049791	CY049792
A/gadwall/Altai/1326/07	CY049801	CY049802	CY049803	CY049804	CY049805	CY049806	CY049807	CY049808
A/gadwall/Altai/1328/07	CY049809	CY049810	CY049811	CY049812	CY049813	CY049814	CY049815	CY049816

Virus Name	PB2	PB1	PA	HA	NP	NA	MP	NS
A/gadwall/NL/1/06	CY043871	CY043870	CY043869	CY043864	CY043867	CY043866	CY043865	CY043868
A/garganey/Altai/1213/07	CY049769	CY049770	CY049771	CY049772	CY049773	CY049774	CY049775	CY049776
A/goose/HongKong/23/78	CY005589	CY005588	CY005587	CY006028	CY005585	CY005584	CY005583	CY005586
A/goose/Italy/296426/03	FJ432785	FJ432784	FJ432783	FJ432778	FJ432781	FJ432780	FJ432779	FJ432782
A/grayteal/Aus/1/79	CY005671	CY005670	CY005669	CY014623	CY005667	CY014624	CY005666	CY005668
A/grayteal/Aus/3/79	CY005685	CY005684	CY005684	CY005679	CY005682	CY005681	CY005680	CY005683
A/grayteal/WesternAus/1840/79	CY045270	CY045269	CY045268	CY045263	CY045266	CY045265	CY045264	CY045267
A/grayteal/WesternAus/1855/79	CY031163	CY031162	CY031161	CY031156	CY031159	CY031158	CY031157	CY031160
A/greylaggoose/NL/4/99	CY060195	CY060196	CY060197	CY060198	CY060199	CY060200	CY060201	CY060202
A/gull/Moscow/3100/06	EU152234	EU152235	EU152236	EU152237	EU152238	EU152239	EU152240	EU152241
A/herringgull/NL/4/06	CY077039	CY077038	CY077037	CY077032	CY077035	CY077034	CY077033	CY077036
A/mallard/Altai/1208/07	CY049761	CY049762	CY049763	CY049764	CY049765	CY049766	CY049767	CY049768
A/mallard/Bavaria/185-26/08	HQ259229	HQ259230	HQ259231	HQ259232	HQ259233	HQ259234	HQ259235	HQ259236
A/mallard/Bavaria/185-8/08	HQ259221	HQ259222	HQ259223	HQ259224	HQ259225	HQ259226	HQ259227	HQ259228
A/mallard/CzechRepublic/15902-17K/09	HQ244427	HQ244428	HQ244429	HQ244430	HQ244431	HQ244432	HQ244433	HQ244434
A/mallard/Denmark/64650/03	DQ251441	DQ251442	DQ251443	HE802063	DQ251444	DQ251445	AY531030	DQ251446
A/mallard/France/061054/06	CY046139	CY046140	CY046141	CY046142	CY046143	CY046144	CY046145	CY046146
A/mallard/France/06964/06	CY046155	CY046156	CY046157	CY046158	CY046159	CY046160	CY046161	CY046162
A/mallard/Gloucestershire/PD374/1985	GQ176121	GQ176122	GQ176123	GQ176128	GQ176125	GQ176126	GQ176127	GQ176124
A/mallard/Hokkaido/24/09	AB530989	AB530990	AB530991	AB530992	AB530993	AB530994	AB530995	AB530996
A/mallard/Italy/3401/05	CY021404	CY021403	CY021402	CY021397	CY021400	CY021399	CY021398	CY021401
A/mallard/Italy/4223-2/06	CY034765	CY034764	CY034763	CY034758	CY034761	CY034760	CY034759	CY034762
A/mallard/Marquenterre/Z237/83	DQ864507	DQ864506	DQ864508	GU066779	DQ864509	GU066780	GU066781	GU066782
A/mallard/NL/02/00	CY076952	CY076951	CY076950	CY076945	CY076948	CY076947	CY076946	CY076949
A/mallard/NL/1/99	CY060238	CY060239	CY060240	CY060241	CY060242	CY060243	CY060244	CY060245
A/mallard/NL/1/06	CY043855	CY043854	CY043853	CY043848	CY043851	CY043850	CY043849	CY043852
A/mallard/NL/1/07	CY043823	CY043822	CY043821	CY043816	CY043819	CY043818	CY043817	CY043820
A/mallard/NL/11/07	CY041409	CY041408	CY041407	CY041402	CY041405	CY041404	CY041403	CY041406
A/mallard/NL/12/00	KF695236	KF695237	KF695238	KF695239	KF695240	KF695241	KF695242	KF695243
A/mallard/NL/12/01	CY060217	CY060218	CY060219	CY060220	CY060221	KF695235	CY060222	CY060223
A/mallard/NL/14/07	CY041241	CY041240	CY041239	CY041234	CY041237	CY041236	CY041235	CY041238
A/mallard/NL/15/99	CY060224	CY060225	CY060226	CY060227	CY060228	CY189928	CY060229	CY060230
A/mallard/NL/17/07	CY043887	CY043886	CY043885	CY043880	CY043883	CY043882	CY043881	CY043884
A/mallard/NL/2/99	CY060258	CY060259	CY060260	CY060261	CY060262	CY060263	CY060264	CY060265
A/mallard/NL/2/05	CY041257	CY041256	CY041255	CY041250	CY041253	CY041252	CY041251	CY041254
A/mallard/NL/22/07	CY043847	CY043846	CY043845	CY043840	CY043843	CY043842	CY043841	CY043844
A/mallard/NL/26/05	CY041425	CY041424	CY041423	CY041418	CY041421	CY041418	CY041419	CY041422
A/mallard/NL/28/06	CY076912	CY076911	CY076910	CY076905	CY076908	CY076907	CY076906	CY076909
A/mallard/NL/29/06	CY043839	CY043838	CY043837	CY043832	CY043835	CY043834	CY043833	CY043836
A/mallard/NL/3/99	CY064939	CY064940	CY064941	CY064942	CY064943	CY064944	CY064945	CY064946
A/mallard/NL/3/05	CY041249	CY041248	CY041247	CY041242	CY041245	CY041244	CY041243	CY041246
A/mallard/NL/30/06	CY076904	CY076903	CY076902	CY076897	CY076900	CY076899	CY076898	CY076901
A/mallard/NL/31/06	CY041233	CY041232	CY041231	CY041226	CY041229	CY041228	CY041227	CY041230

Virus Name	PB2	PB1	PA	HA	NP	NA	MP	NS
A/mallard/NL/33/06	CY041417	CY041416	CY041415	CY041410	CY041413	CY041412	CY041411	CY041414
A/mallard/NL/4/06	CY076959	CY076958	CY076957	CY077064	CY076955	CY076954	CY076953	CY076956
A/mallard/NL/42/06	CY077063	CY077062	CY077061	CY077056	CY077059	CY077058	CY077057	CY077060
A/mallard/NL/5/99	CY064947	CY064948	CY064949	CY064950	CY064951	CY064952	CY064953	CY064954
A/mallard/NL/5/01	CY060340	CY060341	CY060342	CY060343	CY060344	KF695299	CY060345	CY060346
A/mallard/NL/65/06	CY076944	CY076943	CY076942	CY076937	CY076940	CY076939	CY076938	CY076941
A/mallard/NL/71/06	CY041401	CY041400	CY041399	CY041394	CY041397	CY041396	CY041395	CY041398
A/mallard/NL/9/05	CY077015	CY077014	CY077013	CY077008	CY077011	CY077010	CY077009	CY077012
A/mallard/NewZealand/1365-350/05	CY077591	CY077590	CY077589	CY077584	CY077587	CY077586	CY077585	CY077588
A/mallard/NewZealand/1615-17/04	CY045366	CY045365	CY045364	CY045359	CY045362	CY045361	CY045360	CY045363
A/mallard/Postdam/178-4/83	DQ017501	DQ017500	DQ017499	DQ017496	DQ017497	DQ017496	DQ017494	DQ017498
A/mallard/PT/35910-2/06	HM849024	HM849023	HM849022	HM849017	HM849020	HM849019	HM849018	HM849021
A/Mallard/Sweden/102/02	KF695282	KF695283	KF695284	KF695285	KF695286	KF695287	KF695288	KF695289
A/mallard/Sweden/105/02	KF695333	KF695334	KF695335	KF695336	KF695337	KF695338	KF695339	KF695340
A/mallard/Sweden/21/02	KF695267	KF695268	KF695269	KF695270	KF695271	KF695272	KF695273	KF695274
A/mallard/Sweden/24/02	CY060246	CY060247	CY060248	CY060249	KF695204	CY064796	CY060250	CY060251
A/mallard/Sweden/30/05	CY043831	CY043830	CY043829	CY043824	CY043827	CY043826	CY043825	CY043828
A/mallard/Sweden/4/05	CY043879	CY043878	CY043877	CY043872	CY043875	CY043874	CY043873	CY043876
A/mallard/Sweden/45/02	CY060278	CY060279	CY060280	CY060281	CY060282	CY060283	CY060284	KF695311
A/mallard/Sweden/45/05	CY041337	CY041336	CY041335	CY041330	CY041333	CY041332	CY041331	CY041334
A/mallard/Sweden/46/02	CY060285	CY060286	CY060287	CY060288	CY060289	KF695218	CY060290	KF695219
A/mallard/Sweden/48/02	CY060291	CY060292	CY060293	CY060294	CY060295	CY060296	CY060297	CY060298
A/mallard/Sweden/49/02	KF695325	KF695326	KF695327	KF695328	KF695329	KF695330	KF695331	KF695332
A/mallard/Sweden/50/02	CY060305	CY060306	CY060307	CY060308	CY060309	CY060310	CY060311	CY060312
A/mallard/Sweden/52/03	CY060313	CY060314	CY060315	CY060316	CY060317	CY060318	CY060319	CY060320
A/Mallard/Sweden/54/02	KF695341	KF695342	KF695343	KF695344	KF695345	CY189927	KF695346	KF695347
A/Mallard/Sweden/54/03	KF695220	KF695221	KF695222	KF695223	KF695224	KF695225	KF695226	KF695227
A/mallard/Sweden/55/02	CY060321	CY060322	CY064797	CY060323	CY060324	CY060325	CY060326	CY060327
A/mallard/Sweden/56/02	KF695351	KF695352	KF695353	KF695354	KF695355	KF695356	KF695357	KF695358
A/mallard/Sweden/58/03	CY060332	CY060333	CY060334	CY060335	CY060336	CY060337	CY060338	CY060339
A/mallard/Sweden/6/02	CY060363	CY060364	CY060365	CY060366	CY060367	CY060368	CY060369	CY060370
A/mallard/Sweden/62/03	CY060347	CY060348	CY060349	CY060350	CY060351	CY060352	CY060353	CY060354
A/mallard/Sweden/65/02	CY060355	CY060356	CY060357	CY060358	CY060359	CY060360	CY060361	CY060362
A/mallard/Sweden/65/05	CY041361	CY041360	CY041359	CY041354	CY041357	CY041356	CY041355	CY041358
A/mallard/Sweden/7/02	KF695300	KF695301	KF695302	KF695303	KF695304	KF695305	KF695306	KF695307
A/mallard/Sweden/7/03	CY060371	CY060372	CY060373	CY060374	CY060375	CY060376	CY060377	CY060378
A/mallard/Sweden/74/03	CY076936	CY076935	CY076934	CY076929	CY076932	CY076931	CY076930	CY076933
A/mallard/Sweden/81/02	CY060379	CY060380	CY060381	CY060382	CY060383	CY060384	CY060385	CY060386
A/mallard/Sweden/85/02	KF695290	KF695291	KF695292	KF695293	KF695294	KF695295	KF695296	KF695297
A/Mallard/Sweden/91/02	KF695251	KF695252	KF695253	KF695254	KF695255	KF695256	KF695257	KF695258
A/mallard/Sweden/95/05	CY077023	CY077022	CY077021	CY077016	CY077019	CY077018	CY077017	CY077020
A/mallard/Yanchen/05	EU880349	EU880348	EU880347	EU880342	EU880345	EU880344	EU880343	EU880346
A/mallard/ZhaLong/88/04	FJ349254	FJ349253	FJ349252	FJ349247	FJ349249	FJ349248	FJ349251	FJ349250

Virus Name	PB2	PB1	PA	HA	NP	NA	MP	NS
A/muteswan/NL/1/06	CY077047	CY077046	CY077045	CY077040	CY077043	CY077042	CY077041	CY077044
A/northernpintail/Akita/714/06	AB490823	AB490824	AB490825	AB490826	AB490827	AB490828	AB490829	AB490830
A/northernpintail/Sweden/1/03	CY060421	CY060422	CY060423	CY060424	CY060425	CY060426	CY060427	KF695298
A/northernshoveler/NL/1/06	CY077031	CY077030	CY077029	CY077024	CY077027	CY077026	CY077025	CY077028
A/northernshoveler/NL/18/99	CY060414	CY060415	CY060416	CY060417	CY060418	CY189928	CY060419	CY060420
A/pink-footedgoose/NL/1/06	CY041273	CY041272	CY041271	CY041266	CY041269	CY041268	CY041267	CY041270
A/redcrestedpochard/Mongolia/1915/06	GQ907333	GQ907332	GQ907331	GQ907326	GQ907329	GQ907328	GQ907327	GQ907330
A/red-neckedstint/Aus/2/04	CY028266	CY028265	CY028264	CY028259	CY028262	CY028261	CY028260	CY028263
A/red-neckedstint/Aus/3/04	CY034749	CY034748	CY034747	CY034742	CY034745	CY034744	CY034743	CY034746
A/red-neckedstint/Aus/4189/80	CY014632	CY005703	CY005702	CY014630	CY005700	CY014631	CY005699	CY005701
A/red-neckedstint/Aus/4500/80	CY014635	CY005708	CY005707	CY014633	CY005705	CY014634	CY005704	CY005706
A/red-neckedstint/Aus/5/04	CY029896	CY029895	CY029894	CY029889	CY029892	CY029891	CY029890	CY029893
A/red-neckedstint/Aus/5745/81	CY005715	CY005714	CY005713	CY014636	CY005711	CY005710	CY005709	CY005712
A/red-neckedstint/Bunbury/4500/80	CY028282	CY028281	CY028280	CY028275	CY028278	CY028277	CY028276	CY028279
A/red-neckedstint/WesternAus/4094/84	CY035897	CY035896	CY035895	CY035890	CY035893	CY035892	CY035891	CY035894
A/red-neckedstint/WesternAus/4126/80	CY033160	CY033159	CY033158	CY033153	CY033156	CY033155	CY033154	CY033157
A/red-neckedstint/WesternAus/4915/84	CY029904	CY029903	CY029902	CY029897	CY029900	CY029899	CY029898	CY029901
A/red-neckedstint/WesternAus/4923/83	CY028290	CY028289	CY028288	CY028283	CY028286	CY028285	CY028284	CY028287
A/red-neckedstint/WesternAus/5745/82	CY029880	CY029879	CY029878	CY029873	CY029876	CY029875	CY029874	CY029877
A/ruddyshelduck/Mongolia/37/05	GQ907341	GQ907340	GQ907339	GQ907334	GQ907337	GQ907336	GQ907335	GQ907338
A/ruddyshelduck/Mongolia/P52/05	GQ907349	GQ907348	GQ907347	GQ907342	GQ907345	GQ907344	GQ907343	GQ907346
A/sharp-tailedsandpiper/Aus/10/04	CY029888	CY029887	CY029886	CY029881	CY029884	CY029883	CY029882	CY029885
A/sharp-tailedsandpiper/Aus/6/04	CY025204	CY025203	CY025202	CY025197	CY025200	CY025199	CY025198	CY025201
A/shearwater/Aus/1/1973	CY005827	CY005826	CY005825	CY014656	CY014658	CY014657	CY005823	CY005824
A/shearwater/Aus/405/78	CY005665	CY005664	CY005663	CY014621	CY005661	CY014622	CY005660	CY005662
A/shearwater/Aus/751/75	CY045262	CY045261	CY045260	CY045255	CY045258	CY045257	CY045256	CY045259
A/shoveler/NL/19/99	CY005858	CY005857	CY005856	CY014719	CY005854	CY005853	CY005852	CY005855
A/slaty-backedgull/Japan/6KS0185/06	CY079298	CY079297	CY079296	CY079291	CY079294	CY079293	CY079292	CY079295
A/spotbillduck/Xuyi/6/05	GQ203122	GQ203123	GQ203124	GQ184327	GQ169500	GQ184332	GQ219713	GQ219714
A/swan/Slovenia/53/09	HQ283354	HQ283355	HQ283356	HQ283357	HQ283358	HQ283359	HQ283360	HQ283361
A/teal/Germany/WV632/05	CY061882	CY061883	CY061884	CY061885	CY061886	CY061887	CY061888	CY061889
A/teal/Italy/3812/05	CY022652	CY022651	CY022650	CY022645	CY022648	CY022647	CY022646	CY022649
A/teal/Italy/3931-38/05	CY022644	CY022643	CY022642	CY022637	CY022640	CY022639	CY022638	CY022641
A/tern/Aus/752/75	CY077659	CY077658	CY077657	CY077650	CY077655	CY077654	CY077653	CY077656
A/tuftedduck/PT/13771/06	HM849010	HM849009	HM849008	HM849003	HM849006	HM849005	HM849004	HM849007
A/turnstone/NL/1/07	CY041353	CY041352	CY041351	CY041346	CY041349	CY041348	CY041347	CY041350
A/wedge-tailedshearwater/ WesternAus/405/77	CY028659	CY028658	CY028657	CY028652	CY028655	CY028654	CY028653	CY028656
A/whistlingswan/Shimane/468/88	GQ176105	GQ176106	GQ176107	GQ176112	GQ176109	GQ176110	GQ176111	GQ176108
A/whitefrontedgoose/NL/1/99	CY060428	CY060429	CY060430	CY060431	CY060432	CY060433	CY060434	CY060435
A/whitefrontedgoose/NL/2/99	CY060436	CY060437	CY060438	CY060439	CY060440	CY060441	CY060442	CY060443
A/whooperswan/Mongolia/232/05	GQ907357	GQ907356	GQ907355	GQ907350	GQ907353	GQ907352	GQ907351	GQ907354

Submitted as draft sequence, due to low coverage, low quality, ambiguities, frameshifts, gaps, or other problems.

Table S2. Statistically supported migration rates between regions for the whole-genome dataset

	PB2	PB1	PA	HA	NP	NA	MP	NS
East to West	0.64*† (0.01:1.62)		2.61 (0.29:5.35)	1.58 (0.10:3.47)			1.43 (0.03:3.24)	2.05 (0.44:4.16)
East to Central	0.93 (0.07:2.11)	0.87 (0.05:1.99)	0.76 (0.04:1.74)	0.65 (0.00:1.55)	1.48 (0.16:3.08)			0.71 (0.02:1.71)
East to Oceania	2.55 (0.67:4.81)	1.86 (0.40:3.67)	1.45 (0.24:3.01)	1.59 (0.19:3.27)	1.37 (0.16:3.08)	0.89 (0.00:1.97)	1.15 (0.04:2.56)	
West to Central	1.4 (0.25:2.81)	1.16 (0.13:2.40)	1.23 (0.18:2.54)	0.87 (0.12:1.84)	1 (0.01:2.21)	0.97 (0.78:1.98)	1.42 (0.16:2.91)	1.6 (0.37:3.11)
West to East	1.58 (0.35:3.12)	2.12 (0.39:4.27)		2.05 (0.47:3.96)	2.13 (0.11:4.27)	2.52 (0.56:4.69)	1.85 (0.24:3.83)	1.88 (0.39:3.70)
West to Oceania						0.73 (0.00:1.66)		0.87 (0.02:1.90)
Central to West								
Central to East				0.81 (0.00:199)			1.06 (0.00:2.66)	
Central to Oceania								
Oceania to West				1.16 (0.02:2.35)		0.95 (0.16:1.95)		
Oceania to Central								
Oceania to East					0.6 (0.00:1.52)		0.92 (0.00:2.19)	

* Only migration rates with a BF higher than 8 are shown, † Migration rates with BF higher than 100 are depicted in bold

CHAPTER 2.3
Josanne H. Verhagen, Frank Majoor, Pascal Lexmond, Oanh Vuong
Giny Kasemir, Date Lutterop, Ron A.M. Fouchier
Thijs Kuiken

Epidemiology of influenza A virus among black-headed gulls, the Netherlands, 2006 - 2010

Emerging Infectious Diseases (2014), Volume 20, Issue 1: 138-141. DOI:10.3201/eid2001.130984

We sampled 7,511 black-headed gulls (*Chroicocephalus ridibundus*) for influenza virus in the Netherlands during 2006–2010 and found that subtypes H13 and H16 caused annual epidemics in fledglings on colony sites. Our findings validate targeted surveillance of wild waterbirds and clarify underlying factors for influenza virus emergence in other species.

INTRODUCTION

Wild waterbirds of the orders *Anseriformes* (ducks, geese, swans) and *Charadriiformes* (gulls, terns, shore birds) are the ultimate source of influenza A viruses for domestic birds and mammals, including humans (12). Knowledge of the epidemiology of these avian influenza viruses (AIVs) among wild waterbirds is necessary to improve surveillance and better clarify underlying factors in host-switching of AIV. Epidemiology of AIV in wild waterbirds has been studied mainly among ducks (order *Anseriformes*) (207) but is poorly known among gulls, despite their abundance and close association with humans (208). Therefore, we studied the epidemiology of AIV in one of the most common gull species in western Europe, the black-headed gull (*Chroicocephalus ridibundus*).

THE STUDY

Black-headed gulls (n = 7,511) were sampled year- round at multiple locations in the Netherlands during 2006–2010. Birds were captured by hand, leg-noose, or clap net; then, we determined their sex and age (first-year (FY) bird: nestling, fledgling; after first-year (AFY) bird) and weighed them. During the breeding season (April–July), 2,839 FY and 524 AFY birds were sampled at colony breeding sites. Three breeding sites were monitored annually during 2008–2010: Griend, De Kreupel, and Veluwemeer. At Griend, BHGU breeding success was also measured and used to compare breeding chronology to timing of infection (Technical Appendix). Outside the breeding season, 1,200 FY and 2,948 AFY birds were sampled in meadows and cities. Cloacal and oropharyngeal swab samples were collected from each bird and tested for AIV by using matrix (M)–specific reverse transcription PCR (RT-PCR) and, if positive, for H5 and H7 subtypes by using hemagglutinin (HA)-specific RT-PCR. Virus culture was attempted on all M RT-PCR–positive samples by egg inoculation. Virus isolates were classified to HA subtype by hemagglutination inhibition assay and to neuraminidase (NA) subtype by using RT-PCR (151, 157). Blood samples were collected from an arbitrary subset of 134 FY and 214 AFY birds and tested for anti-AIV antibody by nucleoprotein (NP)–specific ELISA (209). Statistics were performed by using software RStudio version 0.95.265 (168, 210). Additional analyses on AIV prevalence among male versus female birds, dead versus live birds, recaptured birds, and capture bias were performed (Technical Appendix).

Our results showed that AIV epidemics in black-headed gulls occurred annually during June and July, with a peak monthly prevalence of 47% during 2008 (Figure 1, Table 1). These epidemics were detected in FY birds only and were limited to subtypes H13 and H16; subtype H13 and H16 viruses represented 100% of all virus isolates and 55% of RT-PCR positive birds. In contrast, no AIVs were detected in 524 AFY birds sampled during the breeding season. Annual epidemics were detected in 2 of 3 colonies sampled annually during 2008–2010 (Technical Appendix, Table S1). More detailed investigation on Griend showed that, although H13 and H16 viruses were detected each year, H13 was the only (2008, 2009) or predominant (2010) subtype detected on the first day of virus detection of each breeding season (Figure 2, Table 2). In 2008 and 2009, H16 was detected the next sampling day, which was 1–2 weeks later. H16 was or became the predominant subtype during 2008–2010; H13 prevalence decreased during that period. The source of H13 and H16 viruses causing these epidemics is unknown. Possible sources are breeding or nonbreeding BHGU, other gull species at the colony sites, and freshwater ponds (if present) at the colony sites. Nonbreeding BHGU tend to wander among colony sites. BHGU that breed north of the Netherlands arrive in the Netherlands from July 1 onwards (F. Majoor, unpublished data).

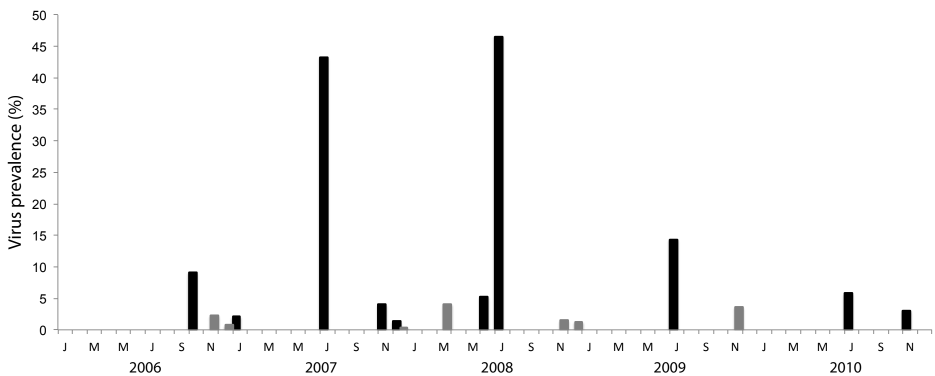


Figure 1. Avian influenza virus prevalence among 7,511 black-headed gulls, the Netherlands, 2006–2010. Cloacal and oropharyngeal samples were collected once from each gull for virus detection. Influenza virus subtypes detected are shown above virus positives. Bars indicate virus prevalence (No. PCR-positive samples/ no. gulls sampled per month). Black bars represent gulls in their first year (FY) of life, comprising nestling and fledgling stages; gray bars represent after-first year (AFY) gulls.

Table 1. Number of black-headed gulls sampled per month for detection of avian influenza virus among 7,511 black-headed gulls, the Netherlands, 2006–2010

Year	No. sampled											
Age	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
2006												
FY	0	0	0	0	6	365	0	0	0	11	74	70
AFY	0	0	0	0	1	0	0	0	1	7	90	138
2007												
FY	96	28	1	0	0	167	37	0	1	6	100	73
AFY	72	39	0	1	34	2	0	1	1	4	153	275
2008												
FY	11	32	33	0	1	632	290	0	0	4	47	108
AFY	37	61	75	0	33	9	42	0	1	5	68	160
2009												
FY	169	43	0	0	0	295	383	0	0	0	45	57
AFY	740	172	3	0	31	82	55	0	0	0	56	288
2010												
FY	60	52	2	0	0	212	451	3	0	2	33	39
AFY	232	135	11	0	45	128	61	7	1	4	40	71

FY, gulls in their first year of life, comprising nestling and fledgling stages; AFY, after first year.

Results from Griend also showed that these epidemics occurred after onset of fledging. The first detection of AIV on Griend (during the last week of June 2008, the first week of July 2009, and mid-July 2010) occurred 1–3 weeks after onset of fledging. Also, of 871 FY birds, AIV was detected only in FY birds with an average length of >200 mm, above which they are considered to be fledged (211). Possible explanations for timing of the epidemic could be increased mobility after fledging and, therefore, increased contact rate; access to water, facilitating more efficient virus transmission; and increased susceptibility of fledglings as a result of immature body condition and loss of maternal antibodies.

Body condition did not differ notably between virus-positive and virus-negative FY birds sampled on the same day ($P > 0.05$, Mann-Whitney Wilcoxon test), except for during the third week of July during 2009 ($P = 0.046$) and 2010 ($P = 0.0004$), when virus-positive birds had lower body condition. This suggests that, overall, H13 and H16 virus infections are nonpathogenic for BHGU. Previous studies found no clinical signs (39) or histological lesions (140) in gulls naturally infected with H13 or H16 virus. No notable differences in virus prevalence were found related to gender, no consistent differences

in virus preva- lence were found related to capture method, and no AIVs in dead BHGU were detected outside epidemics (Technical Appendix).

Outside the breeding season, AIV prevalence was much lower, and no H13 or H16 viruses were isolated; AIV were exclusively isolated from AFY birds and were typed as H1N3, H7N1, and H11N9 (Figure 1, Table 1). Additionally, a single H5 virus was detected by using H5 RT-PCR in an AFY gull sampled in December 2006. H13 viruses have been isolated from ring-billed gulls (*Larus delawarensis*) outside the breeding season (40). The lack of detection of H13 and H16 viruses in BHGU outside the breeding season in our study provides no support for virus circulation at low prevalence in overwintering FY birds. Our sample size of FY birds sampled outside the breeding season (n = 1,200) may be around the theoretical limit to detect the presence of these viruses in the population, assuming a virus prevalence of 0.5% in a homogeneously distributed population (133). However, a nonhomogeneous BHGU population structure outside the breeding season might support a situation in which susceptible FY gulls are present year-round and thus facilitate the circulation of AIV throughout the year at an even lower prevalence.

Table 2. Number of 871 FY black-headed gulls sampled per week, Griend, the Netherlands, June–July 2008–2010

	No. sampled		
Month Week no.	2008	2009	2010
June			
25	0	46	44
26	98	70	33
27	0	71	74
July			
28	0	48	60
29	101	50	71
30	0	47	62

FY, gulls in their first year of life, comprising nestling and fledgling stages.

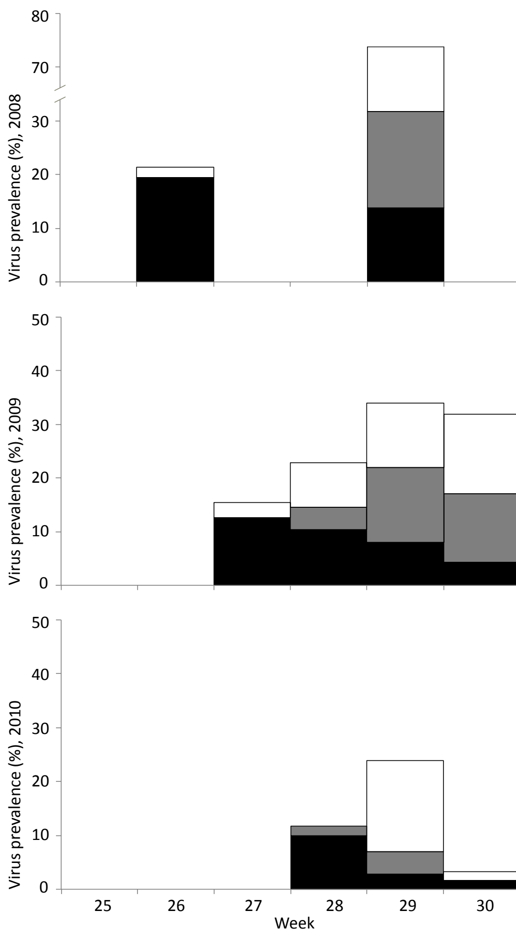


Figure 2. Avian influenza virus prevalence and hemagglutinin subtype (H) distribution of 871 first-year black-headed gulls sampled on the colony site of Griend during 2008–2010. Bars indicate virus prevalence (no. PCR-positive samples/no. sampled per week). Black bar sections, H13; gray bar sections, H16; white bar sections, unknown H subtype.

Prevalence of anti-AIV antibodies detected in FY birds sampled outside the breeding season was statistically more significant (15/59 (25.4%)) than in FY birds sampled during the breeding season (4/75 (5.3%)) ($P < 0.01$, Fisher exact test). The 4 seropositive FY birds were fledglings (n = 55); nestlings (n = 20) were seronegative. There was no statistically significant difference in the seropositivity of AFY gulls sampled during (40/101 (39.6%)) and outside (45/113 (39.8%)) the breeding season ($P > 0.05$, Fisher exact test). These results suggest that FY birds during the breeding season are the most susceptible category to become infected with AIV.

CONCLUSIONS

We describe annual AIV epidemics in BHGU colonies. These epidemics were caused by AIV subtypes H13 and H16 and occurred in FY birds during the second half of the breeding season, with prevalence rates of up to 72% per week. On most sampling days, infected and non-infected FY birds had similar body conditions, suggesting H13 and H16 viruses are nonpathogenic for BHGU. These findings broaden our view on AIV dynamics in populations of gull species often closely associated with humans and facilitate more targeted sampling of colonial nesting waterbirds. Further research is needed to show if the same AIV dynamics apply to other gull species and other geographic areas and to clarify the epidemiology of AIV in wild birds and factors that influence emergence of influenza in domestic animals and humans.

ACKNOWLEDGEMENTS

We thank Judith Guldemeester and Ger van der Water for technical and logistical assistance. This work was sponsored by grants from the Dutch Ministry of Agriculture and the National Institute of Allergy and Infectious Diseases, National Institutes of Health contract HH- SN266200700010C.

TECHNICAL APPENDIX

Sampled Sites

Location and Characteristics of Black-headed Gull (BHGU) Colony Sites

Three breeding colony sites were intensively monitored for AIV: Griend, De Kreupel and Veluwemeer. The island of Griend (lat-y 53.252, long-x 5.254) is home to the largest BHGU colony of the Netherlands, consisting of approximately 30,000 pairs (212). The colony size was estimated at 35,166 breeding pairs in 2008, 32,780 in 2009, and 31,408 in 2010 (213-215). This human-uninhabited and protected island is located in the Wadden Sea. We sampled BHGU on Griend for AIV from 2008 to 2010. De Kreupel consists of a chain of small islands (lat-y 52.797, long-x 5.229) in the IJsselmeer, and is home to the second-largest BHGU colony of the Netherlands, consisting in 2010 of 9231 breeding pairs (216). We sampled BHGU on De Kreupel from 2007 to 2010. Veluwemeer (lat-y 52.399, long-x 5.711) is an inland site with three small islands that are home to a BHGU colony of approximately 1400 pairs (216). We sampled BHGU at Veluwemeer for AIV from 2006 to 2010.

Other bird species than BHGU also use these three sites for breeding. Sandwich Tern (*Thalasseus sandvicensis*), Common Tern (*Sterna hirundo*), Arctic Tern (*Sterna paradisaea*) and Oystercatcher (*Haematopus ostralegus*) breed on Griend (215). Common Tern (*Sterna hirundo*), Tufted Duck (*Aythya fuligula*), Gadwall (*Anas strepera*) and Mallard (*Anas platyrhynchos*) breed on De Kreupel and on the islands of Veluwemeer. Great Cormorant (*Phalacrocorax carbo*) breeds on De Kreupel, and Egyptian Goose *(Alopochen aegyptiaca)* and Mute Swan (*Cygnus olor*) breed on the islands of Veluwemeer. In addition, these colony sites are used as stop-over sites for migrating shorebirds, Griend and De Kreupel more so than the more inland located Veluwemeer (F. Majoor, unpublished data). Vegetation on the colony site of Griend consists of grasses (e.g. *Ammophila arenaria*, *Elymus arenarius*) and nettles (*Urtica dioica*). Vegetation on De Kreupel and the islands of Veluwemeer consists mainly of fireweed (*Chamerion angustifolium*) and nettles.

In addition to the colony sites of Griend, De Kreupel and Veluwemeer, BHGU were sampled on other colony sites within the Netherlands for the presence of AIV in the summer of 2008. These colony sites were located in the Wadden Sea on the island of Ameland (lat-y 53.435, long-x 5.640, 3650 pairs in 2010 (216)), near Bargerveen (lat-y 52.681, long-x 7.032, 829 pairs in 2008 (217)), Blauwestad (lat-y 53.171, long-x 7.012, 1500 pairs in 2009 (218)), Tjeukemeer (lat-y 52.888, long-x 5.800, 1500 pairs, F.Majoor,

unpublished data) and Zoetermeer (lat-y 52.075, long-x 4.532, 500 pairs, F.Majoor, unpublished data). BHGU found dead were sampled at the colony sites listed above, and at Houtribdijk (lat-y 52.626, long-x 5.423), Arnhem (lat-y 51.985, long-x 5.910) and Hilversum (lat-y 52.229, long-x 5.167).

Distinction between coastal and inland colony sites was described previously (219) based on location of the colony site and food collected by BHGU breeding there. Remarkably, breeding at coastal colony sites (Griend, Ameland, De Kreupel and Blauwestad) started 1-2 weeks later than at more inland located colony sites (Veluwemeer, Bargerveen and Tjeukemeer) (F.Majoor, unpublished data).

Measurement of BHGU Breeding Success

The BHGU colony on Griend has been monitored for breeding success since 1964 (212). For this purpose, breeding success enclosures (fences surrounding multiple nests) have been used on Griend since 1994 to monitor nests from egg laying until fledging of the chicks. Between 2008 and 2010, guards of the island monitored three (2008 and 2009) or four (2010) enclosures, which contained on average 7.9 nests (minimum 2, maximum 16). Enclosures 1 to 3 were located on lat-y 53.25232, long-x 5.25131; lat-y 53.25205, long-x 5.25117 and lat-y 53.25205, long-x 5.25104, respectively. Enclosure 4, used in 2010, was adjacent to enclosure 3. Enclosures surrounding multiple nests were visited ≥1 per week to monitor nests from egg laying until fledging of the chicks. Within the enclosures the number of hatchlings per day was estimated based on disappearance of the egg and presence of a chick without a leg ring that subsequently was ringed the same day. Within the enclosures, wing length of all ringed nestlings was monitored. FY gulls were considered fledglings the first day the wing length was ≥ 200mm.

Sampled Black-headed Gulls

Ethical Approval

An independent Animal Ethics Committee of the Erasmus Medical Center (Stichting DEC Consult) approved this study (permit numbers 122-07-09, 122-08-12, 122-09-20, 122-10-20).

Geographical Origin of BHGU

Sampled BHGU were first-year (FY) and after-first-year (AFY) birds of mixed origin, one part originating from breeding colonies in the Netherlands and the other part originating from breeding colonies north/north-east of the Netherlands in Scandinavia, Poland or

the Baltic States (220). Based on sightings, AFY gulls that breed north/north-east of the Netherlands leave the Netherlands by April 1st at the latest to migrate to their breeding colony sites. They may be observed again in the Netherlands from July 1st onwards, followed 1-2 weeks later by FY gulls from those breeding colony sites (F. Majoor, unpublished data).

Determination of Sex, Age and Body Condition of BHGU

Birds were sexed based on total head-bill length and bill depth when captured after fledging (221). Birds were aged based on total head-bill length when captured before fledging (211) and based on plumage when captured after fledging (222). In addition, FY birds were categorized based on wing length as nestling (<200 mm) or fledgling (≥200 mm) (211). To calculate a scaled mass index of body condition (called body condition), body mass and head-bill length as length value were used (223).

Additional Analyses

Analysis of Gender Differences

Gender was determined for 4,356 of 7,511 sampled BHGU (58.0%). Of these 4,356 gulls, 317 (7.3%) were FY birds sampled within the breeding season. Of 4,356 birds, 1,149 birds were female (26.3%) and 3,207 of 4,356 were male (73.6%), suggesting there is a capture bias towards male gulls in the current dataset. Among FY gulls of which the gender was known, 3 of 290 female (1.0%) and 7 of 874 male (0.8%) gulls were M RT-PCR positive. Of AFY gulls, 5 of 859 (0.6%) female gulls and 7 of 2,326 male gulls (0.3%) were M RT-PCR positive. No statistically significant difference in AIV prevalence between genders among FY ($P > 0.05$, Fisher's exact) and AFY birds ($P > 0.05$, Fisher's exact) was observed.

Comparison of Live and Dead Birds

Next to sampling BHGU alive, 158 samples from the cloaca and oropharynx were collected from 113 FY and 45 AFY gulls found dead from 2007 to 2010. In dead FY gulls, viruses were detected in June and July of 2008 on three colony sites (Technical Appendix Table 2). When H13 and H16 viruses were detected in dead FY birds, viruses of the same HA subtypes were detected in FY gulls sampled alive at the same date and location (Additional analyses–Table S2). Samples from cloaca and oropharynx collected on Griend on the day that AIV were detected in multiple dead FY gulls (July 14th, 2008) suggest that more virus was present in dead FY gulls (mean cycle-threshold (Ct) value

of 27, SD = 4.7, n = 20) than in live FY gulls (mean C_T value of 32, SD = 4.5, n = 73) (P = 0.00009, Mann-Whitney Wilcoxon). No viruses were detected in 45 AFY gulls that were found dead in April (n = 1) and June (n = 17) of 2007, in January (n = 1), February (n = 1), April (n = 3), May (n = 2), June (n = 10), July (n = 2), October (n = 1) and November (n = 2) of 2008, in January (n = 1) and June (n = 1) of 2009 and in July (n = 3) of 2010.

Analysis of Recaptured Gulls

FY gulls that were captured and sampled more than once on the colony site of Griend within the breeding season were used to investigate whether an individual FY gull was infected multiple times with AIV within a single breeding season. On the colony site of Griend, 2, 14 and 17 FY gulls were captured and sampled twice within the breeding season of 2008, 2009 or 2010, respectively (Technical Appendix Table 3). AIV were detected in 7 of the 33 recaptured FY gulls. Of the seven FY gulls that tested AIV positive, six were detected positive once and one was detected positive twice with an interval of 1 week. Of the FY gull that tested AIV positive twice, the first time H16N3 AIV was isolated, one week later the sample was M RT-PCR positive, but no virus could be isolated and characterized. Therefore, serial infections with one subtype or cross-infections of H13 and/or H16 viruses within a single host and season could not be demonstrated. In addition to these 33 FY gulls that were captured and sampled twice on the colony site of Griend, 215 gulls were captured and sampled twice at other locations in the Netherlands between 2006 and 2010; none of them tested positive for AIV. In addition, ten gulls were sampled three times; again, none of them tested positive for AIV.

Analysis of Capture Method

To determine whether the decrease in body condition during the course of the breeding season was associated with the capture method used, we compared body condition—as well as wing length and virus prevalence—between birds caught by hand and birds caught by clap net, on 3 days in 2009 and 2010 when methods were used in parallel (Technical Appendix Table 4). Capture method was not associated with a consistent change in body condition. In contrast, wing length of FY birds captured by hand (n = 126) was significantly lower than wing length of FY birds captured by clap net (n = 65) on 3 out of 3 days (P < 0.01, Mann-Whitney Wilcoxon test). Virus prevalence of FY birds captured by hand did not differ from virus prevalence of FY birds captured using a clap net on 2 out of 3 days. However, on 1 day (July 21st, 2010), birds captured by hand (n = 39) had significantly lower wing length (P < 0.05, Mann-Whitney Wilcoxon test), the same mean body condition and significantly higher virus prevalence (P < 0.05, Mann-Whitney

Wilcoxon test) than birds captured using a clap net (n = 32).

Table S1. Avian influenza virus prevalence in first-year Black-headed Gulls sampled at 3 breeding colony sites ((A) Griend, (B) De Kreupel, (C) Veluwemeer) in the Netherlands during the 2006–2010 breeding seasons*†

A

	Griend				
Year	Date	No. sampled	No. PCR positive	No. VI positives	No. each subtype
2006	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND
2007	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND
2008	28-Jun	98	20	19	H13N8 (19)
	14-Jul	100	72	32	H13N8 (14) H16N3 (18)
	ND	ND	ND	ND	ND
2009	17-Jun	46	0	0	NA
	24-Jun	70	0	0	NA
	02-Jul	71	11	9	H13N2 (9)
	09-Jul	48	11	7	H13N2 (4) H13N6 (1) H16N3 (2)
	15-Jul	50	17	11	H13N2 (3) H13N3 (1) H16N3 (7)
	22-Jul	47	15	8	H13N2 (1) H13N6 (1) H16N3 (6)
2010	23-Jun	44	0	0	NA
	30-Jun	33	0	0	NA
	07-Jul	73	0	0	NA
	14-Jul	60	7	7	H13N2 (1) H13N8 (5) H16N8 (1)
	21-Jul	71	17	5	H13N2 (1) H13N8 (1) H16N3 (3)
	26-Jul	60	2	1	H13N2 (1)
Total		871	172	99	

*ND, no data; NA, not applicable. †This sample (n=2,430) is a subset of the 7,511 BHGU sampled once for virus detection and shown in Figure 1, main manuscript. It excludes birds older than 1 year (n=3,472), First-year birds sampled outside the breeding season (n=1,200) and those sampled during the breeding season on colony sites other than the above 3 (n=410). Details of breeding colony sites are described in Technical Appendix Sampled Sites.

B

	De Kreupel				
Year	Date	No. sampled	No. PCR positive	No. VI positive	No. each subtype
2006	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND
2007	05-Jul	36	16	10	H13N3 (1) H13N6 (3) H16N3 (6)
	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND
2008	09-Jun	64	0	0	NA
	24-Jun	77	0	0	NA
	15-Jul	78	23	9	H13N8 (5) H16N3 (4)
2009	15-Jun	47	0	0	NA
	26-Jun	64	0	0	NA
	29-Jun	66	0	0	NA
	03-Jul	63	0	0	NA
	06-Jul	50	0	0	NA
	13-Jul	26	1	1	H13N2 (1)
2010	24-Jun	63	0	0	NA
	02-Jul	43	0	0	NA
	08-Jul	60	0	0	NA
	19-Jul	39	0	0	NA
	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND
Total		776	40	20	

C

	Veluwemeer				
Year	Date	No. sampled	No. PCR positives	No. VI positives	No. each subtype
2006	06-May	6	0	0	NA
	04-Jun	199	0	0	NA
	12-Jun	94	0	0	NA
	23-Jun	72	0	0	NA
2007	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND
	10-Jun	144	0	0	NA
	ND	ND	ND	ND	ND
	30-Jun	23	0	0	NA
2008	07-May	1	0	0	NA
	06-Jun	126	0	0	NA
	25-Jun	18	0	0	NA
2009	ND	ND	ND	ND	ND
	01-Jul	29	0	0	NA
	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND
2010	ND	ND	ND	ND	ND
	05-Jun	30	0	0	NA
	04-Jul	41	0	0	NA
	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND
	ND	ND	ND	ND	ND
Total		783	0	0	

Table S2. Avian influenza virus prevalence in first-year black-headed gulls found dead or captured alive in the Netherlands during 2007– 2010

			Dead		Alive	
Year	Date	Location	No. PCR positive/no. sampled (%)	Subtype	No. PCR positive/no. sampled (%)	Subtype
2007	05-Jun	Houtribdijk	0/4 (0)	NA	0	NA
	06-Jun	Houtribdijk	0/1 (0)	NA	0	NA
2008	22-Jan	Arnhem	0/1 (0)	NA	0	NA
	06-Jun	Veluwemeer	0/3 (0)	NA	0/126 (0)	NA
	09-Jun	De Kreupel	0/3 (0)	NA	0/64 (0)	NA
	20-Jun	Bargerveen	0/4 (0)	NA	0/52 (0)	NA
	24-Jun	De Kreupel	0/10 (0)	NA	0/77 (0)	NA
	25-Jun	Veluwemeer	0/10 (0)	NA	0/18 (0)	NA
	28-Jun	Griend	0/15 (0)	NA	20/98 (20)	H13N8 (19)
	29-Jun	Blauwestad	1/6 (17)	H13N8 (1)	13/81 (16)	H13N8 (4)
	06-Jul	Ameland	1/5 (20)	H13N8 (1)	39/115 (34)	H13N3 (1); H13N8 (9); H16N3 (12)
	08-Jul	Houtribdijk	0/1 (0)	NA	0/6 (0)	-
	14-Jul	Griend	20/30 (67)	H13N8 (4); H16N3 (5); H16N8 (2)	73/101 (72)	H13N8 (14); H16N3 (18)
	15-Jul	De Kreupel	0/7 (0)	NA	23/78 (29)	H13N8 (5); H16N3 (4)
	03-Dec	Hilversum	0/1 (0)	NA	0/5 (0)	NA
2009	31-Jan	Arnhem	0/1 (0)	NA	0	NA
	26-Jun	De Kreupel	0/1 (0)	NA	0/64 (0)	NA
	29-Jun	De Kreupel	0/2 (0)	NA	0/66 (0)	NA
	03-Jul	De Kreupel	0/5 (0)	NA	0/63 (0)	NA
2010	20-Jan	Hilversum	0/1 (0)	NA	0	NA
	03-Feb	Hilversum	0/1 (0)	NA	0/1 (0)	NA
	19-Jul	De Kreupel	0/1 (0)	NA	0/39 (0)	NA

*NA, not applicable

Table S3. Detection of avian influenza virus in avian influenza virus-positive first-year black-headed gulls that were recaptured and sampled within this study period on the colony site of Griend, the Netherlands

FY bird no.	Capture sequence	Sample date	Result PCR	Wing length (mm)	Head-bill length (mm)	Body mass (g)	SMI
1	1	28-Jun-08	–	172	66,8	176	208
	2	14-Jul-08	+	233	71,9	202	206
2	1	28-Jun-08	–	181	70,7	240	228
	2	14-Jul-08	+	259	77,8	218	170
3	1	02-Jul-09	–	236	76,1	273	247
	2	09-Jul-09	+	275	79	234	181
4	1	09-Jul-09	–	251	70	204	248
	2	15-Jul-09	+	265	71	199	234
5	1	14-Jul-10	+	281	79,1	246	199
	2	21-Jul-10	+	289	79,7	167	141
6	1	07-Jul-10	–	260	73,4	193	205
	2	21-Jul-10	+	278	74,5	155	166
7	1	21-Jul-10	+	286	77,1	204	194
	2	26-Jul-10	–	288	77,3	210	221

SMI, scaled mass index of body condition

Table S4. Avian influenza virus prevalence, wing length, and SMI of body condition of first-year black-headed Gulls captured on Griend on the same sampling day

.	Date of sampling			Capture technique		Significance of difference between hand-captured and clap-net-captured birds	
Year			Per sampling day	Hand	Clap net	p-value	Test*
2008	28-Jun	Mean wing length	201	201	-	NA	NA
		Mean SMI	216	216	-	NA	NA
		No. virus positive/No. sampled (%)	20/98 (20)	20/98 (20)	-	NA	NA
	14-Jul	Mean wing length	232	232	-	NA	NA
		Mean SMI	196	196	-	NA	NA
		No. virus positive/No. sampled (%)	72/100 (72)	72/100 (72)	-	NA	NA
2009	17-Jun	Mean wing length	169	169	-	NA	NA
		Mean SMI	237	237	-	NA	NA
		No. virus positive/No. sampled (%)	0/46 (0)	0/46 (0)	-	NA	NA
	24-Jun	Mean wing length	202	202	-	NA	NA
		Mean SMI	247	247	-	NA	NA
		No. virus positive/No. sampled (%)	0/70 (0)	0/70 (0)	-	NA	NA
	02-Jul	Mean wing length	229	229	-	NA	NA
		Mean SMI	238	238	-	NA	NA
		No. virus positive/No. sampled (%)	11/71 (15)	11/71 (15)	-	NA	NA

.	Date of sampling			Capture technique		Significance of difference between hand-captured and clap-net-captured birds	
Year			Per sampling day	Hand	Clap net	p-value	Test*
	09-Jul	Mean wing length	244	244	-	NA	NA
		Mean SMI	204	204	-	NA	NA
		No. virus positive/No. sampled (%)	11/48 (23)	11/48 (23)	-	NA	NA
	15-Jul	Mean wing length	253	252	301	NA	NA
		Mean SMI	195	196	170	NA	NA
		No. virus positive/No. sampled (%)	17/50 (34)	17/49 (35)	0/1 (0)	NA	NA
	22-Jul	Mean wing length	272	267	281	p<0.01	MW
		Mean SMI	188	182	199	p<0.05	MW
		No. virus positive/No. sampled (%)	15/47 (32)	10/31 (32)	5/16 (31)	p=1	FE
2010	23-Jun	Mean wing length	183	183	-	NA	NA
		Mean SMI	217	217	-	NA	NA
		No. virus positive/No. sampled (%)	0/44 (0)	0/44 (0)	-	NA	NA
	30-Jun	Mean wing length	211	211	-	NA	NA
		Mean SMI	246	246	-	NA	NA
		No. virus positive/No. sampled (%)	0/33 (0)	0/33 (0)	-	NA	NA
	07-Jul	Mean wing length	247	239	272	p<0.01	MW
		Mean SMI	240	247	220	p<0.01	MW
		No. virus positive/No. sampled (%)	0/73 (0)	0/56 (0)	0/17 (0)	p=1	FE
	14-Jul	Mean wing length	247	247	-	NA	NA
		Mean SMI	208	208	-	NA	NA
		No. virus positive/No. sampled (%)	7/60 (12)	7/60 (12)	-	NA	NA
	21-Jul	Mean wing length	266	247	283	p<0.01	MW
		Mean SMI	186	183	190	p>0.05	MW
		No. virus positive/No. sampled (%)	17/71 (24)	17/39 (44)	0/32 (0)	p<0.01	FE
	26-Jul	Mean wing length	287	-	287	NA	NA
		Mean SMI	206	-	206	NA	NA
		No. virus positive/No. sampled (%)	2/60 (3)	-	2/60 (3)	NA	NA

SMI, scaled mass index of body condition; NA, not applicable. *MW, Mann-Whitney Wilcoxon; FE, Fisher's exact.

CHAPTER 2.4

Josanne H. Verhagen, Ursula Höfle, Geert van Amerongen
Marco W. van de Bildt, Frank Majoor, Ron A.M. Fouchier
Thijs Kuiken

Long-term effect of serial infections with H13 and H16 low pathogenic avian influenza viruses in black-headed gulls

Journal of Virology (2015), Volume 89, Issue 22:11507-22. DOI: 10.1128/JVI.01765-15

Infections of domestic and wild birds with low-pathogenic avian influenza viruses (LPAIVs) have been associated with protective immunity to subsequent infection. However, the degree and duration of immunity in wild birds from previous LPAIV infection, by the same or a different subtype, are poorly understood. Therefore, we inoculated H13N2 (A/black-headed gull/Netherlands/7/ 2009) and H16N3 (A/ black-headed gull/Netherlands/26/2009) LPAIVs into black-headed gulls (*Chroicocephalus ridibundus*), their natural host species, and measured the long-term immune response and protection against one or two reinfections over a period of >1 year. This is the typical interval between LPAIV epizootics in wild birds. Reinfection with the same virus resulted in progressively less virus excretion, with complete abrogation of virus excretion after two infections for H13 but not H16. However, reinfection with the other virus affected neither the level nor duration of virus excretion. Virus excretion by immunologically naive birds did not differ in total levels of excreted H13 or H16 virus between first- and second-year birds, but the duration of H13 excretion was shorter for

second-year birds. Furthermore, serum antibody levels did not correlate with protection against LPAIV infection. LPAIV-infected gulls showed no clinical signs of disease. These results imply that the epidemiological cycles of H13 and H16 in black-headed gulls are relatively independent from each other and depend mainly on infection of first- year birds.

IMPORTANCE

Low-pathogenic avian influenza viruses (LPAIVs) circulate mainly in wild water birds but are occasionally transmitted to other species, including humans, where they cause subclinical to fatal disease. To date, the effect of LPAIV-specific immunity on the epidemiology of LPAIV in wild birds is poorly understood. In this study, we investigated the effect of H13 and H16 LPAIV infection in black-headed gulls on susceptibility and virus excretion of subsequent infection with the same or the other virus within the same breeding season and between breeding seasons. These are the only two LPAIV hemagglutinin subtypes predominating in this species. The findings suggest that H13 and H16 LPAIV cycles in black-headed gull populations are independent of each other, indicate the importance of first-year birds in LPAIV epidemiology, and emphasize the need for alternatives to avian influenza virus (AIV)-specific serum antibodies as evidence of past LPAIV infection and correlates of protection against LPAIV infection in wild birds.

INTRODUCTION

Wild aquatic birds of the orders Anseriformes (mainly ducks, geese, and swans) and Charadriiformes (mainly gulls and waders) play a major role in the epidemiology of low-pathogenic avian influenza viruses (LPAIVs). Evidence to date indicates that LPAIV infection in these species is mainly a digestive tract infec- tion and causes no clinical disease (77). LPAIVs are categorized into so-called subtypes based on their surface proteins hemagglutinin (HA) (H1 to H16) and neuraminidase (NA) (N1 to N9). From wild birds, these viruses may be transmitted occasionally to domestic animals and sporadically (usually indirectly via poultry) to humans, in which they can cause infections ranging from subclinical infection to fatal disease. For the epidemiology of most LPAIV subtypes, a major role is played by ducks, in which epizootics occur each fall (12, 26). However, there are two subtypes, H13 and H16, for which gulls, such as black-headed gulls (BHGU) (*Chroicocephalus ridibundus*), are the major reservoir (39, 40, 224). BHGU are furthermore special in that they are not commonly infected with other LPAIV subtypes, and epidemics are known to occur annually at the end of each breeding season at colony sites (224). This makes BHGU particularly suited to study the effect of

multiple homologous and heterologous LPAIV infections on immunity.

Despite numerous studies on the epidemiology of LPAIV in wild birds, the effect of immunity on the epidemiology of LPAIV in wild bird populations is poorly understood. Previous studies reported variable levels of protection by the immune system against reinfection with LPAIV in domestic and wild birds. For instance, LPAIV infections followed by exposure to the same (i.e., homologous) LPAIV HA subtype have been shown to induce strong protection in chickens (225) and mallards (*Anas platyrhynchos*) (61) but weak protection in Pekin ducks (225). LPAIV infections followed by exposure to a different (i.e., heterologous) LPAIV HA subtype have been shown to induce no protection in chickens (225) and weak protection in Pekin ducks (225), mallards (59, 61), and quails (226). Susceptibility of birds to LPAIV infection is suggested to vary by age, with, in most cases, decreased virus replication with increasing age, but this has been investigated mainly in very young birds (57, 58). In naturally and experimentally infected mallards, avian influenza virus (AIV)-specific serum antibodies have been detected for a long period of time after infection (227, 228), but little is known about their protective effect.

To clarify the role of immunity in the epidemiology of LPAIV subtypes in wild birds, we investigated the protective effect of LPAIV infection on subsequent infections with the homologous 8 or a heterologous virus in a natural host species over a period of >1 year. Clinical effects of infection were also investigated. This study addresses the following questions. (i) What is the protective effect of LPAIV infection on subsequent exposure to the homologous virus? (ii) What is the protective effect of LPAIV infection on subsequent exposure to a heterologous virus? (iii) Are first- year birds equally susceptible to LPAIV infection as second-year birds? (iv) Does LPAIV cause disease in BHGU? To answer these questions, 2-month-old BHGU were inoculated with either LPAIV H13N2 or H16N3, and inoculation was repeated with one of these viruses after 1 month and after 1 year. The results of experimental infections showed that there was a protective effect after previous infection with the homologous virus but not after previous infection with a heterologous virus. In addition, there was no effect of age on susceptibility to LPAIV infection, and neither H13 nor H16 caused clinical signs in experimentally infected BHGU.

Table 1. Experimental design

		LPAIV subtype at each inoculation		
		I	II	III
		July 6th 2012	August 3rd 2012	July 15th 2013
Group no.	No. of birds	2 months of age	3 months of age	14 months of age
1	6	H13	H16	H16
2	6	H16	H16	H16
3	6	sham	H16	H16
4	6	sham	sham	H16
5	6	H16	H13	H13
6	6	H13	H13	H13
7	6	sham	H13	H13
8	6	sham	sham	H13

MATERIALS AND METHODS

Ethics statement

This study was carried out in accordance with Euro- pean guidelines (European Union directive on animal testing 2010/63/ EU) and Dutch legislation (Experiments on Animals Act). The protocol was approved by the Animal Ethics Committee of the Dutch National Vaccine Institute of the National Institute for Public Health and the Environment (RIVM) (project number 2012-139). The capture of birds prior to the experiment was approved by the Dutch Ministry of Economic Affairs in compliance with the Flora and Fauna Act (permit number FF/ 75A/2010/039).

Collection, housing, and feeding of birds

Fifty BHGU chicks between 1 and 7 days of age were captured by hand at a BHGU breeding colony site on an island at Blauwe Stad (53°10’15’’N, 7°00’43’’E), in the Netherlands, on 22 May 2012. Birds were hand-raised indoors at the RIVM in Bilthoven, the Netherlands. Prior to the experiment and outside the infectious period (i.e., period from the day when cloaca samples of all birds tested negative for viral RNA until 1 week prior to (the next) virus inoculation, equal to May until June 2012, September 2012 until June 2013, and August until October 2013), birds were housed in two animal rooms. During the infectious period (July to August 2012 and July 2013), birds were housed in groups of six birds per glove box. Birds had continuous access to water (water areas

of 2 m^2/animal room with 20 to 24 birds and ~0.30 m^2/glove box with a maximum of 6 birds) for bathing and drinking. Perches and shelves were available to roost and rest. The room temperature varied between 20°C and 22°C, and light was on between 6:30 a.m. and 6:30 p.m. Animal rooms or glove boxes were cleaned, and water was changed daily.

The diet consisted of sand eel (*Ammodytes tobianus*) with additional vitamins (Akwavit; Twilmij BV, Stroe, the Netherlands), ferret pellets (Arie Blok BV, Woerden, the Netherlands), and live earthworms and mealworms (Firma Van der Neut, Groenekan, the Netherlands). Ground shells were available to provide additional calcium.

Experimental design

We chose BHGU as the study species because BHGU are abundant, H13 and H16 epizootics occur in BHGU every year, and LPAIV infections occurring enzootically in BHGU are restricted to these two subtypes. The timing of the inoculations was chosen to be synchronous with the breeding season of BHGU in July and August and to reflect a reasonable interval between exposures during a breeding season (i.e., 1 month between the first inoculation in July 2012 and the second inoculation in August 2012) and between breeding seasons (i.e., 1 year between the second inoculation in August 2012 and the third inoculation in July 2013). A total of 48 birds (28 males and 20 females) were distributed randomly into 8 groups of 6 birds. Each group followed a different schedule of three intraesophageal inoculations (Table 1). This route of inoculation was chosen because virus replication was limited to the intes- tinal tract of BHGU naturally infected with LPAIVs H13 and H16 (140). The inoculum was egg allantoic fluid containing either 106 median egg infectious doses (EID_{50}) of LPAIV H13 or H16 (virus-inoculated birds) or no virus (sham-inoculated birds), diluted with phosphate-buffered saline (PBS) to a volume of 1.5 ml. Birds were weighed and sampled for virus detection daily from day 0 until day 7 and on days 9, 11, 13, 14, 21, and 28 postinoculation. Birds were sampled for antibody detection on days 0, 7, 14, 21, and 28 postinoculation. From 28 days postinoculation (dpi) onwards, birds were weighed and sampled monthly for virus and antibody detection.

Cloacal and oropharyngeal swabs were collected from all 48 gulls at two time points (31 May 2012 and 16 June 2012) prior to the first inoculation, and all swabs tested negative by matrix-specific reverse transcription-PCR (M-RT-PCR). Also, sera from all 48 gulls were collected on the same day but just prior to the first inoculation (6 July 2012) and tested negative for NP-, H13-, and H16-specific antibodies. The exception was the serum of one BHGU from group sham-sham-H13, which tested positive for NP-specific antibodies and negative for H13- and H16-specific antibodies on 16 June 2012, 29 June

2012, 13 July 2012, and 20 July 2012. This BHGU also tested negative for H1- to H12-specific antibodies on 6 July 2012. Therefore, this BHGU was retained in the study.

Virus preparation

Two virus stocks of influenza virus, A/black-headed Gull/Netherlands/7/2009 (H13N2) (collected on 2 July 2009) and A/black-headed Gull/Netherlands/26/2009 (H16N3) (collected on 22 July 2009), were used in this study. Both of these viruses originated from a BHGU breeding colony site at the island of Griend (53°15'07''N, 5°15'14''E), located in the Wadden Sea in the north of the Netherlands. The viruses were isolated from combined oropharyngeal-cloacal swab samples from first-year BHGU and passaged twice in 11-day-old embryonated chicken eggs. Viral titers of stock solutions were 108 EID_{50}/ml. Prior to inoculation, virus stocks were diluted with PBS to 106 EID_{50}/1.5 ml. These viruses were selected as they originated from the same season and colony site and therefore were considered to be good candidates to simulate a natural pair of LPAIV infections on a colony site. The internal gene segments of these two virus isolates showed high levels of sequence identity (for PB2, 97% of 2,322 nucleotides (nt) were identical; for PB1, 100% of 2,314 nt were identical; for PA, 98% of 2,222 nt were identical; for NP, 99% identical of 1,538 nt were identical; and for MA, 93% of 1,017 nt were identical), except for NS (87% of 866 nt).

Sampling for virus detection

For virus detection, samples were taken from the cloaca of birds by using sterile cotton swabs. After sampling, the swab was submerged in 1.2 ml virus transport medium (VTM) (151). Within 2 h, the sample was frozen at -80°C until analysis.

Sampling for antibody detection

For antibody detection, a blood sample of at most 1 ml from the jugular vein was collected. Blood was collected in gel tubes (MiniCollect, Z serum separator tubes; Greiner Bio-One, Kremsmünster, Austria) and centrifuged at 3,000 rpm for 10 min within 2 h of sampling. Serum was stored at -20°C until analysis.

Detection of viruses: RNA isolation and M-RT-PCR

RNA was isolated from 200 µl of sample in VTM by using a MagnaPure LC system with a MagnaPure LC total nucleic acid isolation kit (Roche Diagnostics, Almere, the Netherlands). Subsequently, RNA was tested for the presence of the highly conserved

matrix segment by M-RT-PCR. Amplification and detection were performed by using an ABI 7700 machine (Applied Bio-systems, Foster City, CA, USA) with a TaqMan Fast Virus 1-Step master mix (Applied Biosystems, Nieuwerkerk aan den IJssel, the Netherlands) and 20 µl of RNA eluate in a total volume of 30 µl. Oligonucleotides (5’-CTT-CTR-ACC-GAG-GTC-GAA-ACG-TA-3’ and 5’-TCT-TGT-CTT-TAG-CCA-YTC-CAT-GAG-3’) and labeled probes (5'-FAM (6-carboxyfluorescein)-TCA-GGC-CCC-CTC-AAA-GCC-GAG-A-black hole quencher (BHQ)-3’ and 5’-FAM-TCA-GGC-CCC-CTC-AAA-GCC-GAA-A-BHQ-3’) were used for the detection of the M segment. Samples were considered positive if the cycle threshold (Ct_T value was <40.

Virus isolation and titration

In all specimens, the presence or absence of infectious virus was detected by inoculating an aliquot of 100 µl of VTM into 11-day-old embryonated chicken eggs (4 eggs/specimen). For a subset of specimens, namely, the original specimens after the first inoculation with H13 (i.e., inoculation group H13-H16-H16) and after the first inoculation with H16 (i.e., inoculation group H16-H16-H16), the virus titer was measured. To do so, we made a 10-fold dilution series of VTM in a volume of 100 µl and used these dilutions to inoculate 11-day-old embryonated chicken eggs (4 eggs/dilution). Eggs were incubated at 37°C for 2 days before allantoic fluid was harvested. Next, allantoic fluid was tested in a hemagglutination test for the presence of AIV (151).

The use of the C_T value as a proxy for viral titer was based on comparison of C_T values and viral titers of identical cloacal samples collected daily after the first inoculation with H13 and H16 viruses. Despite the strong overall correlation between the C_T value and viral titer ($P < 0.01$ by the Pearson correlation test) (Figure 1), the use of the C_T value as a proxy for viral titer needs to be used with caution, as viral RNA is detectable longer after inoculation than infectious virus.

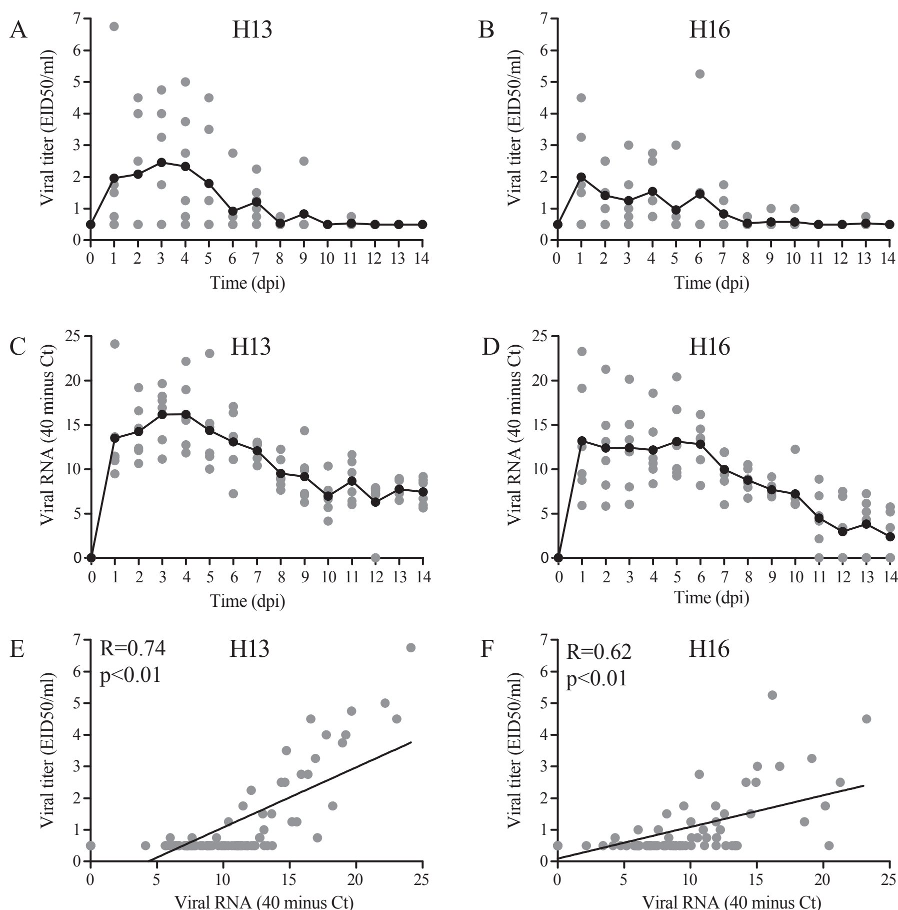


Figure 1. Comparison of viral titers with viral RNA in cloacal swabs after the first LPAIV H13N2 and H16N3 inoculations of black-headed gulls. (A, C, and E) H13; (B, D, and F) H16. Black lines indicate means per sampling day (A to D), and gray dots indicate values for individual birds (n = 6 birds per day) (A to F). Correlation analyses for H13 and H16 based on viral titers and viral RNA from days 0 to 14 postinoculation resulted in R values of 0.74 for H13 ($P < 0.01$) and 0.62 for H16 ($P < 0.01$) (Pearson correlation test). dpi, days post inoculation.

Detection of antibodies

Serum samples were tested for the presence of H13-specific, H16-specific, and NP-specific antibodies. H13- and H16-specific antibodies were detected by using a hemagglutination inhibition (HI) test with H13N2 and H16N3 virus isolates used for inoculation as reference antigens (167). The starting serum dilution in the HI test was 1:6; thus, the minimal detectable antibody titer was 3. Phosphate-buffered saline was included as a serum control. NP-specific antibodies were detected by using a commercial blocking enzyme-linked immunosorbent assay (bELISA) (Idexx FlockChek* AI MultiS-Screen; Idexx

Laboratories BV, Hoofddorp, the Netherlands). Samples were tested according to the manufacturer's instructions. A sample was considered NP positive when the signal-to-noise ratio (i.e., ratio of the mean optical density (ODx) of the sample/ODx of the negative control) was ≤ 0.5.

Clinical signs of infection

Body mass was monitored daily from day 0 to day 7 and on days 9, 11, 13, and 14 postinoculation. After inoculation, each morning, each group was scored qualitatively during 5-min observations for signs of ruffled feathers or decreased movement, feeding, or bathing activity for all individuals. Fecal water content was monitored daily on day 0 until day 7 postinoculation. Per inoculation group, birds were kept for 1 h in a box measuring 45 cm long by 67 cm wide by 20 cm high directly after sampling. Feces fell through a wire mesh grid in the bottom of the box onto a removable polyester sheet (Melinex). After release of the birds into the glove box, the sheet, including feces, was removed and weighed before and after autoclaving in a dry cycle (134°C for 3 min) to evaporate the water in the feces. The mass loss during autoclaving was considered the fecal water content.

As additional methods to measure clinical signs of infection, head movements were measured after the second inoculation, and activity levels were measured after the third inoculation. Head movements (as a proxy for activity) were videotaped for 10 min daily on days 1 to 6 after the second inoculation on 3 August 2012. Activity levels were scored at 3-min intervals during daily observations of 15 min from days -1 to 7 after the third inoculation on 15 July 2013. Activity levels were categorized as active (walking, feeding, preening, and bathing) or passive (standing, sleeping, and sitting).

Statistical analyses

To investigate the correlation between virus ex- cretion based on viral RNA and virus excretion based on viral titer, a Pearson correlation test was performed. To compare virus excretion within and between groups, the area under the curve (AUC) of viral RNA (i.e., based on 40 minus the C_T value as determined by M-RT-PCR) from days 0 to 14 postinoculation was calculated. The mean quantity of virus excreted from cloacae per group (i.e., mean AUC) was based on the AUCs for all birds in the group. To compare the durations of virus excretion within and between groups, the median maximum day of the presence of infectious virus (i.e., positive virus isolation) was used. The median duration of virus excretion per group was based on values from all birds in the group. To investigate whether differences in virus excretion or duration between two groups

or time points were statistically significant, a Mann-Whitney test was performed. To investigate whether differences in virus excretion or duration among three groups or time points were statistically significant, a Kruskal-Wallis test was performed (i.e., for comparisons of H16 virus excretion and durations for three groups of different ages).

To compare the proportions of birds that generated AIV-specific antibodies between groups and between virus subtypes, a Fisher exact test was used. To compare AIV-specific antibody titers within and between groups, the log_2 AUC values for the H13- and H16-specific antibody titers measured weekly from 0 to 28 dpi were calculated. The mean quantity of antibodies generated per group (i.e., mean AUC) was based on AUC values for all birds in the group. To investigate whether differences in anti- body production between two groups or time points were statistically significant, a Mann-Whitney test was performed. To investigate whether differences in antibody production among three groups or time points were statistically significant, a Kruskal-Wallis test was performed. When a statistically significant value was determined ($P < 0.05$), the pairwise difference in levels of antibody production at different time points was analyzed by using the Mann-Whitney test.

To investigate the correlation between virus excretion based on viral RNA and water content of feces, a Pearson correlation test was performed. To investigate the protective effect of homologous AIV-specific antibodies generated after previous virus inoculation, the following values were compared by using a Mann-Whitney test: (i) quantity of virus excretion (i.e., AUC for viral RNA based on 40 minus the C_T value, from days 0 to 14 postinoculation), (ii) peak of virus excretion (i.e., based on viral RNA based on 40 minus the lowest C_T value), (iii) timing of peak of virus excretion (i.e., based on viral RNA, in days postinoculation), and (iv) duration of infectious virus excretion (i.e., based on virus isolation, in days postinoculation) between birds with and those without detectable H16-specific antibody titers on the day of inoculation. Birds that died within 0 to 14 dpi were excluded from analyses.

Nucleotide sequence accession numbers

Full-genome sequences of these viruses are available from GenBank (158) under the following accession numbers: KR087561 to KR087576.

RESULTS

Virus excretion

The first, second, and third inoculations of the different groups, as shown in Table 1, were successful, with the exception of the second inoculation of group sham-H13-H13 (group 7) and group H16-H13-H13 (group 5) (Table 2). Nevertheless, the results obtained based on the remaining groups are still enough to answer the main questions posed above. The unsuccessful inoculation of group sham-H13-H13 was based on the failure to detect virus by M-RT-PCR except in two of six birds on day 1 and day 2 postinoculation and the failure to isolate virus from any bird at any time point, whereas H13 virus replicated well in immunologically naive groups inoculated with H13 virus at the first and third inoculations. The unsuccessful inoculation of group H16-H13-H13 was based on the failure to detect virus by M-RT-PCR except in one of five birds on day 5 postinoculation and the failure to isolate virus from any bird at any time point.

The only known difference in the inoculation procedures between these two inoculations and all other inoculations of the eight groups was the pretreatment of the gavage tubes used for intraesophageal inoculation. Normally, one heat-sterilized gavage tube, wrapped individually in paper, was used per group. However, because there were too few heat-sterilized gavage tubes at inoculation II (3 August 2012), one or two gavage tubes (this information was not recorded) used for H13 inoculation were decontaminated with 80% ethanol, flushed with saline, and introduced loose into the glove box via air locks that had been decontaminated with 4% peracetic acid. Potentially, remnants of peracetic acid on the gavage tubes may have inactivated the virus in the inoculation fluid. Virus titrations of samples of the remaining inoculation fluid after inoculations I (6 July 2012) and III (15 July 2013) were as expected (range, $10^{5.75}$ to $10^{6.25}$ EID_{50}/ml); unfortunately, samples of inoculation fluid after inoculation II were not retained for back titration.

(i) Effect of age on virus excretion

To investigate the effect of age on virus excretion, the quantity (based on AUC from days 0 to 14 postinoculation) and duration of virus excretion between previously uninfected 2-, 3-, and 14-month-old birds were compared. The mean quantity of H13 virus excreted from the cloaca after the first H13 inoculation did not differ significantly between 2-month-old birds (142.8 ± 7.1, i.e., mean for the first inoculation of groups H13-H13-H13 and H13-H16-H16) (Figure 2A and F, black lines) and 14-month-old birds (117.0 ± 12.2) (P = 0.08) (Figure 2H, dashed line). However, the median duration of H13 virus

excretion by 2-month-old birds (6 dpi; range, 1 to 11 dpi) was significantly longer than that for 14-month-old birds (4 dpi; range, 0 to 5 dpi) (P = 0.05) (Table 2 and Figure 3). The mean quantity of H16 virus excreted from the cloaca after the first H16 inoculation did not differ significantly between 2-month-old birds (91.5 ± 13.12, i.e., the mean for the first inoculation of groups H16- H16-H16 and H16-(H13)-H13) (Figure 2B and E, black lines), 3-month-old birds (102.8 ± 13.4) (Figure 2C, gray line), and 14-month- old birds (122.1 ± 21.1) (Figure 2D, dashed line) (P = 0.23). Similarly, the median duration of H16 virus excretion did not differ significantly between 2-month-old birds (4 dpi; range, 0 to 10 dpi), 3-month-old birds (6 dpi; range, 0 to 11 dpi), and 14-month-old birds (4 dpi; range, 0 to 6 dpi) (P = 0.57) (Table 2 and Figure 3).

Table 2. Virus excretion by black-headed gulls after one or more inoculations with LPAIV H13N2, H16N3, or both

		Inoculation no.					
		I		II		III	
		Virus excretion		Virus excretion		Virus excretion	
Group no.	Inoculation schedule	Quantity (AUC of viral RNA) ± SE [b]	Duration (median in days (range))[c]	Quantity (AUC of viral RNA) ± SE [b]	Duration (median in days (range))[c]	Quantity (AUC of viral RNA) ± SE [b]	Duration (median in days (range))[c]
1	H13-H16-H16	151.8 ± 6.7	7 (5-11)	117.6 ± 20.5	5 (0-9)	51.6 ± 28.5	0 (0-3)
2	H16-H16-H16	122.3 ± 9.1	5.5 (4-10)	28.5 ± 6.8	0 (0-3)	13.7 ± 3.4	0 (0-2)
3	sham-H16-H16	0	0	102.8 ± 13.4	6 (0-11)	14.4 ± 7.3	0 (0-2)
4	sham-sham-H16	0	0	0	0	122.1 ± 21.1	4 (0-6)
5	H16-H13[a] -H13	54.6 ± 14.1	3.5 (0-5)	0	0	72.2 ± 10.3	0 (0-3)
6	H13-H13-H13	133.7 ± 11.9	4.5 (1-6)	69.6 ± 8.4	0 (0)	0.8 ± 0.8	0 (0)
7	sham-H13[a] -H13	0	0	1.0 ± 0.6	2 (0-6)	64.0 ± 21.2	0 (0-7)
8	sham-sham-H13	0	0	0	0	117.0 ± 12.2	4 (0-5)

a The second inoculation of groups 5 and 7 was unsuccessful. b The quantity of virus excretion was based on the AUC for viral RNA (i.e., C_T values determined by M-RT-PCR) excreted from the cloaca from days 0 to 14 postinoculation. c Duration of virus excretion was based on the maximum duration of infectious virus excretion based on virus culture from cloaca in days.

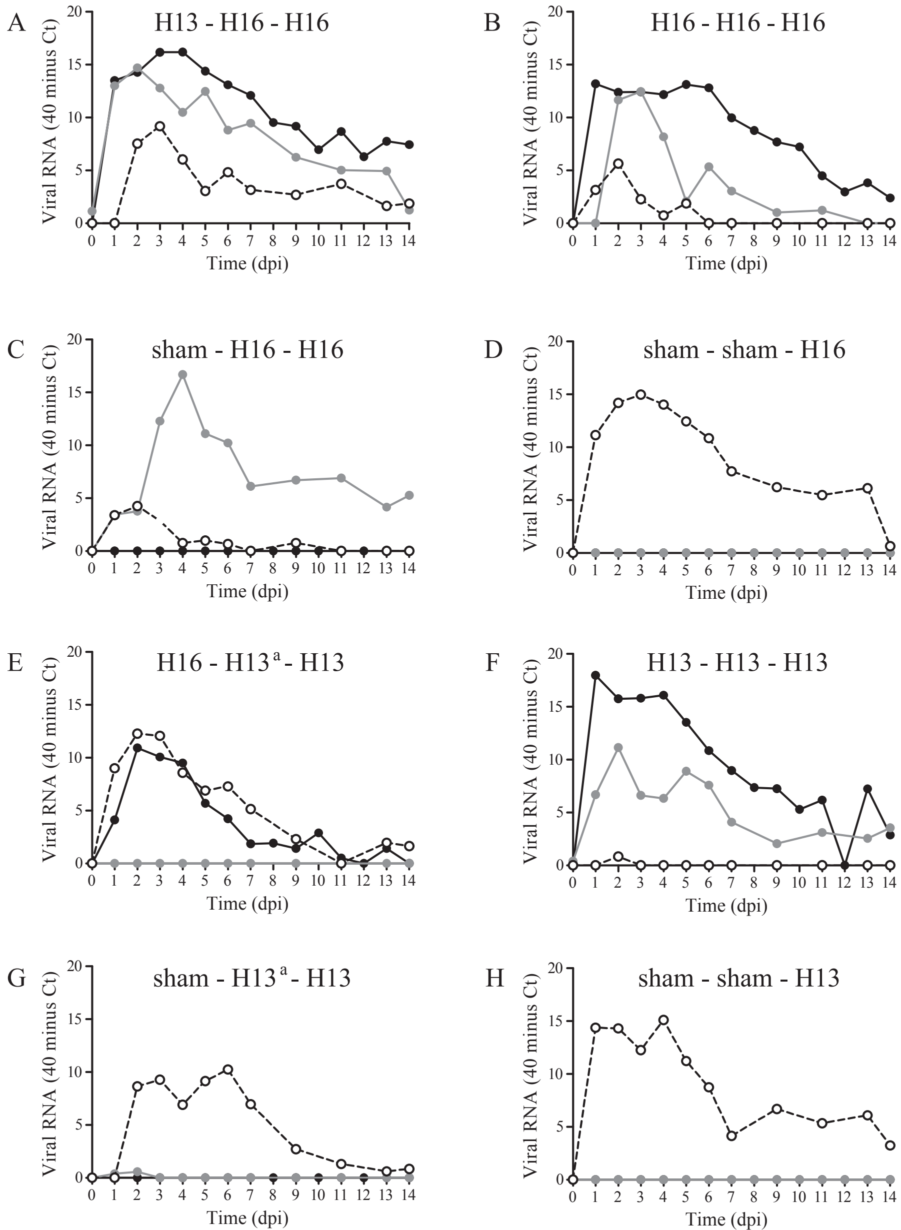


Figure 2. Mean virus excretion from cloaca after experimental infection of black-headed gulls with one or more inoculations of LPAIV H13N2, LPAIV H16N3, or both, based on the quantity of viral RNA (i.e., C_T values determined by M-RT-PCR). Each panel represents data from one group. Mean virus excretion is based on data for all birds in the group. dpi, days post inoculation. Black lines indicate the first inoculation, gray lines indicate the second inoculation, and dashed lines indicate the third inoculation. a, the second inoculation of groups 5 and 7 was unsuccessful.

(ii) Effect of homologous LPAIV infection on virus excretion

To investigate the effect of LPAIV infection on subsequent infection with the same virus, the quantities and durations of virus excretion of homologous inoculation groups were compared.

(a) Group H13-H13-H13. The mean quantity of H13 virus excreted from the cloaca after the second inoculation was significantly lower than that after the first inoculation ($P < 0.01$) (Figure 2F, black and gray lines). Besides as a group, each individual bird excreted less virus after the second than after the first inoculation (Figure 4). Although the median duration of virus excretion appeared to be shorter after the second inoculation (2 dpi; range, 0 to 6 dpi) than after the first inoculation (4.5 dpi; range, 1 to 6 dpi), the difference was not significant ($P = 0.19$) (Table 2). The mean quantity of H13 virus excreted from the cloaca after the third inoculation was significantly lower than that after the second H13 inoculation of the same group ($P < 0.01$) (Figure 2F, black and dashed lines) and was significantly lower than that after H13 inoculation of immunologically naive birds at the third inoculation ($P = 0.01$) (Figure 2F and H, dashed lines). In addition, after the third H13 inoculation, no infectious virus was excreted from the cloaca, and thus, the median duration of virus excretion was shorter after the third H13 inoculation than after the second H13 inoculation (2 dpi; range, 0 to 6 dpi) and than after H13 inoculation of immunologically naive birds at the third inoculation (4 dpi; range, 0 to 5 dpi) (Table 2).

(b) Group sham-(H13)-H13. The mean quantity of H13 virus excreted from the cloaca after the third inoculation appeared to be lower than that after H13 inoculation of immunologically naive birds at the third inoculation, but the difference was not significant ($P = 0.10$) (Figure 2G and H, dashed lines). Also, the median duration of H13 virus excretion after the third inoculation (0 dpi; range, 0 to 7 dpi) appeared to be shorter than that after H13 inoculation of immunologically naive birds at the third inoculation (4 dpi; range, 0 to 5 dpi), but again, the difference was not significant ($P = 0.59$) (Table 2 and Figure 3).

(c) Group H16-H16-H16. The mean quantity of H16 virus excreted from the cloaca after the second inoculation was significantly lower than those after the first H16 inoculation ($P < 0.01$) (Figure 2B, black and gray lines) and after H16 inoculation of immunologically naive birds at the second inoculation ($P < 0.01$) (Figure 2B and C, gray lines). The decrease in virus excretion was consistent for each individual bird of group H16-H16-H16 (Figure 4). Also, the median duration of excretion of infectious virus was significantly shorter after the second inoculation (0 dpi; range, 0 to 3 dpi) than after the first inoculation (5.5

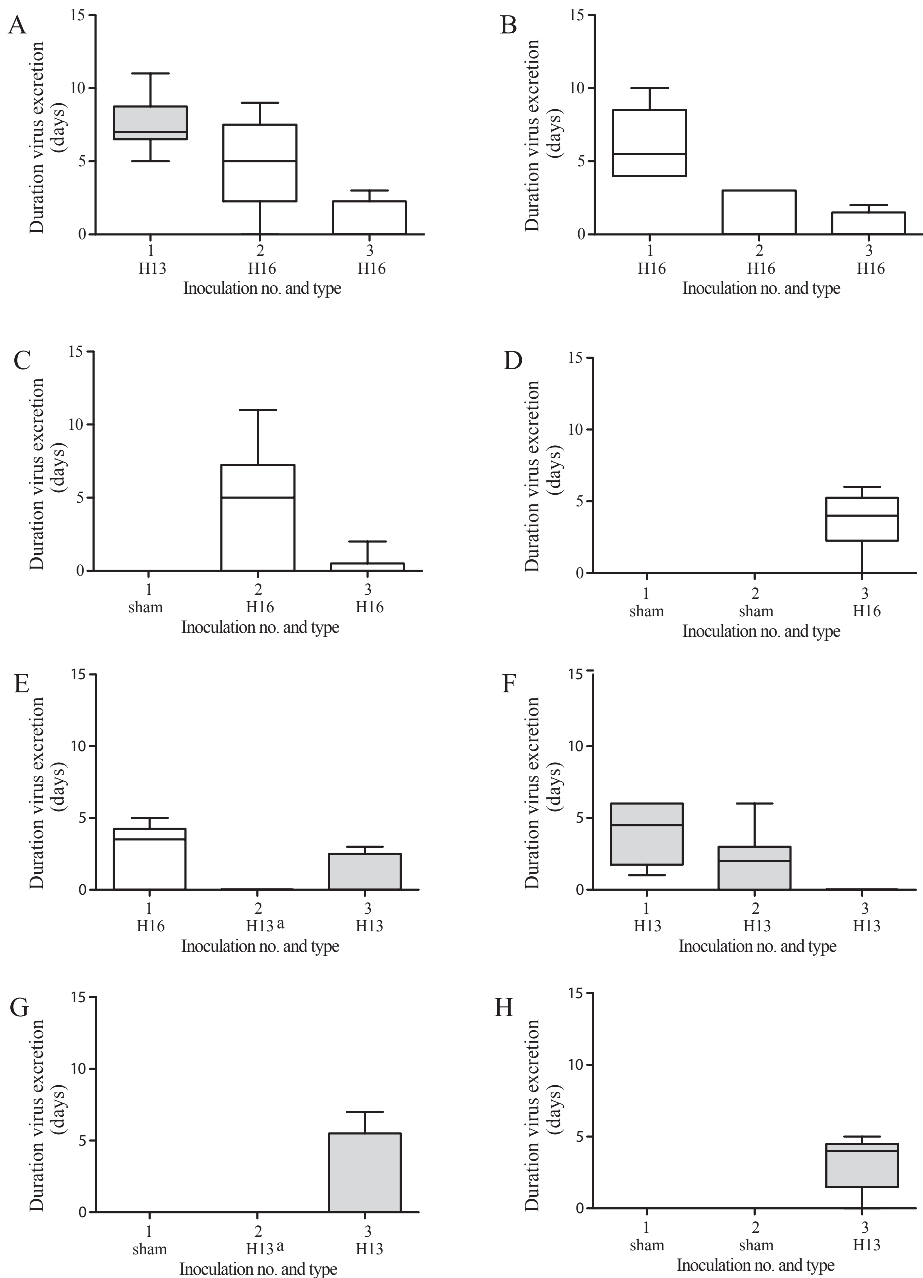


Figure 3. Median duration of excretion of infectious virus from cloacae after experimental infection of black-headed gulls with one or more inoculations of LPAIV H13N2, LPAIV H16N3, or both, based on virus isolation. The median duration of excretion of infectious virus is based on data for all birds in the group. dpi, days post inoculation. Gray boxes indicate H13, and white boxes indicate H16. a, the second inoculation of groups 5 and 7 was unsuccessful.

dpi; range, 4 to 10 dpi) ($P < 0.01$) and than after H16 inoculation of immunologically naive birds at the second inoculation (6 dpi; range, 0 to 11 dpi) ($P = 0.04$) (Table 2 and Figure 3). The mean quantity of H16 virus excreted from the cloaca after the third inoculation did not differ significantly from that after the second inoculation of the same group ($P = 0.22$) (Figure 2B, black and dashed lines) but was significantly lower than that after H16 inoculation of immunologically naive birds at the third inoculation ($P < 0.01$) (Figure 2B and D, dashed lines). Similarly, the median duration of virus excretion after the third inoculation (0 dpi; range, 0 to 2 dpi) did not differ significantly from that after the second inoculation of the same group (0 dpi; range, 0 to 3 dpi) ($P = 0.80$) but was significantly shorter than that after H16 inoculation of immunologically naive birds at the third inoculation (4 dpi; range, 0 to 6 dpi) ($P = 0.04$) (Table 2 and Figure 3).

(d) Group sham-H16-H16. The mean quantity of H16 virus excreted from the cloaca after the third inoculation was significantly lower than that after the second inoculation ($P < 0.01$) (Figure 2C, gray and dashed lines) and was significantly lower than that after H16 inoculation of immunologically naive birds at the third inoculation ($P < 0.01$) (Figure 2C and D, dashed lines). Also, the median duration of excretion of infectious H16 virus was significantly shorter after the third inoculation (0 dpi; range, 0 to 2 dpi) than after the second inoculation (6 dpi; range, 0 to 11 dpi) ($P = 0.04$) and than after H16 inoculation of immunologically naive birds at the third inoculation (4 dpi; range, 0 to 6 dpi) ($P = 0.03$) (Table 2 and Figure 3).

(iii) Effect of heterologous LPAIV infection on virus excretion

To investigate the effect of H13 virus infection on subsequent infection with H16 virus, the quantity and duration of virus excretion after second inoculation of group H13-H16-H16 were compared with those in immunologically naive birds inoculated with H16 at the second inoculation. The effect of H16 virus on subsequent infection with H13 virus could not be investigated due to the unsuccessful inoculation of immunologically naive birds with H13 at the second inoculation and, thus, the lack of a control group (i.e., group sham-(H13)-H13).

(a) Group H13-H16-H16. The mean quantity of H16 virus excreted from the cloaca after the second inoculation did not differ significantly between birds preexposed to H13 and immunologically naive birds ($P = 0.54$) (Figure 2A and C, gray lines). In line with this, the median duration of H16 virus excretion did not differ significantly between birds preexposed to H13 virus (5 dpi; range, 0 to 9 dpi) and immunologically naive birds (6 dpi; range, 0 to 11 dpi) ($P = 0.85$) (Table 2 and Figure 3).

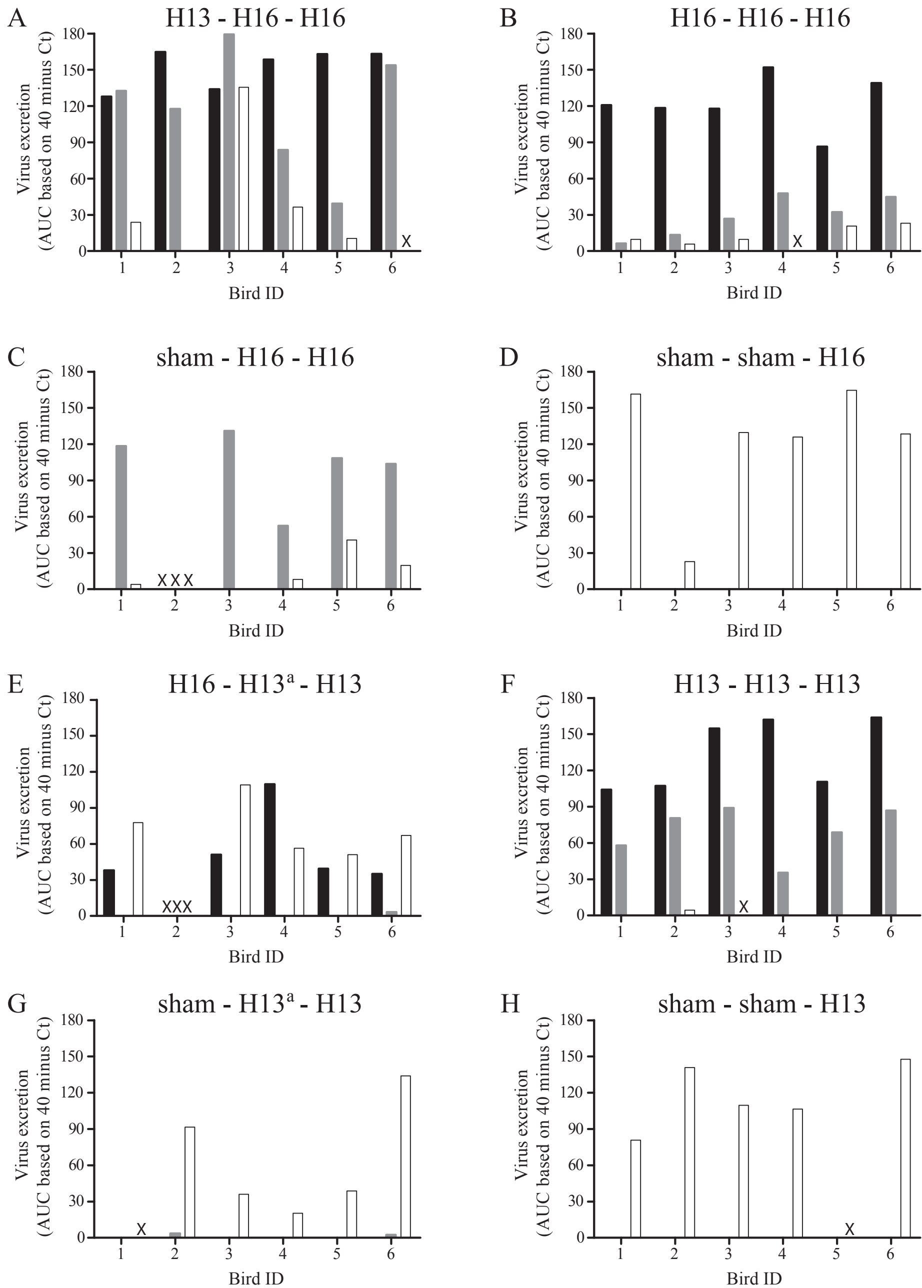


Figure 4. Excretion of LPAIV H13N2 and H16N3 by black-headed gulls, shown per individual bird. Virus excretion was based on the AUC value for viral RNA (i.e., C_T value determined by M-RT-PCR). Black indicates the first inoculation, gray indicates the second inoculation, and white indicates the third inoculation. dpi, days post inoculation; x, the bird died and was excluded from the analysis; a, the second inoculation of groups 5 and 7 was unsuccessful.

(b) Group H16-(H13)-H13. The mean quantity of H13 virus excreted from the cloaca after the second inoculation was low, as H13 virus was detected in the cloaca by M-RT-PCR in only one of six birds on day 7 after the second inoculation, and no virus was isolated from the cloaca. In the same bird, H13-specific antibodies were detected after the second inoculation. Despite the fact that no virus was detected in other birds until 14 days after the second inoculation, the H16-specific antibody titer was boosted in three of five birds after the second inoculation (Figure 5). The mean quantity of H13 virus excreted from the cloaca after the third inoculation was significantly lower than that for immunologically naive birds inoculated with H13 at the third inoculation (P = 0.03) (Figure 2E and H, dashed lines). The median duration of excretion of infectious virus after the third inoculation (0 dpi; range, 0 to 3 dpi) did not differ significantly from that for immunologically naive birds inoculated with H13 at the third inoculation (4 dpi; range, 0 to 5 dpi) (P = 0.08) (Table 2 and Figure 3).

Humoral immune response

(i) Effect of age on AIV-specific antibody production. To investigate the effect of age on the immune response, the proportions of birds that raised AIV-specific antibodies within 1 month after inoculation and the concentrations of these antibodies in serum were compared in 2-, 3- and 14-month-old birds. The proportions of birds that produced H13-specific antibodies after the first inoculation with H13 did not differ significantly between 2-month-old birds (6 of 12 (50%), i.e., the total for the first inoculation of group H13-H16-H16 and group H13-H13-H13) and 14-month-old birds (3 of 5 (60%)) (P = 0.38). The proportions of birds with NP-specific antibodies after the first inoculation with H13 also did not differ significantly between 2-month-old birds (7 of 12 (58%)) and 14-month-old birds (4 of 5 (80%)) (P = 0.32). The mean quantities of H13-specific antibodies generated after the first inoculation with H13 virus did not differ significantly between 2-month-old birds (2.92 ± 0.94; n = 12) and 14-month-old birds (2.20 ± 1.02) (P = 0.74) (Table 3).

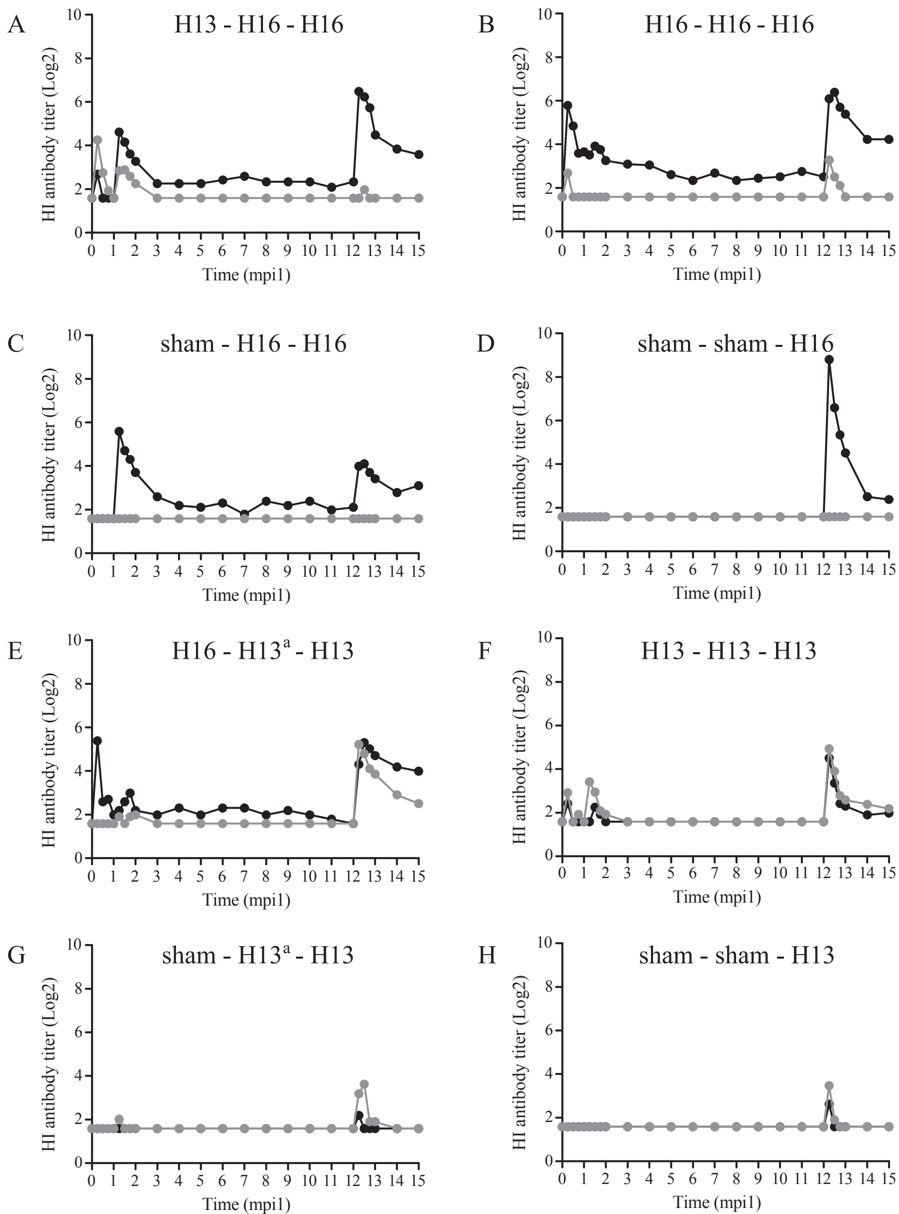


Figure 5. Mean HI antibody titers after one or more inoculations of black-headed gulls with LPAIV H13N2, LPAIV H16N3, or both. Mean antibody titers are based on data for all birds in the group. dpi, days post inoculation. Gray indicates H13-specific antibodies, and black indicates H16-specific antibodies. a, the second inoculation of groups 5 and 7 was unsuccessful.

Table 3. Antibody detection in black-headed gulls after one or more inoculations with LPAIV H13N2, H16N3, or both

			Inoculation no.		
			I		
Group no.	Inoculation schedule	Assay	Antibody titer at 0 dpi	Antibody productionb	No. birds seropositive / totalc
1	H13-H16-H16				
		H13 HI	0	4.17 ± 1.38	4/6
		H16 HI	0	1.10 ± 0.50	3/6
		NP bELISA			3/6
2	H16-H16-H16				
		H13 HI	0	1.10 ± 0.82	2/6
		H16 HI	0	10.49 ± 2.54	6/6
		NP bELISA			4/6
3	sham-H16-H16				
		H13 HI	0	0.00 ± 0.00	0/5
		H16 HI	0	0.00 ± 0.00	0/5
		NP bELISA			0/5
4	sham-sham-H16				
		H13 HI	0	0.00 ± 0.00	0/6
		H16 HI	0	0.00 ± 0.00	0/6
		NP bELISA			0/6
5	H16-H13a-H13				
		H13 HI	0	0.00 ± 0.00	0/5
		H16 HI	0	6.12 ± 2.65	3/5
		NP bELISA			3/5
6	H13-H13-H13				
		H13 HI	0	1.67 ± 1.17	2/6
		H16 HI	0	0.83 ± 0.54	2/6
		NP bELISA			4/6
7	sham-H13a-H13				
		H13 HI	0	0.00 ± 0.00	0/6
		H16 HI	0	0.00 ± 0.00	0/6
		NP bELISA			0/6
8	sham-sham-H13				
		H13 HI	0	0.00 ± 0.00	0/6
		H16 HI	0	0.00 ± 0.00	0/6
		NP bELISA			1/6

NP bELISA, blocking ELISA specific for the nucleoprotein of the influenza A virus; HI, hemagglutination inhibition assay; a The second inoculation of group 5 and 7 was unsuccessful; b Antibody production is based on the AUC on a log_2 scale between 0 and 28 days postinoculation; c Total number of birds that seroconverted between 0 and 28 days postinoculation.

Table 3. continued

Inoculation no.					
II			III		
Antibody titer at 0 dpi	Antibody productionb	No. birds seropositive / totalc	Antibody titer at 0 dpi	Antibody productionb	No. birds seropositive / totalc
0	3.90 ± 2.03	4/6	0	0.40 ± 0.40	1/4
0	8.47 ± 2.69	5/6	2.33 ± 1.50	15.51 ± 6.03	4/4
		4/6			2/4
0	0.00 ± 0.00	0/6	0	3.47 ± 2.49	2/5
3.65 ± 1.73	8.30 ± 2.38	5/6	2.18 ± 0.89	16.52 ± 6.78	4/5
		1/6			3/5
0	0.00 ± 0.00	0/5	0	0.00 ± 0.00	0/5
0	10.89 ± 3.24	4/5	2.10 ± 1.16	8.21 ± 4.60	3/5
		4/5			1/5
0	0.00 ± 0.00	0/6	0	0.00 ± 0.00	0/6
0	0.00 ± 0.00	0/6	0	17.45 ± 1.97	6/6
		0/6			5/6
0	0.83 ± 0.83	1/5	0	10.48 ± 3.61	4/5
1.98 ± 0.89	3.50 ± 1.33	4/5	0	11.43 ± 5.03	3/5
		0/5			4/5
0	3.86 ± 2.19	4/6	0	7.37 ± 4.31	4/5
0	1.00 ± 1.00	1/6	0	5.88 ± 2.74	3/5
		2/6			3/5
0	0.43 ± 0.43	1/6	0	4.11 ± 1.53	4/5
0	0.00 ± 0.00	0/6	0	0.60 ± 0.60	1/5
		0/6			2/5
0	0.00 ± 0.00	0/6	0	2.20 ± 1.02	3/5
0	0.00 ± 0.00	0/6	0	1.03 ± 0.43	3/5
		0/6			4/5

The proportion of birds that produced H16-specific antibodies after the first inoculation with H16 virus did not differ significantly between 2-month-old birds (9 of 11 (82%)) and 3-month- old birds (4 of 5 (80%)) (P = 0.49), between 3-month-old birds and 14-month-old birds (6 of 6 (100%)) (P = 0.45), and between 2-month-old birds and 14-month-old birds (P = 0.40). Also, the proportion of birds with NP-specific antibodies after the first inoculation with H16 virus did not differ significantly between 2-month-old birds (7 of 11 (64%)) and 3-month-old birds (4 of 5 (80%)) (P = 0.38), between 3-month-old birds and 14-month- old birds (5 of 6 (83%)) (P = 0.55), and between 2-month-old birds and 14-month-old birds (P = 0.32). The mean quantities of H16-specific antibodies generated after the first H16 inoculation of 2-month-old birds (8.5 ± 1.9), 3-month-old birds (10.89 ± 3.24), and 14-month-old birds (17.45 ± 1.97) differed significantly (P = 0.05), with a significantly larger quantity of H16-specific antibodies detected in 14-month-old birds than in 2-month-old birds (P = 0.01) (Table 3).

(ii) Detection of AIV-specific cross-reactive antibodies. The detection of cross-reactive antibodies differed between H13 and H16 viruses and by age. After the first H13 inoculation, H16-cross-reactive antibodies were detected on day 7 in 5 of 12 (42%) 2-month-old birds and in 3 of 5 (60%) 14-month-old birds. After the first H16 inoculation, H13-cross-reactive antibodies were detected on day 7 in 2 of 11 (18%) 2-month-old birds and were not detected in 5 3-month-old birds and 6 14-month-old birds (Table 3 and Figure 5).

(iii) AIV-specific antibody production after multiple LPAIV inoculations. Of birds that had been exposed to the same virus more than once (i.e., H13-H13-H13 and H16-H16-H16), no significant differences in the quantities of specific antibody titers after the first, second, and third H13 infections (P = 0.33) or after the first, second, and third H16 infections (P = 0.62) were detected (Table 3).

(iv) Persistence of AIV-specific antibodies between breeding seasons. To investigate the persistence of AIV-specific antibodies in the different inoculation groups, the periods of detection of H13-, H16-, and NP-specific antibodies for the different groups were compared. During the months between the second and third inoculations, AIV-specific antibodies were detected in a limited number of birds for a limited period of time, except for H16-specific antibodies, which stayed detectable until 11 months after the second inoculation. Within this period, H16-specific antibodies were most frequently detected in birds of group H16-H16-H16. In contrast to H16-specific antibodies, H13-specific antibodies were detected only until 1 month after the second inoculation (in groups H13-H16-H16, H16-(H13)-H13, and H13-H13-H13 only). NP-specific antibodies

were detected until 3 months after the second inoculation (i.e., H13- H13-H13) (Table 4). On the day of the third inoculation, H16-specific antibodies were detected (in groups H13-H16-H16, H16-H16-H16, and sham-H16-H16 only), while no H13-specific antibodies were detected on that day (Figure 5).

(v) Link between AIV-specific antibodies and H13 and H16 virus excretion. To investigate if AIV-specific antibodies had a protective effect against subsequent infection, the presence of H13- and H16-specific antibodies on the day of subsequent inoculation was compared with the subsequent excretion of homologous virus. On the day of the second or third inoculation, only H16-specific antibodies were detected (Table 4 and Figure 5). There were no significant differences in the quantity of virus excretion ($P = 0.54$), peak virus excretion ($P = 0.84$), timing of peak virus excretion ($P = 0.14$), and duration of virus excretion ($P = 0.37$) between birds with ($n = 7$) and those without ($n = 9$) H16-specific antibodies belonging to group H16-H16-H16 and sham-H16-H16.

Clinical signs of infection

To investigate clinical signs of infection, body mass, bird behavior, and fecal water content were monitored. Body mass was constant in time from days 0 to 14 postinoculation independent of LPAIV or sham inoculation (Figure 6). After each inoculation, bird behavior, as observed for 5 min per group each morning, varied inconsistently between days (data not shown). After the first inoculations with H13 or H16 virus, the water content of feces, as a proxy for diarrhea, varied inconsistently in time from days 0 to 7 postinoculation and did not correlate with the quantity of virus excretion ($R = 0.02$ and $P = 0.91$). The mass of feces and number of droppings were not associated with the quantity of virus excretion ($R = -0.07$ and $P = 0.62$, and $R = 0.02$ and $P = 0.89$, respectively) (Figure 7).

Head movements as a measure of activity after the second inoculation varied inconsistently among groups and in time. Bird activity, as measured during daily 15-min observations after the third inoculation, varied inconsistently among groups and in time (data not shown).

Table 4. Year-round antibody detection after one or more inoculations of black-headed gulls with LPAIV H13N2, H16N3, or both

			Inoculation no. and months post first inoculation							
			I	II						
Group no.	Inoculation schedule	Assay	0	1	2	3	4	5	6	7
1	H13-H16-H16									
		H13 HI	0/6	0/6	2/6	0/6	0/6	0/6	0/6	0/4
		H16 HI	0/6	0/6	4/6	1/6	3/6	2/6	2/6	1/4
		NP bELISA	0/6	0/6	1/6	1/6	0/6	0/6	0/6	0/4
2	H16-H16-H16									
		H13 HI	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
		H16 HI	0/6	4/6	4/6	3/6	4/6	3/6	3/6	3/6
		NP bELISA	0/6	1/6	1/6	0/6	0/6	0/6	0/6	0/6
3	sham-H16-H16									
		H13 HI	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
		H16 HI	0/5	0/5	3/5	2/5	2/5	2/5	2/5	2/5
		NP bELISA	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
4	sham-sham-H16									
		H13 HI	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
		H16 HI	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
		NP bELISA	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
5	H16-H13a-H13									
		H13 HI	0/5	0/5	1/5	0/5	0/5	0/5	0/5	0/5
		H16 HI	0/5	1/5	1/5	1/5	2/5	1/5	2/5	2/5
		NP bELISA	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
6	H13-H13-H13									
		H13 HI	0/6	0/6	1/6	0/6	0/6	0/6	0/6	0/6
		H16 HI	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
		NP bELISA	0/6	0/6	0/6	0/6	1/6	0/6	0/6	0/6
7	sham-H13a-H13									
		H13 HI	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
		H16 HI	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
		NP bELISA	0/6	0/6	0/6	0/6	0/6	0/6	0/6	0/6
8	sham-sham-H13									
		H13 HI	0/6	0/6	0/6	0/5	0/5	0/5	0/5	0/5
		H16 HI	0/6	0/6	0/6	0/5	0/5	0/5	0/5	0/5
		NP bELISA	0/6	0/6	0/6	0/5	0/5	0/5	0/5	0/5

a The second inoculation of groups 5 and 7 was unsuccessful.

Table 4. continued

			Inoculation no. and months post first inoculation							
			II				III			
Group no.	Inoculation schedule	Assay	8	9	10	11	12	13	14	15
1	H13-H16-H16									
		H13 HI	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4
		H16 HI	1/4	1/4	1/4	1/4	1/4	3/4	2/4	2/4
		NP bELISA	0/4	0/4	0/4	0/4	0/4	1/4	1/4	1/4
2	H16-H16-H16									
		H13 HI	0/6	0/6	0/6	0/6	0/5	0/5	0/5	0/5
		H16 HI	2/6	3/6	3/6	3/6	2/5	3/5	2/5	2/5
		NP bELISA	0/6	0/6	0/6	0/6	0/5	0/5	0/5	0/5
3	sham-H16-H16									
		H13 HI	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
		H16 HI	2/5	2/5	2/5	2/5	1/5	3/5	3/5	3/5
		NP bELISA	0/5	0/5	0/5	0/5	0/5	1/5	1/5	1/5
4	sham-sham-H16									
		H13 HI	0/6	0/6	0/6	0/6	0/6	0/6	0/5	0/4
		H16 HI	0/6	0/6	0/6	0/6	0/6	6/6	2/5	2/4
		NP bELISA	0/6	0/6	0/6	0/6	0/6	0/6	0/5	0/4
5	H16-H13a-H13									
		H13 HI	0/5	0/5	0/5	0/5	0/5	4/5	3/5	2/5
		H16 HI	1/5	2/5	2/5	1/5	0/5	3/5	3/5	3/5
		NP bELISA	0/5	0/5	0/5	0/5	0/5	3/5	1/5	1/5
6	H13-H13-H13									
		H13 HI	0/6	0/5	0/5	0/5	0/5	1/5	1/5	1/5
		H16 HI	0/6	0/5	0/5	0/5	0/5	2/5	1/5	1/5
		NP bELISA	0/6	0/5	0/5	0/5	0/5	2/5	1/5	0/5
7	sham-H13a-H13									
		H13 HI	0/6	0/6	0/6	0/6	0/5	1/5	0/5	0/5
		H16 HI	0/6	0/6	0/6	0/6	0/5	0/5	0/5	0/5
		NP bELISA	0/6	0/6	0/6	0/6	0/5	0/5	0/5	0/5
8	sham-sham-H13									
		H13 HI	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
		H16 HI	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
		NP bELISA	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5

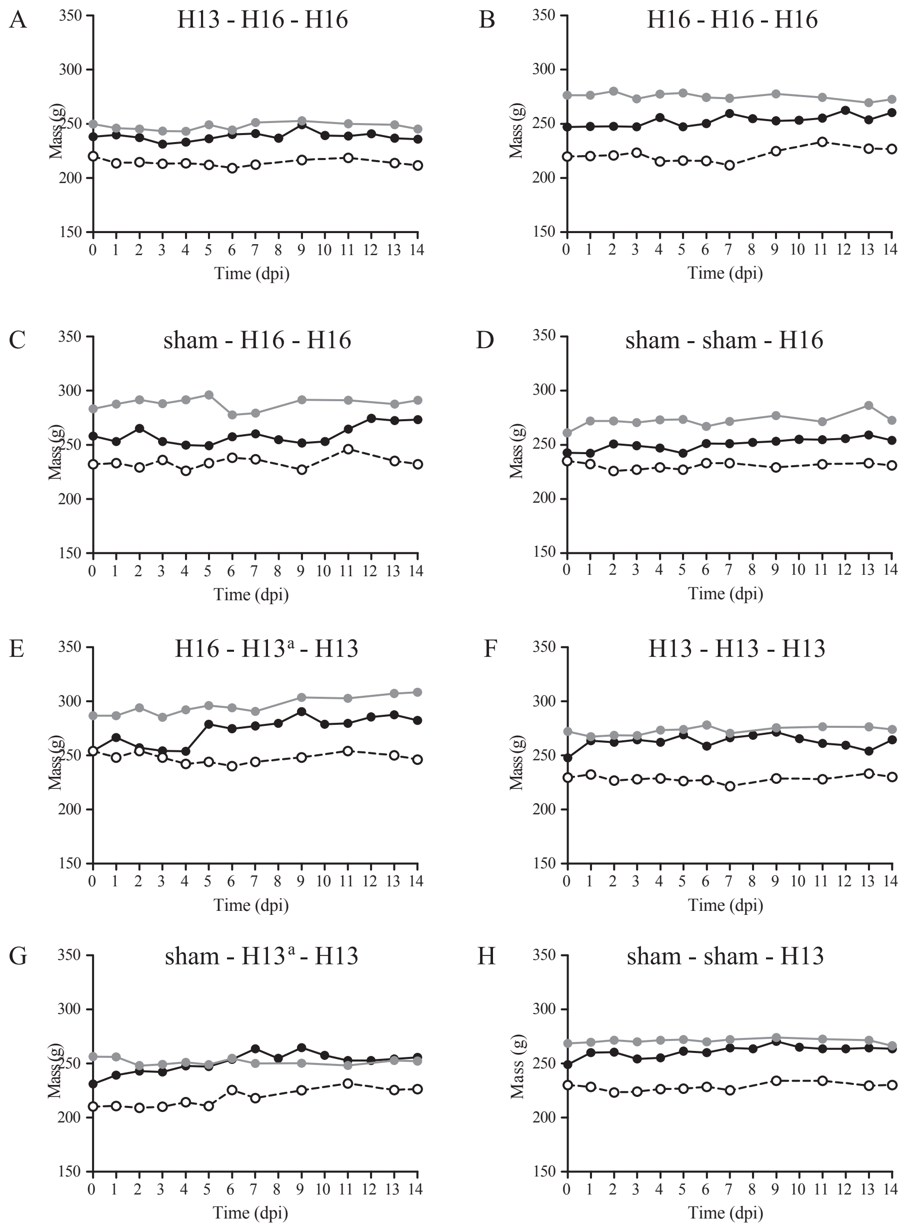


Figure 6. Body mass of black-headed gulls after the first, second, and third LPAIV H13N2 or H16N3 inoculations from days 0 to 14 postinoculation. Black lines indicate the first inoculation, gray lines indicate the second inoculation, and dashed lines indicate the third inoculation. dpi, days post inoculation. a, the second inoculation of groups 5 and 7 was unsuccessful.

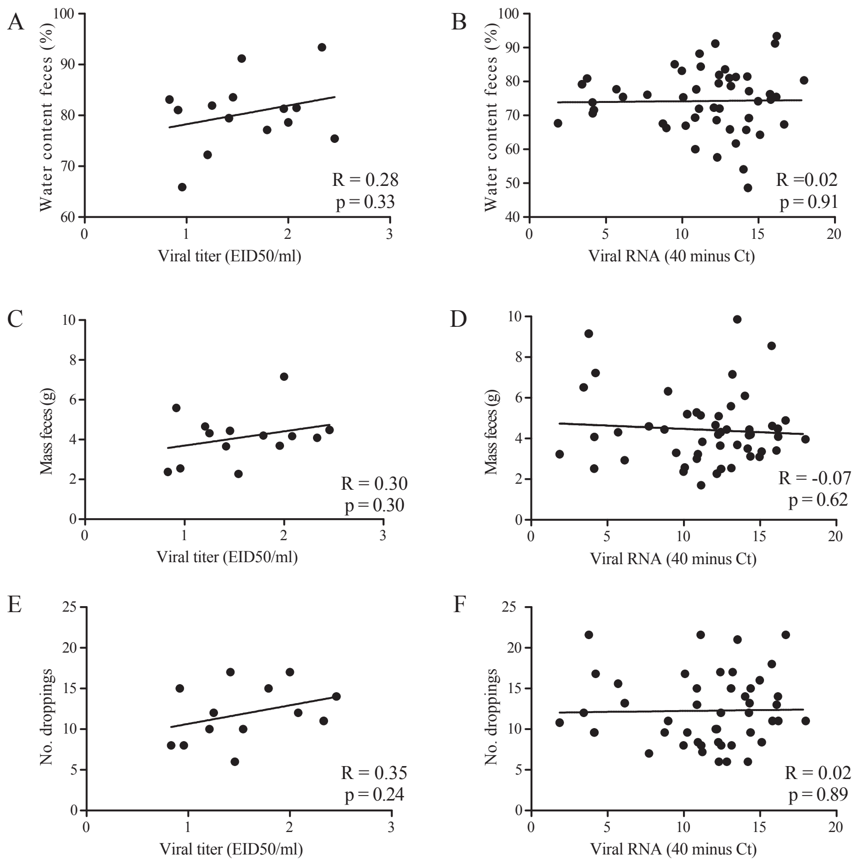


Figure 7. Monitoring of feces and virus excretion from cloacae based on viral titer (A, C, and E) and viral RNA (B, D, and F) from day 0 until day 7 after the first LPAIV H13N2 or H16N3 inoculation of black-headed gulls during the first and second breeding seasons

DISCUSSION

The results of this study on H13 and H16 LPAIV infections in BHGU provided answers to the main questions posed above. LPAIV infection induced long-lasting, partial protection against infection with a homologous virus, which was boosted at each subsequent exposure, but no protection against infection with a heterologous virus. In general, first-year birds and second-year birds were equally susceptible to LPAIV infection. Finally, LPAIV infection did not cause detectable disease in BHGU.

LPAIV infection induced partial protection against subsequent infection with the homologous virus in birds in this study. Furthermore, this protection was boosted upon a second exposure to the homologous virus, with no excretion of infectious virus in H13-

inoculated birds (0 of 5 birds) and only limited excretion in H16-inoculated birds (2 of 5 birds, until 2 dpi). This implies that after two or, at most, three serial infections with a homologous LPAIV, BHGU are no longer productively infected with LPAIV. Although the age at which BHGU will have had two LPAIV infections is unknown, it is likely that BHGU are exposed to LPAIV every year at the end of the breeding season (late summer) at large-colony breeding sites (224). Even if first-year BHGU have not been infected at their breeding colony site, they are most likely to become infected shortly afterwards, when nonbreeding BHGU and BHGU from multiple breeding sites mix abundantly. Thus, BHGU typically will probably have had at least two LPAIV infections after their second summer. Given the long-term protective effect of prior infections with homologous LPAIVs, BHGU older than 1.5 years of age may not be important for the persistence of LPAIV in the population.

LPAIV infection did not induce protection against subsequent infection with the heterologous virus in birds in this study. The level and duration of H16 virus excretion in group H13-H16-H16, 1 month after H13 virus infection, were similar to those of H16 virus excretion by immunologically naive birds (group sham-H16-H16). The effect of H16 virus infection on subsequent H13 virus infection (group H16-(H13)-H13) was ambiguous due to unsuccessful H13 virus inoculation 1 month after H16 inoculation: no H13 virus excretion was detected, although AIV-specific immunity was boosted. However, H13 virus inoculation 1 year after H16 virus inoculation in this group resulted in H13 virus excretion that was significantly smaller in quantity than but similar in duration to those in immunologically naive birds. The absence of a protective effect of H13 virus infection on subsequent H16 virus infection suggests that the epidemiological dynamics of H13 and H16 in BHGU are largely independent of each other; however, a partially protective effect of H16 virus infection on subsequent H13 virus infection cannot be excluded.

Previously, in other aquatic bird species, LPAIV infection induced partial protection against subsequent infection with a homologous virus and partial to nearly complete protection against subsequent infection with a heterologous virus. Similar to our findings in BHGU, LPAIV infection also induced partial protection against reinfection with the homologous virus in mallards (H7N7) (61) and Pekin ducks (H5N3 and H7N2, respectively) (52, 225). In those studies, the time interval between the first and second inoculations was relatively short, ranging from 21 to 84 days. In contrast to our findings in BHGU, LPAIV infection induced partial (H5N3 followed by H7N2 and H3N8 followed by H5N2 and vice versa, respectively) (59, 225) to nearly complete (61) protection against subsequent infection with a heterologous virus in Pekin ducks (225) and mallards (59, 61). The following differences in study design may play a role in this discrepancy. Compared to our study, the LPAIV subtypes used in those studies were different (H5N3 and H7N2

(225), H7N7 and H5N2 (61), and H3N8 and H5N2 (59)); the time interval between the first and second inoculations, 14 (61) or 21 days (59, 225), was shorter; the inoculum dose, 4 X 10^6 PFU (225) or $10^{8.7}$ EID_{50} (61), was higher; and there were two prior infections with a heterologous virus (61) rather than one. In free-living mallards, heterosubtypic LPAIV immunity has been described for different HA subtypes belonging to the same phylogenetic clade (190). For the above-described mallard and Pekin duck studies with homologous as well as heterologous inoculations, the time interval between subsequent infections was relatively short; consequently, it is unknown if protection would have lasted for 1 year, which is the typical interval between epizootics in mallards (26) and BHGU (224).

Overall, the results of this study showed no effect of age on susceptibility to LPAIV infection: there were no differences between immunologically naive 2- and 14-month-old BHGU in the proportion of birds infected, quantity of LPAIV excreted, or du- ration of LPAIV excretion. An exception was the duration of H13 virus excretion, which was significantly longer in 2-month-old than in 14-month-old birds. The latter result needs to be interpreted with caution, because there was already quite a high degree of variability in the virus excretion results between groups of 2-month-old birds inoculated with the same virus (Table 2). These results correspond with those reported previously by Costa et al. (57), who inoculated LPAIV H5N2 or LPAIV H3N8 into mallards ranging from 2 weeks to 4 months of age and found no significant effect of age on the proportion of birds infected or the level of LPAIV excretion. However, these results are in contrast with the results of VanDalen et al. (58), who inoculated LPAIV H4N6 into 3- or 6-month-old mallards and found a significantly larger quantity of excreted viral RNA in 6-month-old birds. Together, these results indicate that the more frequent detection of LPAIV in juvenile than in adult free-living water birds (e.g., see references (26, 32, 229)) cannot be explained by age-dependent susceptibility.

There was no evidence of clinical disease from LPAIV infection in the birds in this study. In order to be able to detect possible clinical signs as sensitively as possible, we measured several parameters (fecal water content, fecal mass, and number of droppings) related to diarrhea, which is often associated with intestinal infections (77). However, none of these parameters were correlated with LPAIV excretion. In addition, there was no loss of body weight, decreased activity level, or any other clinical sign. These results indicate that LPAIV H13 and H16 infections do not cause clinical disease in BHGU. Hypothetically, selection for such a lack of virulence of LPAIV may be driven by the mobility of wild water birds, because any virulence would render the infected bird less mobile, as well as inducing it to separate from the rest of its group and thus reducing the contact rate and therefore the transmission rate (230). However, a caveat

of this study, as for any laboratory infection of wild animals, is that the circumstances were very different from those in the field (77). For example, birds were not exposed to a harsh climate or food scarcity and instead were kept at a constant temperature and fed *ad libitum*. Therefore, a failure to observe clinical signs under laboratory circumstances does not mean that LPAIV is not virulent for BHGU under field circumstances.

Unexpected results of this study were that AIV-specific serum antibodies had little value as a correlate of protection or as evidence of prior infection. First, although LPAIV infection (either H13 or H16) induced partial protection against reinoculation with the homologous virus, this protective effect, at the between-group level, was independent of the presence of AIV-specific antibodies on the day of reinoculation. Moreover, at the within-group level, the presence or titer of H16-specific antibodies on the day of reinoculation was not associated with decreased or shortened H16 virus excretion. Also, at the between-group level (sham-H16-H16 versus H13-H16-H16), the detection of H16-specific antibodies at 1 week post-H13 inoculation was not associated with a protective effect against subsequent H16 infection at 4 weeks post-H13 inoculation. These results show that the presence or titer of AIV-specific serum antibodies in BHGU is not a correlate of protection against LPAIV infection. These results in BHGU correspond to those in mallards (61), where virus excretion after challenge with homologous LPAIV was independent of AIV-specific ELISA antibodies on the day of challenge (61).

Mucosal rather than serum antibodies may be a better correlate of protection, as LPAIV in BHGU (140) and mallards (231) infects the digestive tract. Although already suggested in 1980 by Kida and colleagues (52), mucosal antibodies against virus infections in birds have received little attention, perhaps because of technical difficulties in measurements. AIV-specific antibodies have been detected in bile of ducks infected with AIV (232). Also, mucosal antibodies have been detected in tears of chickens after infection with Newcastle disease virus and infectious bronchitis virus and were associated with partial protection against virus challenge (233, 234). In humans, rotavirus-specific IgA in fecal specimens was directly correlated with protection against rotavirus illness (235, 236). Therefore, the use of mucosal antibodies in feces as a potential correlate of protection of water birds against LPAIV infection in the digestive tract deserves further research.

Second, this study shows that the use of AIV-specific serum antibodies to provide evidence of prior AIV infection is limited. Experimental infections using wild-caught or farm-raised birds often rely on the absence of AIV-specific antibodies to indicate the absence of past infection (49, 61, 118, 237). However, in our study, the vast majority of BHGU did not have NP- or HA-specific antibodies 1 month after primary H13 or H16 infection, with the exception of H16-specific antibodies after H16 infection. Furthermore,

even in the birds that seroconverted, AIV-specific antibodies remained detectable for a maximal period of only 2 to 3 months (for NP- and H13-specific antibodies) or 11.5 months (for H16-specific antibodies) after primary H13 or H16 infection. Thus, our results indicate that a lack of AIV-specific serum antibodies does not exclude past LPAIV infection in BHGU.

The differences in patterns of virus excretion and immunogenicity between the two LPAIV isolates used in this study are most likely due to genetic differences in three gene segments, HA, NA, and NS; the other gene segments were genetically highly similar (sequences are available online (see Materials and Methods)). There were obvious differences in the HA genes (H13 versus H16) and NA genes (N2 versus N3). Therefore, exposure to a LPAIV with the same HA and NA genes may have strengthened the protective effect against reinoculation with the homologous virus. The NS gene segment of H13 belonged to allele B, and that of H16 belonged to allele A (the most common NS allele) (99, 238). The NS1 protein, one of the two proteins encoded by the NS gene segment, is able to inhibit the host innate immune response by antagonizing interferon. Viruses with allele A of the NS1 protein replicated more than did those with allele B in chicken and turkey cells; in contrast, viruses with allele B of the NS1 protein replicated more and to higher titers than did those with allele A in duck cells (239).

In conclusion, we demonstrate that experimental LPAIV infection of BHGU has a protective effect, lasting up to 1 year, on reinfection with a homologous virus but no protective effect on subsequent infection with a heterologous virus. The information generated in this study (e.g., quantity of virus excreted and duration of excretion of infectious virus after the first, second, and third infections with homologous or heterologous viruses) should be useful information to help design surveillance programs of AIV in wild birds and to interpret data generated by these programs. It should also help to build mathematical models to study the epidemiology of LPAIV in BHGU and other free-living aquatic birds. Nevertheless, additional research is needed to show if the same AIV dynamics apply to other bird species and other AIV subtypes and strains. Given the lack of correlation between AIV-specific serum antibodies and protection against LPAIV infection, further research is required to elucidate the mechanism of protection of LPAIV infection and which parameters (e.g., mucosal antibody levels) can be used as correlates of protection. In addition, this study points out that the lack of detectable AIV-specific serum antibodies in birds does not exclude the possibility of a past LPAIV infection. Knowledge on long-term protection against homologous and heterologous LPAIV infections in an aquatic bird species like BHGU, which are annually exposed at its breeding colony sites in West Europe, is essential to understand LPAIV epidemiology and persistence in wild birds.

ACKNOWLEDGEMENTS

We thank Angéla Gommersbach and Tanja Schouten (RIVM animal caretakers), Ellis Mulder and Cor Dijkstra (University of Groningen, for sexing of BHGU), Monique de Vrijer (Vogelklas Karel Schot, for advice on hand-raising and nutrition of birds), and Francisca Velkers (Utrecht University, for advice on hand-raising and nutrition of birds).

U.H. was partly funded by a Netherlands Genomics Institute/Virgo Consortium visiting scientist stipend. This work was sponsored by grants from the Dutch Ministry of Economic Affairs, European Research Council project FLUPLAN (250136), and NIH NIAID contracts HHSN266200700010C (2007-2014) and HHSN272201400008C (2014- 2021).

CHAPTER 3.1

Josanne H. Verhagen, Vincent J. Munster, Frank Majoor, Pascal Lexmond
Oanh Vuong, Job B.G. Stumpel, Guus F. Rimmelzwaan
Albert D.M.E. Osterhaus, Martin Schutten, Roy Slaterus
Ron A.M. Fouchier

Avian influenza A virus in wild birds in highly urbanized areas

PLoS ONE (2012), Volume 7, Issue 6: e38256. DOI: 10.1371/journal.pone.0038256

Avian influenza virus (AIV) surveillance studies in wild birds are usually conducted in rural areas and nature reserves. Less is known of avian influenza virus prevalence in wild birds located in densely populated urban areas, while these birds are more likely to be in close contact with humans. Influenza virus prevalence was investigated in 6059 wild birds sampled in cities in the Netherlands between 2006 and 2009, and compared with parallel AIV surveillance data from low urbanized areas in the Netherlands. Viral prevalence varied with the level of urbanization, with highest prevalence in low urbanized areas. Within cities virus was detected in 0.5% of birds, while seroprevalence exceeded 50%. Ring recoveries of urban wild birds sampled for virus detection demonstrated that most birds were sighted within the same city, while few were sighted in other cities or migrated up to 2659 km away from the sample location in the Netherlands. Here we show that urban birds were infected with AIVs and that urban birds were not separated completely from populations of long-distance migrants. The latter suggests that wild birds in cities may play a role in the introduction of AIVs into cities. Thus, urban bird populations should not be excluded as a human-animal interface for influenza viruses.

INTRODUCTION

Wild aquatic birds are frequently infected with influenza A viruses. Wild birds are assumed to be the original source of influenza A viruses currently circulating in the animal and human population, as wild birds are often infected with all known influenza A virus hemagglutinin (H1–H16) and neuraminidase (N1–N9) subtypes (12, 16). In most cases wild birds are infected with low pathogenic avian influenza (LPAI) viruses that cause no or only mild disease symptoms in their natural hosts. LPAI viruses can occasionally be transmitted to domestic bird and mammalian species in which they can cause mild to severe disease. Since the first discovery of influenza A viruses in wild birds in 1961 (A/Tern/South Africa/1961) (124), wild birds have been monitored for the presence of influenza A viruses (240, 241). However, wild bird sampling activities were intensified (133) after the emergence of highly pathogenic avian influenza (HPAI) H5N1 viruses in South-East Asia, and the detection of HPAI H5N1 viruses in migrating wild birds since 2005 (21, 114, 127). The increase of wild bird sampling activities worldwide resulted in the expansion of the number of sampled species and locations, with most species sampled belonging to the orders Anseriformes (ducks, geese and swans) and Charadriiformes (shorebirds and gulls). In addition to the early detection of HPAI viruses, these studies are important to understand the global circulation of both HPAI and LPAI viruses (1). In most cases, avian influenza virus (AIV) surveillance studies in wild birds were conducted in rural areas and nature reserves characterized by low human densities. AIVs, including HPAI viruses, have sporadically been reported from wild birds in highly urbanized areas (125, 242, 243), but very little is known about the frequency of AIV infection in wild birds in cities and the risk these birds could pose to domestic animal and human health. Since 2007 the majority of the global human population is more urban than rural, and the number of people living in urbanized areas is expected to continue growing in the next decade (244). In many countries, highly urbanized areas contain canals and large city parks with ponds, housing a wide variety of wild and semi-domesticated wild birds. We hypothesized that AIVs are present in wild aquatic birds present in these cities, with prevalence varying with the level of urbanization. We further hypothesized that wild birds sampled near closed water bodies (stagnant water, not connected to other water sources) will be infected with AIV, suggesting these birds play a role in the introduction of AIVs into cities. Here we addressed the questions whether wild aquatic birds present in cities are infected with AIVs and if so, if viral prevalence corresponds with the level of urbanization and connections with closed and open waters.

METHODS

Cloacal and oropharyngeal swabs and blood samples were collected from free-living birds in highly urbanized areas–defined here as cities with >1500 addresses per km2– in the Netherlands from 2006 to 2009. In most cases birds were located in city parks in close proximity to surface waters, in mixed age and species groups. Most sample locations were described either as being located in the centre or in the periphery of a highly urbanized area, and/or being located near open flowing water (in connection with larger water facilities, e.g. canals) or closed stagnant water (not connected to other water sources, e.g. city park ponds). Ducks, geese, gulls and coots were captured by an experienced ornithologist, either individually using a rope with a loop, or with multiple birds at one time using a clap net. All sampled birds were marked individually with a metal leg ring, and bird movements were recorded based on the recoveries of these bands. For comparison of the data obtained from the highly urbanized areas, we used data collected during ongoing AIV surveillance studies in rural, low urbanized areas with little human activity in the Netherlands during the same years. An independent Animal Ethics Committee of the Erasmus Medical Center (Stichting DEC Consult) approved these studies (permit number 122-09-20), in accordance with national and international guidelines. RNA was isolated from cloacal and oropharyngeal samples and analyzed using a real-time reverse transcriptase-PCR (RRT-PCR) assay targeting the matrix gene. All matrix RRT-PCR positive samples were used for detection of H5 and H7 influenza A viruses by using hemagglutinin (HA) specific RRT-PCR tests and for virus isolation in embryonated chicken eggs as described elsewhere (151, 157). The HA subtype of virus isolates was characterized using a hemagglutination inhibition assay and the neuraminidase (NA) subtype was determined by RT-PCR as described (151). Blood collected from the brachial vein of birds was centrifuged at 3000 g for 10 minutes in 0.8 ml gel separation tubes (MiniCollectH tubes, Roche). Serum was tested in a multispecies blocking ELISA specific for the nucleoprotein (NP) of influenza A viruses (IDEXX FlockChek* AI MultiS-Screen) according to the manufacturers instructions. To test the statistical significance of the results the Chi-square test, or the Fisher's exact test if appropriate, was performed using the software from the R project for statistical computing (168).

RESULTS

Avian Influenza Virus and Antibody Detection in Wild Birds in Cities

Cloacal and oropharyngeal samples were collected from 6059 wild birds of 7 species in highly urbanized areas. During the same years, samples were collected from 18660 birds

of the same 7 species in rural areas (Table 1). Birds were sampled year round in both highly and low urbanized areas, but in highly urbanized areas the largest proportion (65%) of samples was obtained in January, November and December, while in low urbanized areas the largest proportion (49%) of samples was obtained in June, September and October. The number of sampled hatch year (HY) and after hatch year (AHY) birds were distributed equally in high and low urbanized areas, with the exception of HY black-headed gulls that were intensively sampled in June and July at their breeding colonies in rural areas. In highly urbanized areas, influenza A viruses were most frequently detected by RRT-PCR in mallards (*Anas platyrhynchos*). Less frequently, viruses were detected in black-headed gulls (*Chroicocephalus ridibundus*), common gulls (*Larus canus*), herring gulls (*Larus argentatus*), and lesser black-backed gulls (*Larus fuscus*), and no viruses were detected in Egyptian geese (*Alopochen aegyptiaca*) and common coots (*Fulica atra*) (Table 1) in highly urbanized areas. No viruses of the H5 subtype were detected, and one LPAI virus of the H7 subtype was isolated. Viruses were isolated from 5/30 RRT-PCR positive samples, including viral subtypes H6N8, H7N1, H11N1 and H11N9. In rural areas, influenza A viruses were most frequently detected in mallards and black-headed gulls. Less often, viruses were detected in common gulls, herring gulls, and Egyptian geese, and no viruses were detected in lesser black-backed gulls and common coots in low urbanized areas. Major differences in virus prevalence between birds in highly and low urbanized areas were found in mallards, black-headed gulls and herring gulls only ($P < 0.05$).

Overall, influenza A virus antibodies were detected in 183/348 (52.6%) of birds sampled in highly urbanized areas, and in 68/132 (51.5%) of birds in rural areas (Table 1). In highly and low urbanized areas, antibodies were detected in 8/50 (16.0%) and 5/15 (33.3%) of HY birds respectively, while antibodies were detected in 175/298 (58.7%) and 63/117 (53.8%) of AHY birds ($P > 0.05$). Thus the seroprevalence in highly and low urbanized areas was similar. In contrast to the seroprevalence data, virus detection rates decreased with increasing levels of urbanization (Figure 1). Nevertheless, avian influenza viruses were even detected in the centers of densely populated cities, in 29/3264 (0.9%) of birds tested.

Table 1. Avian influenza prevalence and seroprevalence in wild bird species sampled in highly and low urbanized areas in the Netherlands between 2006 and 2009

	Highly urbanized areas[a]				Low urbanized areas[b]			
Species	Virology		Serology		Virology		Serology	
	Sampled	Virus positive (%)	Sampled	Seropositive (%)	Sampled	Virus positive (%)	Sampled	Seropositive (%)
Mallard	515	10 (1.9)	101	66 (65.3)	14080	1181 (8.4)	34	21 (61.8)
Egyptian goose	122	0	7	3 (42.9)	298	4 (1.3)	0	0
Black-headed gull	3789	16 (0.4)	98	34 (34.7)	3653	270 (7.4)	78	38 (48.7)
Common gull	609	2 (0.3)	81	68 (84.0)	65	0	6	6 (100)
Lesser black-backed gull	479	1 (0.2)	1	0	72	0	1	0
Herring gull	314	1 (0.3)	17	9 (52.9)	325	8 (2.5)	3	2 (66.7)
Common coot	231	0	43	3 (7.0)	167	0	10	1 (10.0)
Total	6059	30 (0.5)	348	183 (52.6)	18660	1463 (7.8)	132	68 (51.5)

a >1500 addresses/km^2; b <1500 addresses/km^2

Role of Migrating Urban Birds in Introduction of Avian Influenza Viruses in Cities

A total of 430 birds of 6 different species sampled in cities were subsequently sighted on various locations. Of the 430 sighted birds 300 birds (69.8%) were only reported back at the same location as where they were ringed initially and 94 birds (21.9%) were sighted at different water bodies in the same city. However, 5/206 mallards, 6/45 Egyptian geese, 2/123 common coots, 10/37 herring gulls, 11/11 common gulls and 2/8 lesser-black backed gulls (36/430 birds (8.4%)) migrated between cities and remote areas. The most extreme cases were common gulls and mallards ringed in cities in the Netherlands that were reported back up to 1125 km away in Lithuania and 2659 km away in Russia, respectively. These data indicate that the populations of long distance migrants and birds in urbanized areas are connected and that migrating populations may introduce avian influenza viruses into densely populated urban areas. In agreement with this suggestion we found that influenza viruses were even detected in 21/1847 (1.1%) birds living in closed water bodies in cities thus excluding the possibility of introduction of influenza virus by water flow.

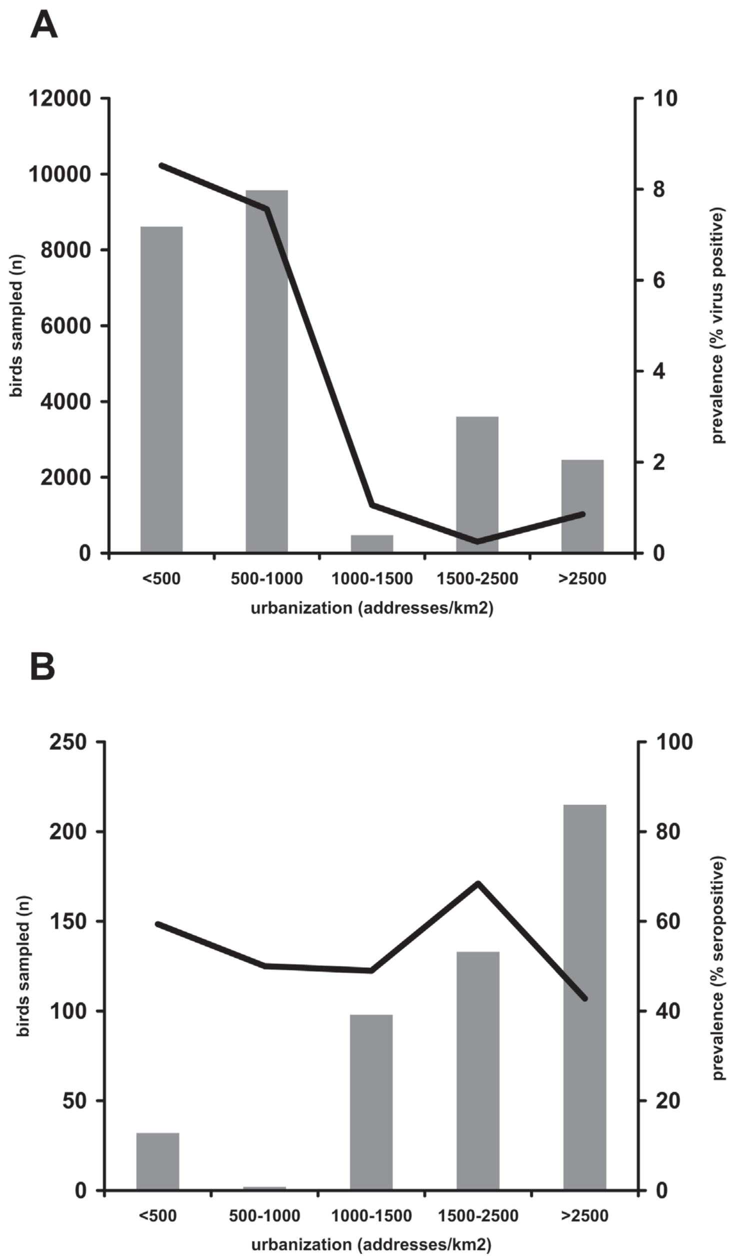


Figure 1. Prevalence of avian influenza virus and antibodies in wild birds based on level of urbanization. Avian influenza virus prevalence (A) and seroprevalence (B) in 7 wild bird species sampled in the Netherlands between 2006 and 2009 in relation to the level of urbanization. Grey bars indicate number of birds sampled (left Y-axes) and triangles indicate prevalence (right Y-axes).

DISCUSSION

In highly urbanized areas in the Netherlands, AIVs were found to circulate in ducks and gulls. Although the overall AIV prevalence in highly urbanized areas was significantly lower as compared to rural areas, it was certainly not negligible. In addition, most Mallards in rural areas were sampled in September and October during virus peak prevalence in this species, while most mallards in highly urbanized areas were sampled in November when virus prevalence was decreasing. If more Mallards in cities were sampled more intensively during virus peak prevalence, possibly more viruses would have been detected in urban mallards. We show that the AIV prevalence was inversely correlated with the level of urbanization, while AIV seroprevalence was approximately constant for the different levels of urbanization. The latter may suggest that birds in rural and urban areas have similar likelihood of experiencing influenza virus infection at least once, but that birds in rural areas may be exposed more frequently. Although some birds breed in highly urbanized areas, large flocks of immunologically naïve birds most likely primarily aggregate in rural areas whereby facilitating transmission as compared with urban populations that consist more often of single individuals or small groups of a single family.

For AHY barnacle geese and greater white-fronted geese it was shown that seroprevalence increases with age (unpublished data). Although the level of antibodies in AHY birds sampled in highly and low urbanized areas was similar, it is possible that the group of urban AHY birds consisted of older birds compared with birds sampled in low urbanized areas. Older birds had a longer window of exposure to viruses that may result in a detectable antibody response. It is further possible that birds in urban environments live longer than birds in rural areas because of e.g. high food availability. The availability of food in highly urbanized areas possibly also makes the bird less susceptible to infections, and might leave more energy to produce a strong long lasting antibody response.

Since AIV were detected in birds residing in both closed and open water bodies, we suggest that wild birds rather than water flow acted as vector for introduction of AIV into cities. Indeed, analysis of the movements of the sampled birds indicated that city populations were not separated completely from populations of long-distance migrants, and that populations moved between different water bodies within cities. Together, our data indicate that viral epizootics in wild migrating birds may directly impact bird populations in urbanized areas, and that urban bird populations should not be excluded as a source of influenza virus infection for humans and animals.

ACKNOWLEDGEMENTS

We thank Judith Guldemeester for technical assistance. This work was sponsored by grants from the Dutch Ministry of Agriculture, the EU DG Sanco monitoring program, EU Framework six program NewFluBird (044490), and contract NIAID NIH HHSN266200700010C. VJM is currently supported by the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Josanne H. Verhagen, Pascal Lexmond, Oanh Vuong, Martin Schutten
Judith Guldemeester, Albert D.M.E. Osterhaus, Armin R.W. Elbers
Roy Slaterus, Menno Hornman, Guus Koch
Ron A.M. Fouchier

Discordant detection of avian influenza virus subtypes in time and space between poultry and wild birds; Towards improvement of surveillance programs

Submitted

Avian influenza viruses from wild birds can cause outbreaks in poultry, and occasionally infect humans upon exposure to infected poultry. Identification and characterization of viral reservoirs and transmission routes is important to prevent infection of poultry, and subsequently virus transmission between poultry holdings and to humans. Based on spatial, temporal and phylogenetic analyses of data generated as part of intense and large-scale influenza surveillance programs in wild birds and poultry in the Netherlands from 2006 to 2011, we demonstrate that LPAIV subtype distribution differed between wild birds and poultry, suggestive of host-range restrictions. LPAIV isolated from Dutch poultry were genetically most closely related to LPAIV isolated from wild birds in the Netherlands or occasionally elsewhere in Western Europe. However, a relatively long time interval was observed between the isolations of related viruses from wild birds and poultry. Spatial analyses provided evidence for mallards (*Anas platyrhynchos*) being more abundant near primary infected poultry farms. Detailed year-round investigation of virus prevalence and wild bird species

distribution and behavior near poultry farms should be used to improve risk assessment in relation to avian influenza virus introduction and retarget avian influenza surveillance programs.

INTRODUCTION

Avian influenza A virus (AIV) outbreaks may have a high impact on animal health and welfare. Moreover, influenza virus subtypes A(H5N1), A(H7N2), A(H7N3), A(H7N7), A(H7N9), A(H9N2), A(H10N7) and A(H10N8) can form a human health risk as they have been isolated from humans upon exposure to poultry (157, 245-252). Infection with these subtypes was associated with mild to severe disease in humans. To prevent infection of poultry, virus spread in poultry holdings and transmission to humans, viral reservoirs and transmission routes into poultry holdings need to be identified and characterized.

Wild birds are the reservoir of AIV subtypes H1-H16 (hemagglutinin, HA) and N1-N9 (neuraminidase, NA) (12, 21). More recently, influenza A virus subtypes H17N10 and H18N11 have been identified in fruit bats (13, 14). It has been suggested that wild birds, especially waterfowl, are the source of avian influenza outbreaks in poultry (21, 253, 254) as a close genetic relationship of AIV in wild and domestic birds has been documented for several outbreaks (78, 157, 253, 255, 256).

Most of the studies that link AIV in poultry and wild birds are based on single highly pathogenic avian influenza (HPAI) H5 or H7 virus outbreaks and limited attention has been paid to the species or temporal and spatial aspects of detection of closely related wild bird viruses. Similarly, most studies that define physical and anthropogenic environmental risk factors associated with poultry have been based on H5 HPAIV outbreaks with no or limited attention paid to wild bird distribution (257, 258). Wild birds are frequently infected with low pathogenic avian influenza viruses (LPAIV), and LPAIV infections in poultry may often go unnoticed and probably occur more frequently than previously assumed (34, 259, 260). In addition, LPAIV of diverse origin may be ancestral to HPAIV causing outbreaks in poultry (78). Wild bird species of importance to poultry with respect to AIV infection can be identified based on genetic analyses of their LPAIV, and information on temporal and spatial variation of LPAIV in wild birds can be useful for disease management purposes and for development of targeted surveillance programs.

From 2005 onwards, many countries have implemented or intensified AIV surveillance programs in wild birds and poultry after inter-regional spread of H5N1 HPAIV. These programs aimed at the real-time detection of H5 and H7 viruses as an early warning system for outbreaks in poultry and to provide definitive proof for the role of wild birds in spreading the disease (133, 261). The AIV surveillance programs

in the Netherlands are among the most intensive surveillance programs in the world, encompassing a relatively small surface area with high numbers of water birds and poultry farms.

Here we describe the host species, temporal and spatial aspects of LPAIV detected in poultry and wild birds in the Netherlands between 2006 and 2011. Genetic analyses were performed on LPAIV isolated from poultry and wild birds. In addition, we made an attempt to define wild bird related environmental risk factors of LPAIV introductions into poultry holdings.

MATERIAL AND METHODS

Study population poultry

Chicken is the dominant species on poultry farms in the Netherlands (in 2010, 1806/2161, 84%), followed by duck (59/2161, 3%) and turkey (53/2161, 2%). The majority of chicken farms are commercial egg layer farms (1126/2161, 52%) that predominantly keep layers indoors (840/1126, 75%) and to a lesser extend outdoors (286/1126, 25%) (262). Farms with less than 250 birds were excluded from the analyses. Poultry farms were located throughout the Netherlands with highest poultry farm densities (predominantly chicken layer farms) located in the center and southeastern part of the Netherlands (Figure S1).

Study population wild birds

The Netherlands forms an important region for breeding, staging and wintering of wild birds. Over 500 species have been sighted, of which 213 breed in the Netherlands with mallard (*Anas platyrhynchos*) as most common breeding aquatic bird species (263). Annually at least 130 aquatic bird species winter regularly in the Netherlands (264). Mallards are distributed more diffuse year round, while Eurasian wigeon (*Anas penelope*) and greater white-fronted geese (*Anser albifrons*) winter in dense groups more locally. Birds were captured manually or using duck decoys, duck traps, clap nets, cannon nets, mist nets or wader funnel traps. The capturing of wild birds was approved by the Dutch Ministry of Economic Affairs based on the Flora and Fauna Act (permit number FF/75A/2009/067). The handling and sampling of wild birds was approved by the Animal Experiment Committee of the Erasmus MC (permit number 122-07-09, 122-08-12, 122-09-20, 122-10-20 and 122-11-31). Sites of wild bird sampling were mainly located in water-rich areas or along main rivers (Figure S1).

Influenza A virus surveillance programs poultry

In the Netherlands a serological surveillance program has been initiated in 2003, based on Council directive 2005/94/EC (29), but which is much more intensive compared to the basic program in other European countries: all farms are sampled once a year, but layer farms with outdoor facilities are sampled 4 times per year, and on turkey farms, every production cycle is sampled (25). This program focuses on the detection of subclinical infection of H5 and H7 LPAIV in poultry, while serving the detection of LPAIV of other subtypes. Clinical surveillance targets the early detection of diseases like avian influenza, supported by the Early Warning System (EWS) based on recommended clinical thresholds (265). Samples for virus detection were collected if farms tested positive for H5- or H7-specific antibodies within the serological surveillance program, or if AIV infection was suspected based on clinical signs. These samples consisted of oropharyngeal and cloacal swab specimens and/or trachea or lung tissues in case of increased mortality. In this study, farms were considered AIV positive if AIV-specific antibodies were detected in more than one bird per farm and/or if the HA subtype was characterized based on antibodies detected or viruses isolated within the study period (2006 – 2011).

Categorization of poultry farms into primary or secondary AIV infected farms

AIV-positive farms of known HA subtype were categorized into most likely infected by wild birds directly (i.e. primary infected farm) or most likely infected as the result of virus spread between farms (i.e. secondary infected farm). Categorization of primary and secondary farms builds on the study of Gonzales and colleagues (260). In addition, for the purpose of this study a more conservative approach was used based on HA subtype, date of virus or antibody detection and genetic analyses. Genetic analyses suggested—irrespective of farm location—that if the time interval between detections of identical AIV subtypes was more than one year, a new AIV introduction was more likely (this study). Thus, a farm was categorized as primary infected farm (n = 18), if the time interval between current and previous poultry AIV detection of the same subtype was at least one year. A farm was categorized as secondary infected farm (n = 47), if the time interval between current and previous AIV detection of the same subtype was less than one year. If a farm was infected multiple times with different HA subtypes and was listed at least once as primary case, this farm was categorized as primary infected farm. Poultry farms categorized as AIV negative farms consisted of farms that tested AIV negative before and during the course of the study period (2006 – 2011).

Antibody detection

Routinely, poultry serum samples collected for AIV-specific antibody detection were analyzed at the Dutch Animal Health Service. Before January 1st 2009, chicken and turkey sera were tested using an indirect AIV-specific ELISA (FlockChek AIV Antibody Test Kit, IDEXX, Hoofddorp, the Netherlands) and duck sera were tested using an in-house developed NP blocking ELISA (266) or directly with the hemagglutination inhibition (HI) assay using H5 and H7 antigens (254). After January 1st 2009, chicken, turkey and duck sera were tested using a nucleoprotein (NP)-specific multispecies blocking ELISA (bELISA, FlockChek AI MultiS-Screen Antibody Test Kit, IDEXX). If AIV-specific antibodies were detected, AIV subtype was determined using an HI assay and neuraminidase inhibition (NI) assay at the Central Veterinary Institute (254, 267). AIV subtype could not be determined for some of the AIV NP positive sera due to bad quality and/or insufficient amount of sera.

Influenza A virus surveillance programs wild birds

In the Netherlands a surveillance program has been initiated in 1998 in which live wild birds were sampled for virus detection. The aim of this program was to detect H5 and H7 HPAIV and LPAIV in wild birds, and to study the epidemiology and evolution of LPAIV of all subtypes. To detect viruses, swab samples were collected from cloaca and from 2006 onwards from both cloaca and oropharynx. Samples were stored in virus transport medium (151) at 4°C for less than a week or at -80°C or -20°C if more than a week until analysis in the laboratory. Birds were considered AIV positive if cloaca and/or oropharynx tested virus positive.

In addition to the sampling of live birds, wild birds found dead were sampled for virus detection since 2006. Data on LPAIV prevalence in dead wild birds was not included in this study.

Virus detection

Wild bird samples collected for virus detection were analyzed at the Erasmus MC as described previously (151). In short, RNA was isolated, and analyzed using a reverse transcriptase–polymerase chain reaction assay targeting the matrix gene (M-RT-PCR) on an ABI 7500 machine. Next, M-RT-PCR positive samples (i.e. cycle threshold value <40) were analyzed using a RT-PCR targeting the H5 and H7 gene (151, 157). Poultry samples collected for virus detection were analyzed at the Central Veterinary Institute in accordance with the Diagnostic Manual of the Council Directive 2005/94/EC (267).

Virus isolation and characterization

Wild bird M-RT-PCR positive samples were used for virus isolation and characterization as described previously (151). Briefly, M-RT-PCR positive samples were inoculated in the allantoic cavity of 11-day old embryonated chicken eggs. The allantoic fluid was harvested after two days and AIV was detected using hemagglutination assays with turkey erythrocytes. The HA subtype of the virus isolates was characterized using an HI assay with turkey erythrocytes and hyper-immune rabbit- and ferret antisera raised against 16 HA subtypes (H1-H16). The NA subtype of virus isolates was characterized by PCR and sequencing (159) and identified with the basic local alignment search tool (BLAST) available from GenBank (158). Poultry viruses were isolated and characterized at the Central Veterinary Institute in accordance with the Diagnostic Manual of the Council Directive 2005/94/EC (267).

Sequence analyses and genetic analyses

Nucleotide sequences of the HA and NA segments of poultry and wild bird LPAIV were obtained. Upon RNA isolation, cDNA was synthesized using the oligonucleotide (5’-AGCAAAAGCAGG-3’). PCR was performed using the AmpliTaq Gold mix (Applied Biosystems, Bleiswijk, the Netherlands). PCR products separated by gel electrophoresis were purified with the QIAquick gel extraction kit (Qiagen, Leusden, the Netherlands). Sequencing was performed on an ABI Prism 3100 using the Big Dye Terminator sequencing kit version 3.1 (Applied Biosystems). Primers specific for the noncoding regions of HA and NA segments were used as described previously (i.e. HA forward primer (5’-AGCAAAAGCAGGGG-3’) and HA reverse primer (5’-AGTAGAAACAAGGGTGGTTT-3’); NA forward primer (5’-GTTGAAGATGAATCCAAATC-3’) and NA reverse primer (5’-AGTAGAAACAAGGAGTTTTTT-3’)) (159) and additional HA-specific primers that are available on request.

Poultry nucleotide sequences were supplemented with sequences that displayed high sequence identity, selected using BLAST available from GenBank (158) and GISAID EpiFlu (160). For each poultry HA or NA sequence, a maximum of 100 sequences with the highest percentage sequence identity were selected. For each HA and NA subtype, BLAST results were merged and duplicates removed. Identical sequences (100% nucleotide identity) were removed if isolated from the same host species, country and year. Full-length and partial sequences were included and the alignments were adjusted manually to include the highest number of sequences in the analysis. Sequences were aligned using MAFFT version 7 (161). The best-fit model of nucleotide substitution was

determined with jModelTest (162). Phylogenetic maximum likelihood (ML) trees were generated with the PhyML package version 3.1 (268) using the General Time Reversible model of nucleotide substitution with accounting for estimates of invariable sites and the gamma distribution parameter (GTR+I+G) and subtree pruning and regrafting (SPR) searches. The reliability of the phylogenetic groupings of each tree was assessed with a nonparametric bootstrap re-sampling analysis using PhyML. Trees were visualized using the Figtree version 1.4.0 (163). Nucleotide sequences generated within this study are online available under the numbers as listed in Table S2.

Landscape analyses of poultry farms in relation to wild birds

Primary infected, secondary infected and AIV-negative poultry farms were compared with respect to numbers of wild birds sighted near farms and wild bird related landscape characteristics. Number of birds counted was based on systematic annual mid-winter counts in bird count units near farms from 2006 to 2010 and was part of a long-term national bird breeding and wintering monitoring program carried out by Sovon since 1975 (263). The selected bird species reside in the Netherlands year round (i.e. mallard) or stage during fall/winter only (i.e. Eurasian wigeons and greater white-fronted geese), and have been shown to host AIV (21). For each farm included in the analysis, the number of birds per species was based on bird counts in one or more counting unit(s) located within 1000-meter radius around the farm. The number of birds per species per farm was extrapolated to the total surface of the circle with radius 1000 meter around the farm from (the bird density per hectare of counting unit)*(surface counting unit within 1000-meter radius around farm). Poultry farms were included in the analysis if at least 10% of the circle with radius 1000 meter was located within bird counting units (i.e. 703 of 2,064 farms, 34%).

Landscape characteristics presumably associated with wild bird distribution (i.e. water, forest and farmland) were investigated. The total surface of water (with at least 6 meter in length or width as determined by the topographic basemap), forest and farmland within 100 and within 1000 meter around each farm (n = 2,064) was derived from a Dutch topographic basemap (TOP10NL) (269) in the program ArcGIS version 10.2.2.

Statistics

Differences in LPAIV subtype distribution between poultry and wild birds were investigated using the Fisher's exact test (for all subtypes of wild birds and poultry) or Chi-square test (for H5- and H7 PCR positives wild birds) using GraphPad Prism 5.

Differences in presence or absence of the different wild bird species near poultry farms were compared using the Fisher's exact test. Wild bird counts and surface of water, forest and farmland near primary infected farms were compared with wild bird counts and surface of water, forest and farmland near secondary infected and AIV-negative poultry farms using the Mann-Whitney test.

RESULTS

Avian influenza virus surveillance in wild birds

From 2006 to 2011, 68,637 live birds belonging to 139 species, 40 families and 18 orders were sampled for AIV detection in the Netherlands. Most birds sampled belong to the order *Anseriformes* (mainly ducks, geese and swans; 50,993 birds; 74%) and *Charadriiformes* (mainly gulls and waders; 16,017 birds; 23%). Sampling intensity varied in time and space with the annual cycle of the wild bird species, with general high sampling intensity in water rich areas and during fall migration and winter staging, and low sampling intensity in areas with less surface water and during spring migration and the breeding season (Table 1).

Influenza A virus prevalence varied in time and space among species. In birds of the order *Anseriformes*, most viruses were detected by M-RT-PCR in mallards (2,466 of 24,192 birds; 10%) and other ducks (478 of 8,258; 6%), and fewer viruses were detected in geese (648 of 14,749 birds; 4%) and swans (31 of 3,794 birds; 1%). In birds of the order *Charadriiformes*, most viruses were detected in gulls (423 of 14,190 birds; 3%), and fewer viruses were detected in waders (23 of 1,827 birds; 1%). In ducks, highest LPAIV prevalence was detected at aggregation sites in fall (August to December, with a maximum of 14% M-RT-PCR positives in October). In geese, highest LPAIV prevalence was detected at staging areas in winter (December to February, with a maximum of 7% M-RT-PCR positives in December). Lowest LPAIV prevalence was detected in spring, when viruses were detected almost exclusively in ducks (April and May, with a minimum of 1% birds M-RT-PCR positive in April). In gull colonies, highest LPAIV prevalence was detected at their breeding sites in summer (June and July, with 11% birds M-RT-PCR positive in July) (Table 1). Of the total of 4,070 M-RT-PCR positive birds, 542 virus isolates were recovered and characterized, yielding an overall recovery rate of 13%. Within the order *Anseriformes*, most viruses were isolated from mallards (n = 250 of 542; 46%), and fewer viruses were isolated from geese (n = 40; 7%), other ducks (n = 20; 4%) and swans (n = 16; 3%). Within the order *Charadriiformes*, most viruses were isolated from gulls (n = 201; 37%), and fewer viruses were isolated from waders (n = 15; 3%).

Avian influenza virus surveillance in poultry

From 2006 to 2011, all poultry farms in the Netherlands were sampled for AIV-specific antibody detection. Farm sampling frequency varied among poultry types as described previously, with turkeys and outdoor layers sampled more frequently than ducks, indoor layers and broilers (260). For the different poultry types, timing of sampling was more or less consistent during the year (Table 1, timing of sampling shown for period 2007-2009).

Influenza A virus seroprevalence varied between poultry types (260) and in time. Highest seroprevalence was detected on turkey and duck farms, followed by mixed, outdoor layer farms and indoor layer farms (Table 1). No AIV-specific antibodies were detected on broiler farms. Most AIV-seropositive farms were detected from May until August with 20 of 35 seropositive cases (57%) from 2007 to 2009 (Table 1).

From 2006 to 2011, in total 82 poultry farms (with unique address) tested positive for AIV and/or antibodies. Of the 82 AIV sero- and/or virus positive poultry farms, 16 virus isolates were obtained. Most virus isolates were obtained from chickens (11 of 16), fewer from turkeys (5 of 16) and none from ducks (Table 2). The HA subtype of the viruses that circulated on 65 of 82 AIV positive poultry farms was identified. A single HA subtype was detected on 63 poultry farms, two different HA subtypes were detected on two poultry farms and four different HA subtypes were detected on one single poultry farm, resulting in 70 HA subtypes on 65 poultry farms. The NA subtype of the viruses that circulated on 29 of 82 AIV positive poultry farms was identified. A single NA subtype was detected on 27 poultry farms, two different NA subtypes were detected on one poultry farm and three different NA subtypes were detected on one poultry farm, resulting in 32 NA subtypes on 29 poultry farms.

Prevalence of influenza A virus HA subtypes in poultry and wild birds

In poultry, the most frequently detected HA subtypes were H7 (21%) and H8 (21%), followed by H1 (16%), H5 (13%), H6 (14%), H9 (7%), H10 (4%) and H2 (3%) (Figure 1A, Table 3, Table S1). Viruses of the H1 subtype were primarily detected in turkeys (8 of 11), even though only 2% of Dutch poultry farms house turkeys. Due to follow-up investigation of all AIV-(sero)positive poultry farms for H5- and H7 AIV or antibodies, HA subtypes other than H5 or H7 may be under represented among the HA subtypes detected in poultry. In wild birds, H13 (20%), H3 (16%), H16 (15%) and H4 (11%) were the most abundantly isolated HA subtypes, followed by H6 (10%), H1 (6%), H10 (6%), H5 (5%), H7 (5%), H2 (2%), H11 (2%), H8 (1%), H9 (1%) and H12 (1%). Viruses of the H3 and H4 subtype were primarily isolated from dabbling ducks, while H13 and H16 subtypes

Table 1. Avian influenza virus prevalence in wild birds and poultry in the Netherlands. (A) Total number of wild birds sampled for virus detection in time from 2006 to 2011. (B) Total number of poultry farms sampled for antibody detection in time from 2007 to 2009.

A

Month	Anseriformes							
	Duck species				Goose species		Swan species	
	Mallard		Other duck species					
	Sampled	Virus (%)	Sampled	Virus (%)	Sampled	Virus (%)	Sampled	Virus (%)
January	3210	96 (3)	1101	10 (1)	5088	227 (4)	661	12 (2)
February	1363	58 (4)	862	4 (0)	1549	88 (6)	473	1 (0)
March	952	49 (5)	1291	5 (0)	451	6 (1)	66	3 (5)
April	614	11 (2)	152	0 (0)	143	1 (1)	66	0 (0)
May	557	11 (2)	142	0 (0)	401	0 (0)	2	0 (0)
June	742	71 (10)	128	0 (0)	564	0 (0)	0	0 (0)
July	728	26 (4)	139	1 (1)	73	1 (1)	2	0 (0)
August	1161	184 (16)	164	6 (4)	18	0 (0)	1226	3 (0)
September	4203	530 (13)	994	72 (7)	29	1 (3)	264	0 (0)
October	4375	601 (14)	1325	201 (15)	778	0 (0)	104	0 (0)
November	3377	473 (14)	1058	108 (10)	1353	32 (2)	474	1 (0)
December	2910	356 (12)	902	71 (8)	4302	292 (7)	456	11 (2)
Total	24192	2466 (10)	8258	478 (6)	14749	648 (4)	3794	31 (1)

B

Month	Chicken					
	Layer-indoor		Layer-outdoor		Broiler	
	Sampled	Antibody (%)	Sampled	Antibody (%)	Sampled	Antibody (%)
January	217	0 (0)	181	1 (0.6)	277	0 (0)
February	201	1 (0.5)	160	0 (0)	225	0 (0)
March	261	0 (0)	226	0 (0)	180	0 (0)
April	230	1 (0.4)	170	0 (0)	195	0 (0)
May	233	1 (0.4)	211	2 (0.9)	299	0 (0)
June	232	3 (1.3)	288	2 (0.7)	177	0 (0)
July	181	0 (0)	182	0 (0)	292	0 (0)
August	160	0 (0)	171	3 (1.8)	164	0 (0)
September	194	0 (0)	209	0 (0)	155	0 (0)
October	157	0 (0)	196	0 (0)	165	0 (0)
November	203	0 (0)	209	0 (0)	187	0 (0)
December	225	1 (0.4)	279	1 (0.4)	159	0 (0)
Total	2494	7 (0.3)	2482	9 (0.4)	2475	0 (0)

Table 1A continued

Month	Charadriiformes				Non-Anseriformes or Charadriiformes		Total	
	Gull species		Wader species					
	Sampled	Virus (%)	Sampled	Virus (%)	Sampled	Virus (%)	Sampled	Virus (%)
January	2693	3 (0)	2	0 (0)	117	0 (0)	12872	348 (3)
February	1182	7 (1)	1	0 (0)	132	0 (0)	5562	158 (3)
March	576	7 (1)	62	0 (0)	129	0 (0)	3527	70 (2)
April	634	0 (0)	668	2 (0)	88	0 (0)	2365	14 (1)
May	368	0 (0)	297	0 (0)	61	0 (0)	1828	11 (1)
June	3075	106 (3)	192	0 (0)	60	0 (0)	4761	177 (4)
July	2442	270 (11)	57	0 (0)	146	0 (0)	3587	298 (8)
August	83	1 (1)	152	2 (1)	211	0 (0)	3015	196 (7)
September	10	0 (0)	160	1 (1)	194	0 (0)	5854	604 (10)
October	60	1 (2)	207	14 (7)	258	0 (0)	7107	817 (11)
November	882	19 (2)	29	4 (14)	130	1 (1)	7303	638 (9)
December	2185	9 (0)	0	0 (0)	101	0 (0)	10856	739 (7)
Total	14190	423 (3)	1827	23 (1)	1627	1 (0)	68637	4070 (6)

Table 1B continued

Month	Turkey		Duck		Mixed		Total	
	Sampled	Antibody (%)	Sampled	Antibody (%)	Sampled	Antibody (%)	Sampled	Antibody (%)
January	56	2 (3.6)	36	0 (0)	25	0 (0)	792	3 (0.4)
February	52	1 (1.9)	30	0 (0)	27	0 (0)	695	2 (0.3)
March	65	0 (0)	15	0 (0)	26	0 (0)	773	0 (0)
April	49	0 (0)	8	0 (0)	18	0 (0)	670	1 (0.1)
May	58	1 (1.7)	6	0 (0)	22	0 (0)	829	4 (0.5)
June	49	0 (0)	4	0 (0)	29	1 (3.4)	779	6 (0.8)
July	66	4 (6.1)	3	0 (0)	19	0 (0)	743	4 0.5)
August	51	2 (3.9)	6	1 (16.7)	15	0 (0)	567	6 (1.1)
September	53	2 (3.8)	5	0 (0)	17	0 (0)	633	2 (0.3)
October	48	0 (0)	5	1 (20.0)	19	0 (0)	590	1 (0.2)
November	49	0 (0)	6	0 (0)	23	2 (8.7)	677	2 (0.3)
December	49	0 (0)	34	1 (2.9)	37	1 (2.7)	783	4 (0.5)
Total	645	12 (1.9)	158	3 (1.9)	277	4 (1.4)	8531	35 (0.4)

Table 2. Avian influenza viruses isolated from poultry in the Netherlands between 2006 and 2011 with their genetically closest relatives based on genetic analyses of the hemagglutinin and neuraminidase gene segment. Seg., segment; HA, hemagglutinin; NA, neuraminidase; NL, the Netherlands; Ty, turkey; Ch, chicken; * = exact collection date not available. Seg., segment.

Poultry LPAI virus	Closest relative of poultry LPAI virus			time interval (days)	sequence identity (%)	length sequence (nt)
Name, Location, Date	Seg.	Name	Location, time			
A/Ty/ Netherlands/06001571/06 (H6N5)	HA	A/White-Fronted Goose/ Netherlands/1/2006 (H6N2)	Oud-Alblas (NL), 14-Jan-2006	2	0,996	1576
Dinteloord, 16-Jan-2006	NA	A/Mallard/Switzerland/ WV4060167/2006 (H3N5)	Switzerland, 15-Dec-2006	325	0,987	1310
A/Ch/ Netherlands/06022003/06 (H7N7)	HA	A/Mallard/ Netherlands/60/2008 (H7N1)	Wieringen (NL), 15-Oct-2008	806	0,993	1560
Voorthuizen, 01-Aug-2006	NA	A/Mallard/ Sweden/5944/2005 (H7N7)	Ottenby (Sweden), 23-Nov-2005	252	0,987	1238
A/Ty/ Netherlands/07016245/07 (H1N5)	HA	A/Bewick's swan/ Netherlands/1/2007 (H1N5)	Friesland (NL), 5-Jan-2007	168	0,988	1587
Weert, 22-Jun-2007	NA	A/Black-backedGull/ Netherlands/1/2006 (H4N5)	Schiermonnikoog (NL), 14-Feb-2006	493	0,985	1310
A/Ty/ Netherlands/09006938/09 (H10N7)	HA	A/Mallard/ Netherlands/53/2008 (H10N7)	Wieringen (NL), 2-Oct-2008	196	0,993	1571
Deurne, 16-Apr-2009	NA	A/Mallard/ Netherlands/82/2008 (H7N7)	Oudeland van Strijen (NL), 17-Dec-2008	120	0,997	1238
A/Ch/ Netherlands/10007882/10 (H7N4)	HA	A/Mallard/ Netherlands/60/2008 (H7N1)	Wieringen (NL), 15-Oct-2008	578	0,987	1560
Deurne, 16-May-2010	NA	A/Ch/ Netherlands/10009401/10 (H8N4)	Hiaure (NL), 4-Jun-2010	19	0,989	1345
A/Ch/ Netherlands/10008427/10 (H10N7)	HA	A/Mallard/ Netherlands/67/2008 (H10N7)	Oud-Alblas (NL), 13-Dec-2008	523	0,992	1571
Drachtstercompagnie, 20-May-2010	NA	A/Mallard/ Netherlands/74/2008 (H10N7)	Oud-Alblas (NL), 13-Dec-2008	523	0,991	1238
A/Ch/ Netherlands/10010413/10 (H6N1)	HA	A/Mallard/ Netherlands/18/2010 (H6N8)	Oud-Alblas (NL), 3-Sep-2010	105	0,99	1576
Idsegahuizum, 21-May-2010	NA	A/Mallard/ Bavaria/185-26/2008 (H1N1)	Bavaria (Germany), 22-Sep-2008	606	0,987	1306
A/Ch/ Netherlands/10009401/10 (H8N4)	HA	A/Ch/ Netherlands/11004004/11 (H8N4)	Vreeland (NL), 9-Mar-2011	278	0,984	1644
Hiaure, 4-Jun-2010	NA	A/Ch/ Netherlands/10007882/10 (H7N4)	Deurne (NL), 16-May-2010	19	0,989	1345

Poultry LPAI virus	Closest relative of poultry LPAI virus			time interval (days)	sequence identity (%)	length sequence (nt)
Name, Location, Date	Seg.	Name	Location, time			
A/Ch/ Netherlands/10020245/10 (H9N2)	HA	A/Duck/Italy/260/2004 (H9N8)	Italy, 1-Jan-2004*	2532	0,969	1588
Pijnacker, 7-Dec-2010	NA	A/Mallard/ Netherlands/7/2007 (H4N2)	Krimpen aan den IJssel (NL), 27-Sep-2007	1167	0,977	1284
A/Ch/ Netherlands/11004004/11 (H8N4)	HA	A/Mallard/ Sweden/99377/2009 (H8N4)	Ottenby (Sweden), 3-Sep-2009	553	0,989	1644
Vreeland, 10-Mar-2011	NA	A/Mallard/ Sweden/100546/2009	Ottenby (Sweden), 22-Oct-2009	503	0,991	1345
A/Ch/ Netherlands/11004875/11 (H7N1)	HA	A/Mallard/Poland/446/09 (H7N7)	Pomeranian Voivodeship (Poland), 27-Dec-2009	452	0,996	1560
Schore, 24-Mar-2011	NA	A/Mallard/ Netherlands/51/2010 (H1N1)	Oud-Alblas (NL), 3-Dec-2010	111	0,995	1306
A/Ch/ Netherlands/11008327/11 (H7N7)	HA	A/Ty/ Netherlands/11011530/2011 (H7N7)	Creil (NL), 26-Jun-2011	45	0,998	1560
Kootwijkerbroek, 12-May-2011	NA	A/Ty/Germany/R1775/2011 (H7N7)	Germany, 1-Jan-2011*	131	0,995	1238
		A/Ch/Germany/R1801/2011 (H7N7)	Germany, 1-Jan-2011*	131	0,995	1238
A/Ch/ Netherlands/11009919/11 (H1N1)	HA	A/White-fronted Goose/ Netherlands/4/2011 (H1N1)	Lith (NL), 17-Jan-2011	133	0,987	1587
Stolwijk, 30-May-2011	NA	A/White-fronted Goose/ Netherlands/4/2011 (H1N1)	Lith (NL), 17-Jan-2011	133	0,999	1306
A/Ch/ Netherlands/11011326/11 (H7N7)	HA	A/Ty/11011530/ Netherlands/2011 (H7N7)	Creil (NL), 26-Jun-2011	4	0,999	1560
Creil, 22-Jun-2011	NA	A/Ty/11011530/ Netherlands/2011 (H7N7)	Creil (NL), 26-Jun-2011	4	0,998	1238
A/Ty/ Netherlands/11011530/11 (H7N7)	HA	A/Ch/ Netherlands/11011326/2011 (H7N7)	Creil (NL), 22-Jun-2011	4	0,999	1560
Creil, 26-Jun-2011	NA	A/Ch/ Netherlands/11011326/2011 (H7N7)	Creil (NL), 22-Jun-2011	4	0,998	1238
A/Ty/ Netherlands/11015452/11 (H9N2)	HA	A/Teal/Finland/10529/2010 (H9N2)	Söörmarkku (Finland), 5-Oct-2010	330	0,985	1588
Deurne, 31-Aug-2011	NA	A/Mallard/ Sweden/99820/2009 (H11N2)	Ottenby (Sweden), 27-Sep-2009	703	0,991	1284

Table 3. Avian influenza virus HA and NA subtype combinations detected in wild birds and poultry, the Netherlands, 2006 to 2011. For wild birds, subtypes were based on virus isolates. For poultry, subtypes were based on antibody detection, virus detection and/or virus isolation. Numbers refer to wild birds and numbers between brackets refer to poultry farms. Subtype combinations indicated with an asterisk were significant more frequently detected in poultry than in wild birds, with * = P <0.05 and ** = P <0.01 (Fisher's exact test).

Subtype	N1	N2	N3	N4	N5	N6	N7	N8	N9	Nx	Total
H1	26 (1)	1	1	1	1(6)**					(4)	30 (11)
H2		3	8(1)							(1)	11 (2)
H3	1	16	1			11		59	1		89 (0)
H4		5			7	41		5			58 (0)
H5		16	8			1				(9)	25 (9)
H6	11(3)	11			3(2)			28(1)		(4)	53 (10)
H7	13(1)	1	4(1)	1(2)*			6(5)**	1		(6)	26 (15)
H8	1			4(4)**						(11)	5 (15)
H9		5(2)								(3)	5(5)
H10	3			6		4	21(3)				34 (3)
H11	2							1	6		9 (0)
H12					3						3 (0)
H13		21	3			5		82			111 (0)
H16			80					3			83 (0)
Total	57 (5)	79 (2)	105 (2)	12 (6)	14 (8)	62 (0)	27 (8)	179 (1)	7 (0)	(38)	542 (70)

were isolated from gulls. HA subtype diversity based on viruses detected in poultry was highest in May and June (Figure 1E), and in wild birds in September to January (Figure 1C). Of the HA subtypes detected in poultry, H5 and H6 were significantly more frequently isolated from geese, while H10 was significantly more frequently isolated from waders. All HA subtypes isolated from geese were detected in poultry (Figure 1, Table S1).

No H5 or H7 HPAIV were detected in poultry or wild birds within the study period. In addition to virus isolations, in wild birds H5 and H7 viruses were detected using HA-specific RT-PCR assays. Of 4,070 M-RT-PCR positive birds, 96 birds tested positive for H5 viruses and 36 birds tested positive for H7 viruses. H5 viruses were detected from August until March with most H5 virus detections in October (26 of 96, detected in October in 6 of 6 years). H7 viruses were detected from July until April with most H7 virus detections in December (12 of 36, detected in December in 3 of 6 years). Of M-RT-PCR positive birds, H5 viruses were detected in swans significantly more frequently (3 of 31; 10%) than in all wild birds combined (96 of 4,070; 2%) (P <0.05, Chi-square test), whereas in gulls H5 viruses were detected significantly less frequently (1 of 423; 0.2%) by H5-specific RT-PCR (P < 0.01, Chi-square test). Furthermore, H5 viruses were detected in mallards (62 of 2,466; 3% of M-RT-PCR positive birds), other ducks (9 of 478; 2%) and geese (21 of 648; 3%). No H5 viruses were detected in M-RT-PCR positive waders (0 of 23; 0%).

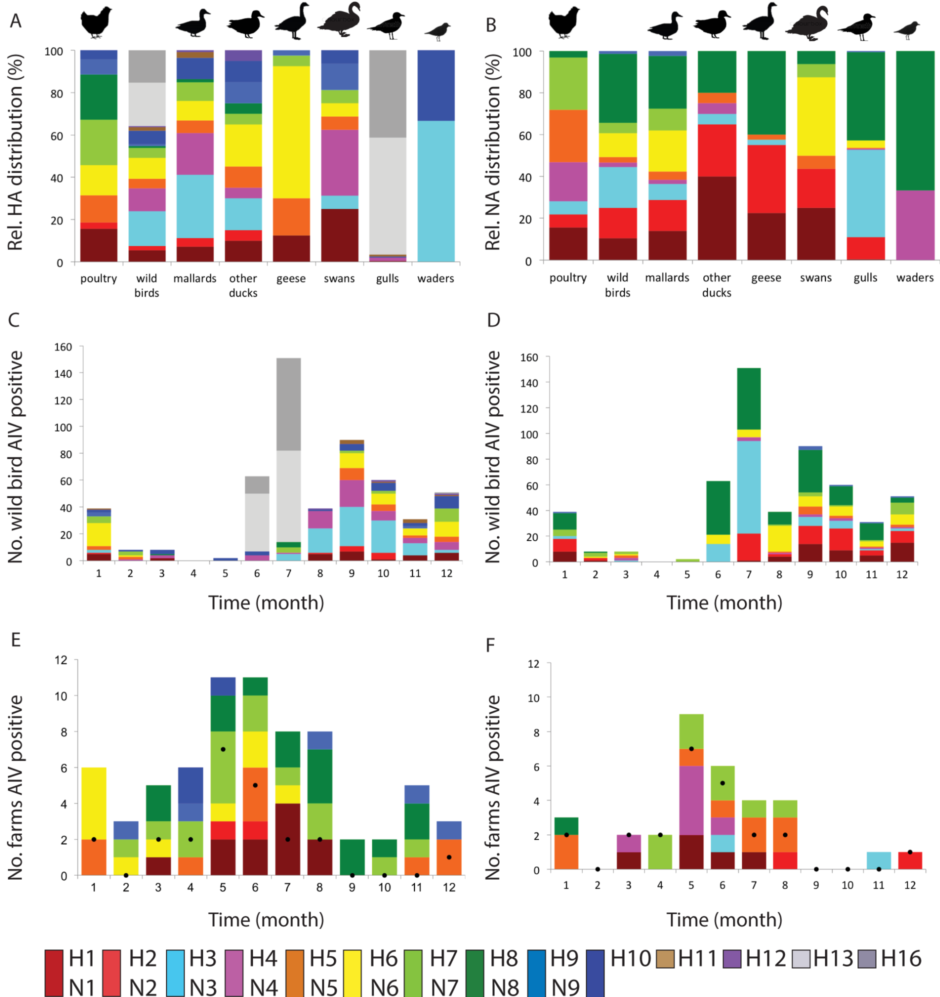


Figure 1. Avian influenza virus subtype distribution in wild birds and poultry, the Netherlands, 2006–2011. Subtype distribution shown for poultry and wild bird species for the hemagglutinin, HA (A) and neuraminidase, NA (B). Distribution based on 70 poultry cases (70 HA and 32 NA known) and 542 wild bird virus isolates (i.e. 250 mallards, 20 other ducks, 40 geese, 16 swans, 201 gulls and 15 waders). Subtype distribution in wild birds in time shown for the HA (C) and NA (D) was based on virus isolates. Subtype distribution in poultry in time shown for the HA (E) and NA (F) was based on antibody detection, virus detection and/or isolation. Black dots indicated number of virus positive farms per month.

Of M-RT-PCR positive birds, H7 viruses were detected in mallards (17 of 2,466; 1%), other ducks (6 of 478; 1%) and geese (3 of 648; 0.5%). No H7 viruses were detected in swans (0 of 31, 0%), gulls (0 of 423; 0%) or waders (0 of 23; 0%) by H7-specific RT-PCR.

Prevalence of influenza A virus NA subtypes in poultry and wild birds

In poultry, N5 (25%), N7 (25%) and N4 (19%) were most frequently detected, followed by N1 (16%), N2 (6%), N3 (6%) and N8 (3%) (Figure 1B, Table 3, Table S1). Viruses of the N5 subtype were more frequently detected in turkeys, and in most cases linked to H1 that was also mostly detected in turkeys. In wild birds, N8 (33%) and N3 (19%) were most frequently detected, followed by N2 (15%), N1 (11%), N6 (11%), N7 (5%), N5 (3%), N4 (2%) and N9 (1%). Viruses of the N3 and N8 subtype were most frequently isolated from gulls and combined with H13 or H16 subtype. NA subtypes detected in poultry differed from the NA subtypes as detected in wild birds (Figure 1, Table S1), but all NA subtypes isolated from other duck species, geese and waders were detected in poultry. Highest NA subtype diversity based on virus detection was detected in June in poultry (Figure 1F), and in September to January in wild birds (Figure 1D).

Difference in influenza A virus HA and NA subtypes in poultry and wild birds

The subtype of AIV that circulated on poultry farms was characterized for 32 out of 82 AIV infected poultry farms, resulting in 13 different HA/NA combinations (Table 3). All HA and NA subtype combinations detected in poultry were detected in wild birds in the Netherlands. In poultry, most frequently detected LPAIV subtypes were H1N5 (6 of 32, 19%), H7N7 (5 of 32, 16%) and H8N4 (4 of 32, 12%) (Table 3). These subtype combinations and H7N4 were significantly more frequently detected in poultry than in wild birds ($P < 0.05$, Fisher's exact test). Part of detections of these subtypes (i.e. H1N5 and H6N5) were epidemiologically linked (e.g. described contact between farms during introduction or infectious period potentially explaining spread between farms). In wild birds most frequently isolated LPAIV subtypes were H13N8 (82 of 542, 15%), H16N3 (80 of 542, 15%), H3N8 (59 of 542, 11%) and H4N6 (41 of 542, 8%). The 13 different LPAIV subtype combinations detected in poultry were of the same subtype as 131 of 542 (24%) of LPAIV isolated from wild birds, with 86 from 250 (34%) of mallards, 9 of 20 (45%) of other ducks, 27 of 40 (67%) of geese, 9 of 16 (56%) of swans, 0 of 201 (0%) of gulls and 0 of 15 (0%) of waders.

Genetic links of poultry and wild bird influenza A viruses

A total of 16 LPAIV isolated from poultry between 2006 and 2011 (Table 2) were included in the genetic analyses. For most poultry HA and NA nucleotide sequences, the closest relatives as identified by BLAST and phylogeny were wild bird LPAIV (11 of 16 poultry HA and NA genes, Table 2, Figure S2). Poultry LPAIV that were most closely related to other poultry LPAIV were of subtypes less commonly or rarely detected in wild birds within the study period (i.e. H7, H8, N4 and N7) (Table 3, Table S1).

Based on genetic analyses of the HA and NA segments, the majority of poultry LPAIV isolates were most closely related to HA and NA of two different LPAIV (Table 2), with one poultry LPAIV isolate genetically most closely related to a single wild bird LPAIV isolate (i.e. A/Ch/Netherlands/11009919/11 (H1N1) and A/White-fronted goose/Netherlands/4/2011 (H1N1)). A second poultry LPAIV was genetically most closely related to H10N7 LPAIV isolated from two mallards sampled at one site on one day (i.e. A/Ch/Netherlands/10008427/10 (H10N7) with HA of A/Mallard/Netherlands/67/2008 (H10N7) and NA of A/Mallard/Netherlands/74/2008 (H10N7)).

Although all poultry HA and NA subtypes were detected in viruses isolated from wild birds in the Netherlands within the study period, several of the 16 poultry isolates were genetically most closely related to LPAIV isolated from wild birds sampled outside the Netherlands but within Western Europe (Table 2). The time interval between detection of genetically closely related LPAIV varied considerably, from 2 days until 2,532 days (Table 2). This time interval was shorter for more common wild bird HA subtypes like H6 (2 to 105 days) than for more rarely detected wild bird HA subtypes like H9 (805 to 2,532 days). The time interval for more common wild bird NA subtypes like N1 (111 to 606 days) and N2 (703 to 1,167 days) did not differ from more rarely detected wild bird NA subtypes like N7 (245 to 523 days) and N5 (325 to 493 days) (Table 2, Table 3).

Landscape analyses of poultry farms in relation to wild birds

Mallards were observed significantly more frequently near poultry farms (675 of 703 farms; 96%) than Eurasian wigeons (490 of 703; 70%, $P < 0.0001$, Fisher's exact test) or greater white-fronted geese (512 of 703; 73%, $P < 0.0001$, Fisher's exact test). However, presence of mallards, Eurasian wigeons or greater white-fronted geese did not differ significantly between primary infected, secondary infected or AIV-negative farms ($P > 0.05$, Fisher's exact test). Despite the fact that mallards were observed more frequently within 1000 meter around poultry farms, Eurasian wigeons and greater white-fronted geese were, if observed, counted in significantly higher numbers than mallards (mean

mallards 63 birds per farm, mean Eurasian wigeons 154 birds per farm (P < 0.0001, Mann-Whitney test) and greater white-fronted geese 226 birds per farm (P < 0.05, Mann-Whitney test). Overall, mean number of mallards counted near primary infected farms (n = 73) was significantly higher than near secondary (n = 39, P < 0.05, Mann-Whitney test) or near AIV-negative farms (n = 61, P < 0.05, Mann-Whitney test) (Figure 2). Mean number of Eurasian wigeons and greater white-fronted geese was higher near primary infected farms than near secondary infected or AIV-negative farms, however not significantly (respectively 673, 45, 104 Eurasian wigeons and 499, 139, 163 greater white-fronted geese) (P > 0.05, Mann-Whitney test) (Figure 2). Water surface within 100 meter around a poultry farm was higher near primary infected farms (0.24 ha, n = 18) than near secondary infected (0.09 ha, n = 47, P > 0.05, Mann-Whitney test) or than AIV-negative farms (0.09 ha, n = 1999, P > 0.05, Mann-Whitney test), however not significantly (Figure 2). Surface of forest or farmland within 100 meter around poultry farm did not differ significantly between primary, secondary and AIV-negative poultry farms (P > 0.05, Mann-Whitney test) (Figure 2). Water, farmland and forest surface within 1000 meter around poultry farms did not differ significantly between primary infected, secondary infected and AIV-negative farms (data not shown) (all P > 0.05, Mann-Whitney test).

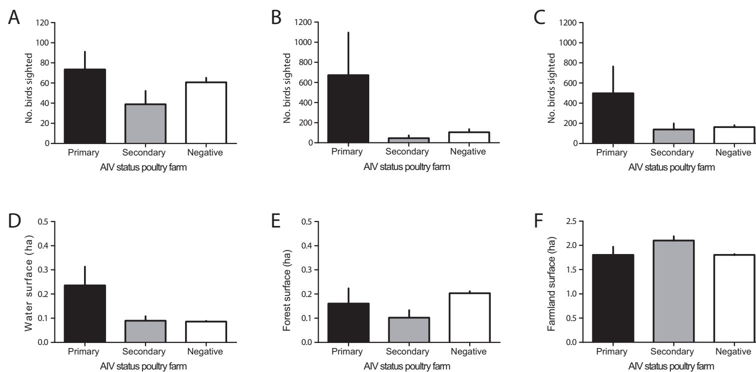


Figure 2. Wild bird distribution and environmental characteristics near primary infected, secondary infected and avian influenza virus negative poultry farms in the Netherlands. For poultry farms located near bird monitoring areas (n = 703: consisting of 6 primary infected, 19 secondary infected and 678 AIV negative farms) number of mallards (A), Eurasian wigeons (B) and greater white-fronted geese (C) within 1000 meters around farms (mean±SE) was investigated. For all poultry farms (n = 2,064: consisting of 18 primary infected, 47 secondary infected and 1,999 AIV negative farms) surface of water (D), forest (E) and farmland (F) within 100 meters around farms (mean±SE) was investigated. Black bars indicates primary infected farms, grey indicates secondary infected farms and white indicates AIV negative farms.

DISCUSSION

Within this 6-year study in the Netherlands, LPAIV subtype distribution differed between poultry and wild birds and indicated apparent differences in host susceptibility to LPAIV subtypes and lineages. LPAIV of some subtypes (i.e. H1, H5, H7, H8, H9, N4, N5 and N7) were significantly more frequently detected in poultry than in wild birds, while LPAIV of other subtypes (i.e. H3, H4, H13, H16, N6 and N8) were significantly more frequently detected in wild birds than in poultry. Given the significant differences, random subtype distribution in wild birds and poultry seems unlikely. Within this study, poultry LPAIV subtype combinations were most frequently detected within wild geese (27 of 40 virus isolates, 67%), followed by swans and ducks. Whether geese acted as so called bridge species for introduction of LPAIV into poultry farms, or whether they are susceptible to the same LPAIV subtypes as chickens and turkeys but do not act as bridge species, or whether they were infected with LPAIV strains that have a broader host range in general—and therefore are more likely to infect poultry—needs to be determined. Some of the LPAIV subtypes detected in poultry (e.g. H8N1, H8N4, H9N2) were detected in ducks and geese in the Netherlands rarely, and exclusively outside LPAIV peak prevalence in autumn, whereas H8 and H9 were isolated relatively frequently from wild birds in North America and Asia respectively (160). Remarkably, none of the common HA subtypes in *Anseriformes*, like H3 and H4 were detected in poultry. Previous studies showed that these virus subtypes were isolated from chickens and turkeys to a limited extent (34). Potential explanations for differences in host susceptibility may be related to the virus strain (e.g. virus tropism, replication, immune evasion) and/or related to modes of transmission (e.g. respiratory, uptake fecal material, water-dependent). It may be worthwhile to experimentally test a variety of LPAIV subtypes and lineages in poultry, to investigate if particular viruses are indeed more prone to cause infections in chickens and turkeys. In addition, observed difference in LPAIV subtype distribution between poultry and wild birds may partly be explained by the considerable spatial discordance between sampling locations of wild birds and locations of poultry farms (Figure S1).

In this study, a long time-interval between LPAIV detection in wild birds and poultry was detected suggesting that the conditions for LPAIV introduction into poultry rely on more than just LPAIV peak prevalence in wild birds. Despite LPAIV peak prevalence in e.g. wild ducks in fall and winter, LPAIV may not reach farms at that time of year due to foraging and aggregation behavior of ducks elsewhere. In addition to wild bird behavior and distribution, seasonal changes in poultry behavior in outdoor farms potentially affect the exposure to LPAIV. It has been suggested that outdoor layers spend more time outside when precipitation is low. In the Netherlands, spring is the driest season, which

may explain increased LPAIV detections in outdoor poultry at the end of spring and early summer, however published data supporting this is lacking. Furthermore, a specific wild bird species may be at the source of introduction into poultry that is currently not identified. Most sampling activities in live wild birds focus on mallards—and high LPAIV prevalence and diversity has been demonstrated in this species—whereas a different avian species may be infected with LPAIV more relevant to poultry. Also, the spatial scale at which the surveillance program is being carried out may affect the time interval between LPAIV detection in wild birds and poultry. For instance, a relatively short time interval (i.e. 6-8 weeks) was reported for LPAIV detection in sentinel ducks and domestic turkeys in a 4-year study in Minnesota, USA (34). Thus, in addition to LPAIV prevalence, data on wild bird species distribution and behavior directly near poultry farms year-round would be valuable information to define risk species and periods of AIV introduction.

The majority of poultry LPAIV isolates most likely originated from independent introductions from wild birds, but such independent wild bird origin can not be inferred with confidence for some HA and NA subtype viruses, i.e. H7, H8 and N4. The long time interval between the detection of poultry LPAIV and their most closely related LPAIV in wild birds as detected by genetic analyses of HA and NA segments, may indicate that the wild bird surveillance program as implemented in the Netherlands is of insufficient intensity or focus if it were to provide “early warning signals” for outbreaks in poultry. Also, a relatively large proportion of poultry HA or NA segments were most closely related to LPAIVs detected outside the Netherlands, in most cases Western European countries. To better facilitate studies like this one, organisations involved in avian influenza surveillance programs should be encouraged to release LPAIV sequence data—for poultry and wild bird viruses—more routinely into public databases.

In our study, mallards were observed more frequently near poultry farms than Eurasian wigeons or greater white-fronted geese. This is not surprising, given the more continuous distribution of mallards in winter, whereas wigeons and geese tend to aggregate in large flocks locally. Consequently, if wigeons or geese were found, the number of birds was much higher than for mallards. However, of these three bird species, only mallards were sighted in statistically significantly higher numbers near AIV primary infected farms. Increased water surface directly surrounding the poultry farms was associated with AIV primary infected farms, however not statistically supported. Although annual bird counts cover a large part of the Netherlands, the counts were skewed towards water rich and poultry poor areas and therefore a minority of farms was covered by these counts. Detailed case-control studies on year-round wild bird distribution and behavior near AIV-positive and -negative poultry farms may identify wild bird related risk factors in relation to AIV introduction.

Despite relatively intensive avian influenza surveillance programs established in the Netherlands, it is still difficult to link wild bird and poultry LPAIV with certainty in time and space. To better target wild bird surveillance programs, a more detailed multi-disciplinary study is needed that includes year-round data on virus prevalence and wild bird distribution and behavior near poultry farms and data on poultry like timing of seroconversion, age at sampling, seasonality of placing new flocks, biosecurity and presence of other disease(s). Virus isolation and virus sequencing of both wild birds and poultry is crucial to identify potential bridge and/or reservoir wild bird species, as well as to support experimental studies on the identification of viruses more prone to cause infections in chickens and turkeys. Our findings establish that evaluation of the design of current large-scale AIV surveillance programs in wild birds and poultry is needed to improve for risk assessment of AIV introduction and minimize the costs.

ACKNOWLEDGEMENTS

The authors thank Ger van der Water and Dries Oomen for logistical and technical assistance. We thank Teun de Vaal, Bert Pellegrom, Hans Zantinge, Arie Keijzer, Jan Berkhouwer, Jan Slijkerman, Dick Jonkers, Engbert van Oort, Gerard Müskens, David Kleijn, Frank Majoor, Kees Oosterbeek for sampling wild birds, and veterinarians of various practices for sampling poultry, and Sjaak de Wit of the Animal Health Service (GD Deventer) for providing farm data. The Dutch waterbird census scheme is part of a governmental ecological surveillance (NEM), carried out in association with Dutch Statistics and supported by Dutch Ministry of Economic Affairs and the Ministry of Infrastructure and the Environment. This study was sponsored by grants from the Dutch Ministry of Economic Affairs contract no. TRCDR/2006/7406, European Research Council project FLUPLAN (250136) and NIAID/NIH contract no. HHSN272201400008C.

SUPPORTING INFORMATION

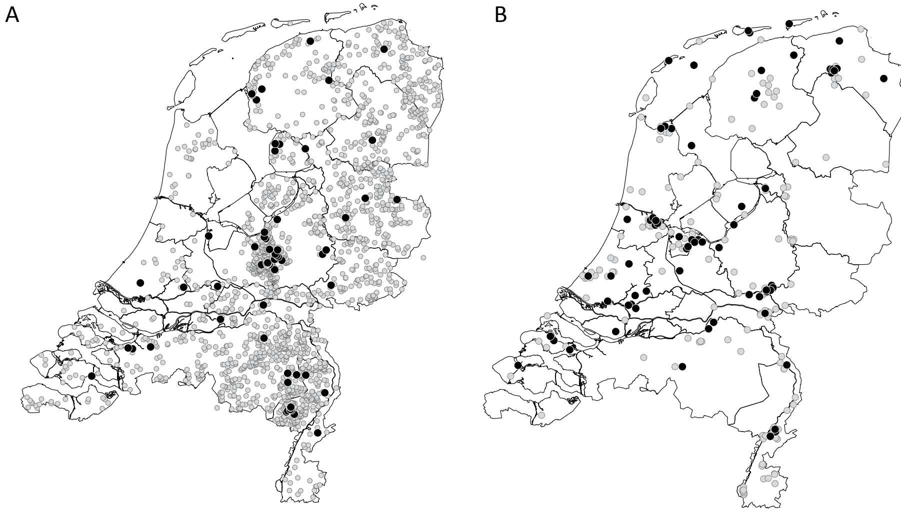
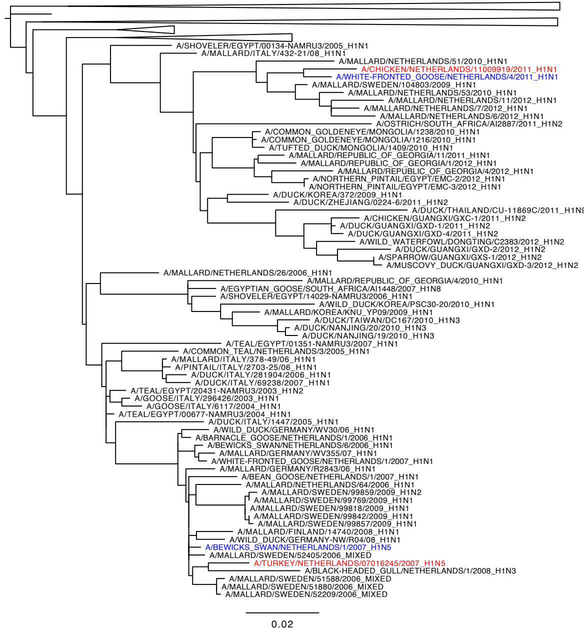
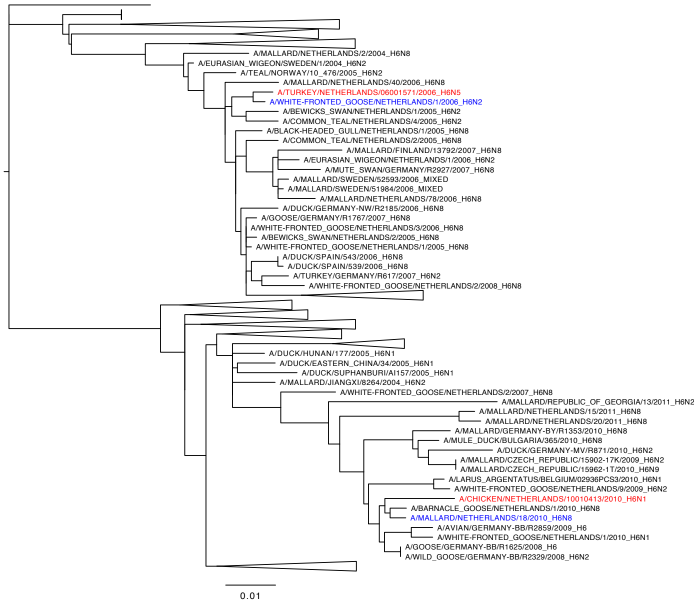


Figure S1. Distribution of (A) poultry farms and (B) sites of wild bird sampling within the Netherlands, 2006 to 2011. Black indicates poultry farms or wild birds that tested positive for avian influenza viruses, grey indicates poultry farms or wild birds that tested negative for avian influenza viruses.

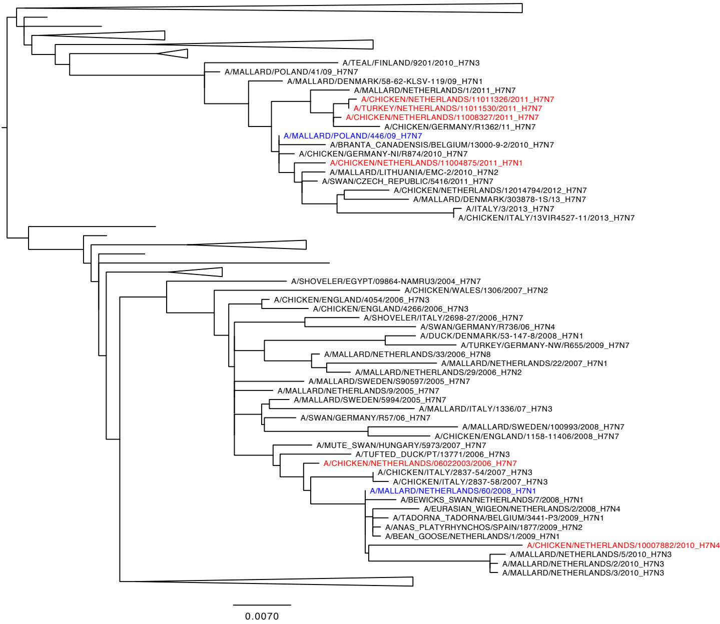
A



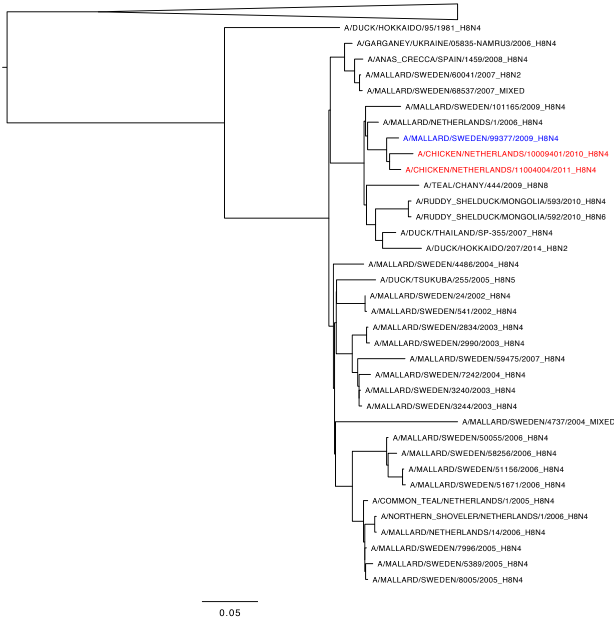
B



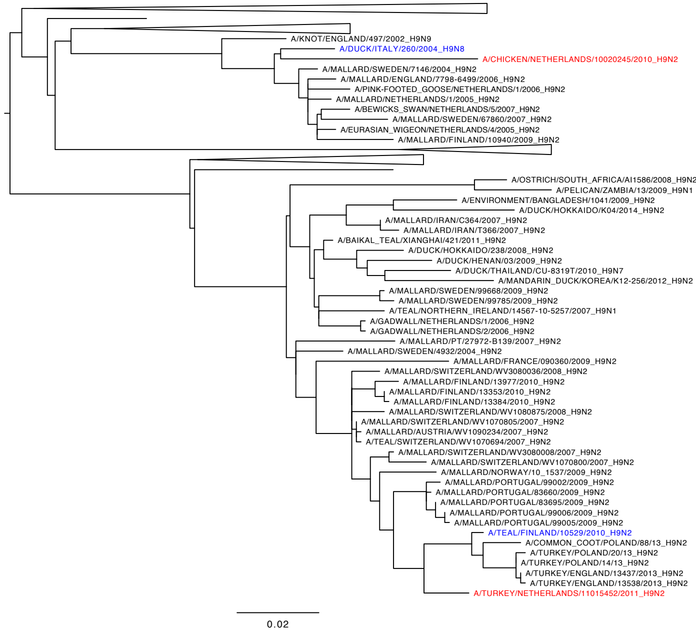
C



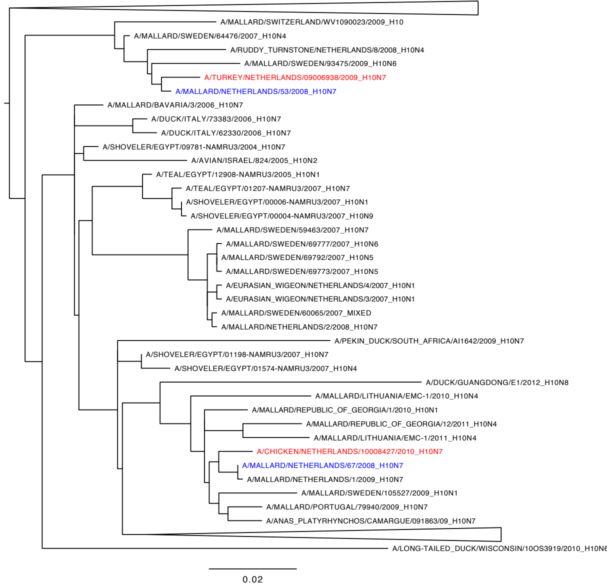
D



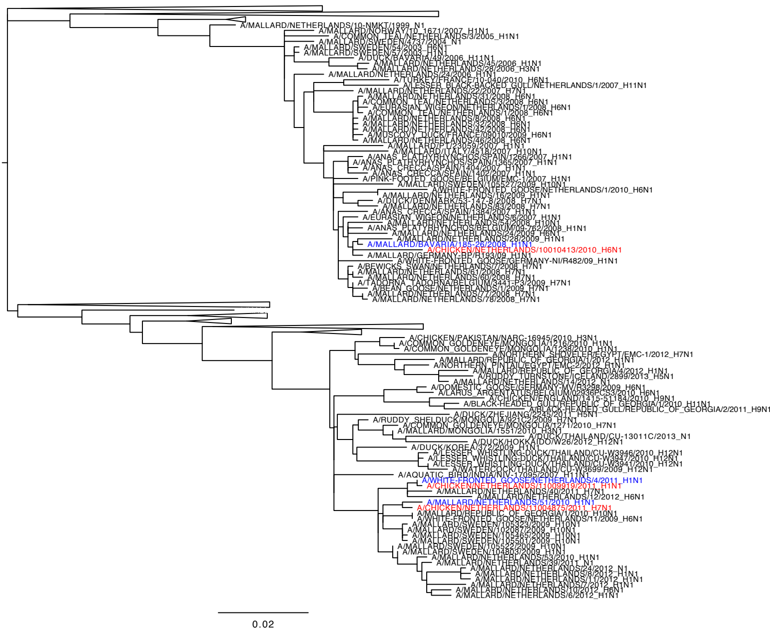
E



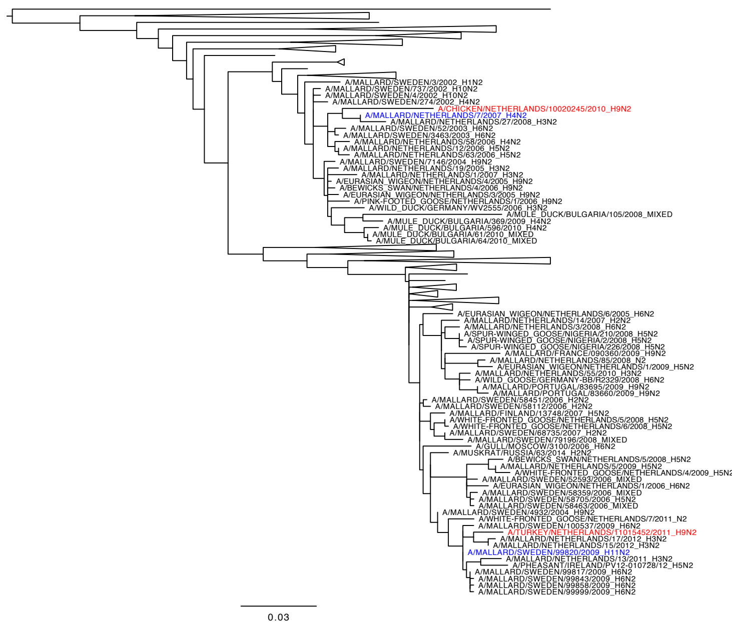
F



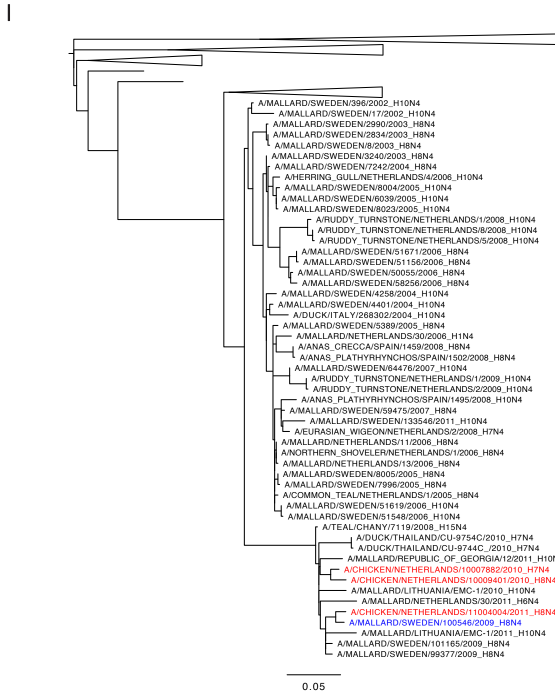
G



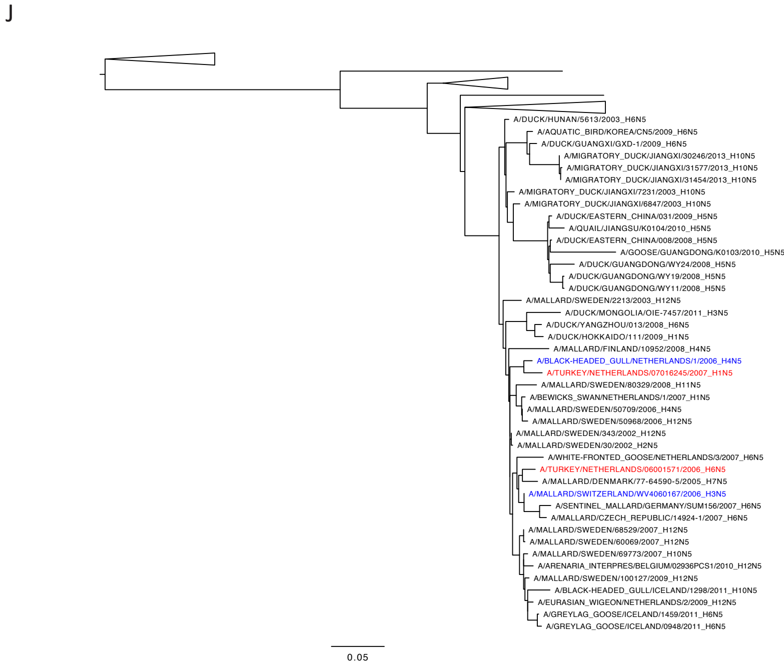
H



I



J



K

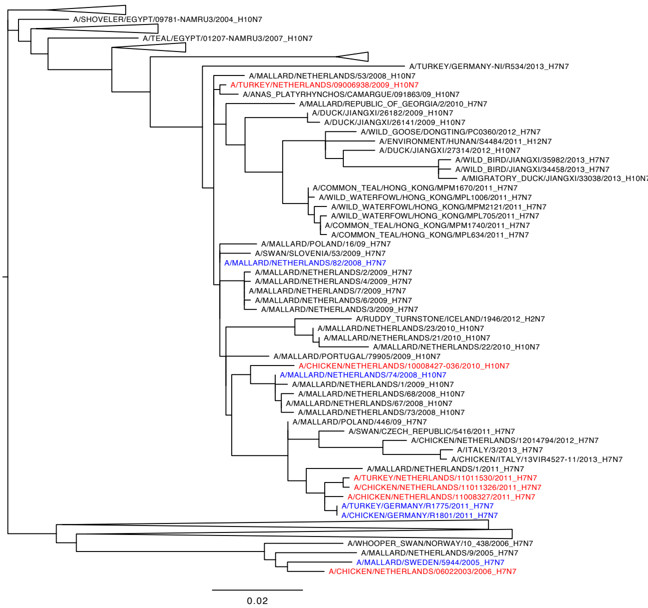


Figure S2. Maximum Likelihood trees of influenza A virus HA and NA subtypes as detected in poultry, the Netherlands, 2006–2011. H1 (A), H6 (B), H7 (C), H8 (D), H9 (E), H10 (F), N1 (G), N2 (H), N4 (I), N5 (J) and N7 (K). Red indicates influenza viruses isolated from poultry in the Netherlands within this study period and blue indicates the genetically closest influenza virus isolated from wild birds.

Table S1. Avian influenza virus hemagglutinin and neuraminidase subtype distribution among poultry and wild bird species, the Netherlands, 2006–2011. Number and percentage (between brackets) of hemagglutinin (A) and neuraminidase (B) subtypes are shown for poultry and wild bird species. Poultry subtypes are shown for primary and secondary cases (i.e. all combined) and separate for primary cases only (i.e. primary cases). Subtypes indicated with an asterisk were significantly more or less frequently detected in the corresponding group than in all wild birds combined, with * = P <0.05 and ** = P <0.01 (Fisher's exact test).

A

	Poultry		Wild birds						
				Anseriformes				Charadriiformes	
Subtype	All combined	Primary cases	All combined	Mallard	Other duck species	Goose species	Swan species	Gull species	Wader species
Total	70	20	542	250 (46)	20 (4)	40 (7)	16 (3)	201 (37)	15 (3)
H1	11 (16)**	2 (10)	30 (6)	18 (7)	2 (10)	5 (13)	4 (25)	1 (<1)	-
H2	2 (3)	1 (5)	11 (2)	10 (4)	1 (5)	-	-	-	-
H3	-	-	89 (16)	75 (30)**	3 (15)	-	1 (6)	-	10 (67)**
H4	-	-	58 (11)	49 (20)**	1 (5)	-	5 (31)*	3 (1)	-
H5	9 (13)**	1 (5)	25 (5)	15 (6)	2 (10)	7 (18)**	1 (6)	-	-
H6	10 (14)	3 (15)	53 (10)	23 (9)	4 (20)	25 (63)**	1 (6)	-	-
H7	15 (21)**	6 (30)	26 (5)	22 (9)*	1 (5)	2 (5)	1 (6)	-	-
H8	15 (21)**	1 (5)	5 (1)	4 (2)	1 (5)	-	-	-	-
H9	5 (7)**	4 (20)	5 (1)	-	2 (10)*	1 (3)	2 (13)*	-	-
H10	3 (4)	2 (10)	34 (6)	25 (10)	2 (10)	-	1 (6)	1 (<1)	5 (33)**
H11	-	-	9 (2)	7 (3)	-	-	-	2 (1)	-
H12	-	-	3 (1)	2 (1)	1 (5)	-	-	-	-
H13	-	-	111 (20)	-	-	-	-	111 (55)**	-
H16	-	-	83 (15)	-	-	-	-	83 (41)**	-

B

	Poultry		Wild birds						
				Anseriformes				Charadriiformes	
Subtype	All combined	Primary cases	All combined	Mallard	Other duck species	Goose species	Swan species	Gull species	Wader species
Total	32	11	542	250 (46)	20 (4)	40 (7)	16 (3)	201 (37%)	15 (3)
N1	5 (16)	2 (18)	57 (11)	35 (14)	8 (40)**	9 (23)*	4 (25)	1 (<1)**	-
N2	2 (6)	2 (18)	79 (15)	37 (15)	5 (25)	13 (33)**	3 (19)	21 (10)	-
N3	2 (6)	2 (18)	105 (19)	19 (8)**	1 (5)	1 (3)**	-	84 (42)**	-
N4	6 (19)**	1 (9)	12 (2)	5 (2)	1 (5)	-	-	1 (<1)	5 (33)**
N5	8 (25)**	1 (9)	14 (3)	10 (4)	1 (5)	1 (3)	1 (6)	1 (<1)	-
N6	-	-	62 (11)	49 (20)**	-	-	6 (38)**	7 (3)**	-
N7	8 (25)**	3 (27)	27 (5)	26 (10)**	-	-	1 (6)	-	-
N8	1 (3)**	-	179 (33)	63 (25)*	4 (20)	16 (40)	1 (6)*	85 (42)*	10 (67)*
N9	-	-	7 (1)	6 (2)	-	-	-	1 (<1)	-

Table S2. The low pathogenic avian influenza viruses (LPAIV) and the accession numbers of the segments used in this study as listed in online databases GenBank (158) and GISAID EpiFlu (160)

Viral Name	Source	Accession number HA segment	Accession number NA segment	Viral Name	Source	Accession number HA segment	Accession number NA segment
A/White-fronted_goose/Netherlands/1/2007_H1N1	GenBank	KR862410	KR862542	A/Teal/Switzerland/WV1070694/2007_H9N2	GenBank	GU194479	
A/Bean_goose/Netherlands/1/2007_H1N1	GenBank	KR862411	KR862543	A/Turkey/Germany/EK224/1995_H9N2	GenBank	JX273569	
A/Mallard/Netherlands/26/2006_H1N1	GenBank	KR862413		A/Turkey/Netherlands/11015452/2011_H9N2	GenBank	pending	pending
A/Mallard/Netherlands/64/2006_H1N1	GenBank	KR862414	KR862551	A/Duck/Tsukuba/574/2006_H10N1	GISAID	EPI356629	
A/Mallard/Netherlands/23/2006_H1N1	GenBank	KR862412		A/Shoveler/Egypt/00006-NAMRU3/2007_H10N1	GISAID	EPI372442	
A/Bewicks_swan/Netherlands/6/2006_H1N1	GenBank	KR862416		A/Teal/Egypt/12908-NAMRU3/2005_H10N1	GISAID	EPI372481	EPI372480
A/Pink-footed_goose/Belgium/EMC-1/2007_H1N1	GenBank	KR862369	KR862390	A/Wild_bird/Korea/A323/2009_H10N1	GISAID	EPI387876	
A/Eurasian_wigeon/Netherlands/2/2007_H1N1	GenBank	KR862417		A/Duck/Hokkaido/W87/2007_H10N2	GISAID	EPI161527	
A/Black-headed_gull/Netherlands/1/2008_H1N3	GenBank	KR862418		A/Duck/Hunan/S11205/2012_H10N3	GISAID	EPI461563	
A/White-fronted_goose/Netherlands/4/2011_H1N1	GenBank	KR862419	KR862558	A/Duck/Thailand/LM-CU4747/2009_H10N3	GISAID	EPI314742	
A/Common_teal/Netherlands/3/2005_H1N1	GenBank	KR862408	KR862539	A/Duck/Thailand/LM-CU4753/2009_H10N3	GISAID	EPI314746	
A/Barnacle_goose/Netherlands/1/2006_H1N1	GenBank	KR862409	KR862540	A/Muscovy_duck/Thailand/CU-LM4754/2009_H10N3	GISAID	EPI256770	
A/Eurasian_wigeon/Netherlands/6/2007_H1N1	GenBank	KR862420	KR862560	A/Duck/Italy/268302/2004_H10N4	GISAID	EPI178493	EPI178495
A/Mallard/Netherlands/16/2009_H1N1	GenBank	KR862421	KR862577	A/Pied_avocet/Ukraine/05848-NAMRU3/2006_H10N4	GISAID	EPI372496	EPI372495
A/Mallard/Netherlands/53/2010_H1N1	GenBank	KR862422	KR862581	A/Shoveler/Egypt/01574-NAMRU3/2007_H10N4	GISAID	EPI372458	EPI372457
A/Mallard/Netherlands/6/2012_H1N1	GenBank	KR862423	KR862582	A/Long-tailed_duck/Wisconsin/10OS3919/2010_H10N6	GISAID	EPI419336	
A/Mallard/Netherlands/7/2012_H1N1	GenBank	KR862424	KR862586	A/Mallard/Denmark/16109-4/2011-11-14_H10N6	GISAID	EPI541472	
A/Mallard/Netherlands/11/2012_H1N1	GenBank	KR862426	KR862590	A/Anas_platyrhynchos/Camargue/091863/09_H10N7	GISAID	EPI332944	EPI332955
A/Mallard/Netherlands/51/2010_H1N1	GenBank	KR862427	KR862595	A/Avian/Israel/201/2001_H10N7	GenBank	JN564724	JN575025
A/Northern_pintail/Egypt/EMC-2/2012_H1N1	GenBank	KR862370	KR862392	A/Avian/Israel/218/2000_H10N7	GenBank	JN564725	JN575026
A/Northern_pintail/Egypt/EMC-3/2012_H1N1	GenBank	KR862371		A/Avian/Israel/232/2001_H10N7	GISAID	EPI456969	
A/Chicken/Netherlands/11009919/2011_H1N1	GenBank	pending	pending	A/Avian/Israel/297/2001_H10N7	GenBank	JN564728	JN575029
A/Teal/Egypt/20431-NAMRU3/2003_H1N2	GISAID	EPI372275		A/Avian/Israel/445/2001_H10N7	GenBank	JN564730	JN575031
A/Shoveler/Egypt/00134-NAMRU3/2005_H1N1	GISAID	EPI372331		A/Avian/Israel/457/2001_H10N7	GenBank	JN564731	JN575032
A/Shoveler/Egypt/14029-NAMRU3/2006_H1N1	GISAID	EPI372378	EPI372377	A/Duck/Italy/62330/2006_H10N7	GISAID	EPI178528	

Viral Name	Source	Accession number HA segment	Accession number NA segment	Viral Name	Source	Accession number HA segment	Accession number NA segment
A/Teal/Egypt/01351-NAMRU3/2007_H1N1	GISAID	EPI372466	EPI372465	A/Duck/Italy/73383/2006_H10N7	GISAID	EPI174777	
A/Teal/Egypt/00677-NAMRU3/2004_H1N1	GISAID	EPI372528		A/Duck/Shimane/45/1997_H10N7	GenBank	AB296078	AB296079
A/Goose/Italy/6117/2004_H1N1	GISAID	EPI178520	EPI178522	A/Harbor_seal/Denmark/14-5061-1lu/2014-07_H10N7	GISAID	EPI541474	
A/Mallard/Germany/R2843/06_H1N1	GISAID	EPI222781		A/Mallard/Egypt/EMC-4/2012_H10N7	GISAID	EPI552755	
A/Mallard/Germany-RP/R193/09_H1N1	GISAID	EPI248501	EPI248500	A/Mallard/Netherlands/1/2012_H10N7	GISAID	EPI552756	
A/Wild_duck/Germany/WV30/06_H1N1	GISAID	EPI248514	EPI248512	A/Mallard/Netherlands/1/2014_H10N7	GISAID	EPI552751	
A/Mallard/Germany/WV355/07_H1N1	GISAID	EPI248519	EPI248517	A/Mallard/Netherlands/47/2010_H10N7	GenBank	KR862524	KR862722
A/Wild_duck/Germany-NW/R04/08_H1N1	GISAID	EPI248521		A/Mallard/Netherlands/50/2010_H10N7	GenBank	KR862527	KR862727
A/Anas_platyrhynchos/Belgium/09-762/2008_H1N1	GISAID	EPI257212	EPI257214	A/Northern_pintail/Egypt/EMC-1/2012_H10N7	GISAID	EPI552754	
A/Wild_duck/Korea/CSM38/2004b_H1N1	GISAID	EPI296244		A/Seal/Sweden/SVA0546/2014_H10N7	GISAID	EPI545212	
A/Duck/Italy/7686-11/10_H1N1	GISAID	EPI301849		A/Shoveler/Egypt/00600-NAMRU3/2004_H10N7	GISAID	EPI372291	
A/Pintail/Italy/2703-25/06_H1N1	GISAID	EPI301856		A/Shoveler/Egypt/01198-NAMRU3/2007_H10N7	GISAID	EPI372402	
A/Mallard/Italy/378-49/06_H1N1	GISAID	EPI301857		A/Shoveler/Egypt/09781-NAMRU3/2004_H10N7	GISAID	EPI372339	EPI372338
A/Teal/Italy/6323-5/07_H1N1	GISAID	EPI301858		A/Teal/Egypt/01207-NAMRU3/2007_H10N7	GISAID	EPI372426	EPI372425
A/Mallard/Italy/432-21/08_H1N1	GISAID	EPI301859		A/Seal/Sweden/SVA0824/2014_/H10N7_H10N7	GISAID	EPI547696	
A/Shoveler/Italy/6965-6/07_H1N3	GISAID	EPI301860		A/Chicken/77/Jiangxi/2014_H10N8	GISAID	EPI537463	
A/Turkey/Netherlands/07016245/2007_H1N5	GenBank	pending	pending	A/Chicken/Jiangxi/102/2013_H10N8	GISAID	EPI530542	
A/Anas_crecca/Spain/1384/2007_H1N1	GenBank	FN386464	FN386472	A/Environment/Dongting_Lake/Hunan/3-9/2007_H10N8	GISAID	EPI221966	
A/Anas_crecca/Spain/1404/2007_H1N1	GenBank	FN386465	FN386474	A/Environment/Jiangxi/03366/2013_H10N8	GISAID	EPI530386	
A/Anas_plathyrhynchos/Spain/1365/2007_H1N1	GenBank	FN386463	FN386471	A/Environment/Jiangxi/03367/2013_H10N8	GISAID	EPI530394	
A/Bewicks_swan/Netherlands/1/2007_H1N5	GenBank	CY076976	CY076978	A/Environment/Jiangxi/03413/2013_H10N8	GISAID	EPI530402	
A/Chicken/Guangxi/GXc-1/2011_H1N2	GenBank	KF013910		A/Environment/Jiangxi/03489/2013_H10N8	GISAID	EPI530410	
A/Common_goldeneye/Mongolia/1216/2010_H1N1	GenBank	KF501071	KF667692	A/Environment/Jiangxi/10615/2014_H10N8	GISAID	EPI530418	
A/Common_goldeneye/Mongolia/1238/2010_H1N1	GenBank	KF501070	KF667690	A/Environment/Jiangxi/10721/2014_H10N8	GISAID	EPI530426	
A/Common_teal/Netherlands/10/2000_H1N1	GenBank	CY060178	CY060180	A/Environment/Jiangxi/10738/2014_H10N8	GISAID	EPI530434	
A/Duck/Guangxi/GXd-1/2011_H1N2	GenBank	KF013918		A/Jiangxi-Donghu/346/2013_H10N8	GISAID	EPI497477	
A/Duck/Guangxi/GXd-2/2012_H1N2	GenBank	KF013934		A/Jiangxi/09037/2014_H10N8	GISAID	EPI530450	
A/Duck/Guangxi/GXd-4/2011_H1N2	GenBank	KF013926		A/Mallard/Sweden/7/2003_H10N8	GISAID	EPI251793	
A/Duck/Hebei/843/2005_H1N2	GenBank	FJ536843		A/Northern_shoveler/Hong_Kong/MPC657/2006_H10N9	GISAID	EPI469806	
A/Duck/Hokkaido/111/2009_H1N5	GenBank	AB560963	AB560964	A/Northern_shoveler/Hong_Kong/MPE2531/2008_ H10N9	GISAID	EPI469805	
A/Duck/Hokkaido/327/2009_H1N3	GenBank	AB560965		A/Northern_shoveler/Hong_Kong/MPE2984/2008_ H10N9	GISAID	EPI469808	
A/Duck/Italy/1447/2005_H1N1	GenBank	HF563054		A/Shoveler/Egypt/00004-NAMRU3/2007_H10N9	GISAID	EPI372434	
A/Duck/Italy/281904/2006_H1N1	GenBank	FJ432770		A/Ruddy_turnstone/Netherlands/8/2008_H10N4	GenBank	KR862508	KR862683
A/Duck/Italy/69238/2007_H1N1	GenBank	FJ432754		A/Mallard/Netherlands/1/2007_H10N7	GenBank	KR862510	
A/Duck/Korea/372/2009_H1N1	GenBank	KJ764732	KJ764734	A/Eurasian_wigeon/Netherlands/3/2007_H10N1	GenBank	KR862511	
A/Duck/Korea/U11-1/2007_H1N2	GenBank	KJ764772		A/Ruddy_turnstone/Netherlands/2/2009_H10N4	GenBank	KR862515	KR862690
A/Duck/Korea/U11/2007_H1N2	GenBank	HQ014832		A/Mallard/Lithuania/EMC-1/2011_H10N4	GenBank	KR862380	KR862396
A/Duck/Korea/U14/2007_H1N3	GenBank	HQ014840		A/Mallard/Lithuania/EMC-1/2010_H10N4	GenBank	KR862381	KR862397
A/Duck/Nanjing/19/2010_H1N3	GenBank	HQ336713		A/Mallard/Netherlands/16/2006_H10N7	GenBank	KR862507	
A/Duck/Nanjing/20/2010_H1N3	GenBank	HQ336721		A/Mallard/Netherlands/2/2008_H10N7	GenBank	KR862517	KR862710
A/Duck/Shimane/188/1999_H1N1	GenBank	CY091592	CY091594	A/Mallard/Netherlands/67/2008_H10N7	GenBank	KR862518	KR862711
A/Duck/Taiwan/DC167/2010_H1N3	GenBank	KC693623		A/Mallard/Netherlands/1/2009_H10N7	GenBank	KR862522	KR862715
A/Duck/Thailand/CU-11869C/2011_H1N9	GenBank	KJ161957		A/Mallard/Netherlands/53/2008_H10N7	GenBank	KR862523	KR862716
A/Duck/Tsukuba/718/2005_H1N1	GenBank	AB670330	AB472014	A/Mallard/Netherlands/30/2009_H10	GenBank	KR862525	
A/Duck/Zhejiang/0224-6/2011_H1N2	GenBank	JN605372		A/Mallard/Egypt/EMC-1/2012_H10N7	GenBank	KR862382	
A/Duck/Zhejiang/0607-13/2011_H1N2	GenBank	JN605373		A/Chicken/Netherlands/10008427/2010_H10N7	GenBank	pending	pending

Viral Name	Source	Accession number HA segment	Accession number NA segment	Viral Name	Source	Accession number HA segment	Accession number NA segment
A/Duck/Zhejiang/0611-15/2011_H1N3	GenBank	JN716320		A/Turkey/Netherlands/09006938/2009_H10N7	GenBank	pending	pending
A/Duck/Zhejiang/0611-17/2011_H1N3	GenBank	JN605375		A/Mallard/Netherlands/06013952/2006_H10N7	GenBank	pending	
A/Duck/Zhejiang/0611-24/2011_H1N3	GenBank	JN716323		A/Mallard/Netherlands/06014516/2006_H10N8	GenBank	pending	
A/Duck/Zhejiang/0611-8/2011_H1N3	GenBank	JN605374		A/Avian/Israel/543/2008_H10N7	GenBank	JN564732	JN575033
A/Duck/Zhejiang/473/2013_H1N4	GenBank	KF357774		A/Avian/Israel/824/2005_H10N2	GenBank	JN564733	
A/Duck/Zhejiang/475/2013_H1N4	GenBank	KF357775		A/Chicken/Jiangsu/RD5/2013_H10N9	GenBank	KF006414	
A/Duck/Zhejiang/476/2013_H1N4	GenBank	KF357776		A/Common_teal/Hong_Kong/MPK630/2009_H10N9	GenBank	KF259197	
A/Duck/Zhejiang/477/2013_H1N4	GenBank	KF357777	KF357766	A/Duck/Guangdong/E1/2012_H10N8	GenBank	JQ924786	
A/Egyptian_goose/South_Africa/AI1448/2007_H1N8	GenBank	GQ404705		A/Duck/Hunan/S1496/2011_H10N8	GenBank	KP862019	
A/Environment/Korea/CSM12/2007_H1N2	GenBank	KJ764740		A/Duck/Hunan/S3137/2009_H10N8	GenBank	KP862027	
A/Environment/Korea/UPO218/2008_H1N6	GenBank	KJ764780		A/Duck/Hunan/S4280/2009_H10N8	GenBank	KP862035	
A/Goose/Italy/296426/2003_H1N1	GenBank	FJ432778	FJ432780	A/Duck/Huzhou/4233/2013_mixed	GenBank	KP413924	
A/Mallard/Bavaria/185-8/2008_H1N1	GenBank	HQ259224		A/Duck/Jiangxi/13875/2005_H10N3	GenBank	KP287830	
A/Mallard/Bavaria/42/2006_H1N1	GenBank	GU046744	GU046745	A/Duck/Jiangxi/13946/2005_H10N3	GenBank	KP287838	
A/Mallard/Finland/14740/2008_H1N1	GenBank	KF183609		A/Duck/Jiangxi/15846/2013_H10N3	GenBank	KP285477	
A/Mallard/Korea/KNU_YP09/2009_H1N1	GenBank	HQ897965		A/Duck/Jiangxi/2039/2005_H10N8	GenBank	KP287926	
A/Mallard/Netherlands/10/1999_H1N8	GenBank	CY060206		A/Duck/Jiangxi/24570/2009_mixed	GenBank	KP287794	
A/Mallard/Netherlands/30/2006_H1N4	GenBank	CY076897	CY076899	A/Duck/Jiangxi/26141/2009_H10N7	GenBank	KP287966	KP287968
A/Mallard/Republic_of_Georgia/1/2012_H1N1	GenBank	CY185593	CY185595	A/Duck/Jiangxi/26182/2009_H10N7	GenBank	KP287974	KP287976
A/Mallard/Republic_of_Georgia/11/2011_H1N1	GenBank	KC190172		A/Duck/Jiangxi/26281/2009_H10N7	GenBank	KP287982	
A/Mallard/Republic_of_Georgia/4/2010_H1N1	GenBank	CY185441		A/Duck/Jiangxi/26331/2009_H10N7	GenBank	KP287990	
A/Mallard/Republic_of_Georgia/4/2012_H1N1	GenBank	CY185641	CY185643	A/Duck/Jiangxi/27314/2012_H10N7	GenBank	KP287665	KP287667
A/Mallard/Sanjiang/390/2007_H1N1	GenBank	CY077076	CY077078	A/Duck/Jiangxi/302/2006_H10N3	GenBank	KP287846	
A/Mallard/Sweden/104803/2009_H1N1	GenBank	JX566076	JX566260	A/Duck/Jiangxi/6450/2013_H10N8	GenBank	KP285589	
A/Mallard/Sweden/3/2002_H1N2	GenBank	CY060268	CY060270	A/Duck/Jiangxi/6544/2013_H10N8	GenBank	KP285661	
A/Mallard/Sweden/51588/2006_mixed	GenBank	CY164649		A/Duck/Jiangxi/6556/2013_H10N8	GenBank	KP285677	
A/Mallard/Sweden/51880/2006_mixed	GenBank	CY164144		A/Duck/Jiangxi/6613/2013_mixed	GenBank	KP287044	
A/Mallard/Sweden/52209/2006_mixed	GenBank	CY164872		A/Duck/Jiangxi/6648/2013_H10N8	GenBank	KP285733	
A/Mallard/Sweden/52405/2006_mixed	GenBank	CY164156		A/Duck/Jiangxi/860/2006_H10N3	GenBank	KP287886	
A/Mallard/Sweden/57/2003_H1N1	GenBank	CY060329	CY060330	A/Duck/Mongolia/149/03_H10N5	GenBank	AB450456	AB270599
A/Mallard/Sweden/98/2002_H1N6	GenBank	CY060410		A/Duck/Zhejiang/6D20/2013_H10N2	GenBank	KP063197	
A/Mallard/Sweden/99769/2009_H1N1	GenBank	JX565989		A/Eurasian_wigeon/Netherlands/4/2007_H10N1	GenBank	CY077048	
A/Mallard/Sweden/99818/2009_H1N1	GenBank	JX565992		A/Goose/Guizhou/829/2012_H10N7	GenBank	KF259194	
A/Mallard/Sweden/99842/2009_H1N1	GenBank	JX566000		A/Harbour_seal/Germany/1/2014_H10N7	GenBank	KP137835	KP137832
A/Mallard/Sweden/99857/2009_H1N1	GenBank	JX566013		A/Herring_gull/Netherlands/4/2006_H10N4	GenBank	CY077032	CY077034
A/Mallard/Sweden/99859/2009_H1N2	GenBank	JX566015		A/Mallard/Bavaria/3/2006_H10N7	GenBank	FJ183474	FJ183475
A/Muscovy_duck/Guangxi/GXd-3/2012_H1N2	GenBank	KF013942		A/Mallard/Korea/1203/2010_H10N8	GenBank	JN817572	
A/Ostrich/South_Africa/AI2887/2011_H1N2	GenBank	JX069105		A/Mallard/Korea/1242/2010_H10N6	GenBank	JN817576	
A/Pintail/Akita/1265/2008_H1N2	GenBank	AB546162		A/Mallard/Netherlands/02/2000_H10N7	GenBank	CY076945	CY076947
A/Pintail/Aomori/1130/2008_H1N3	GenBank	AB546180		A/Mallard/Portugal/79940/2009_H10N7	GenBank	CY116612	
A/Pintail/Aomori/422/2007_H1N1	GenBank	AB546149	AB546151	A/Mallard/Republic_of_Georgia/1/2010_H10N1	GenBank	KC190171	KC190180
A/Pintail/Aomori/794/2008_H1N1	GenBank	AB546153	AB546155	A/Mallard/Republic_of_Georgia/12/2011_H10N4	GenBank	CY185505	CY185507
A/Pintail/Miyagi/1472/2008_H1N1	GenBank	AB546157	AB546159	A/Mallard/Republic_of_Georgia/14/2011_H10N7	GenBank	CY185689	CY185691
A/Pintail/Shimane/324/98_H1N9	GenBank	AB274304		A/Mallard/Republic_of_Georgia/15/2011_H10N7	GenBank	CY185385	CY185387
A/Sparrow/Guangxi/GXs-1/2012_H1N2	GenBank	KF013902		A/Mallard/Sweden/102087/2009_H10N1	GenBank	CY183839	CY183841
A/Swan/Hokkaido/55/1996_H1N1	GenBank	AB271115	AB271116	A/Mallard/Sweden/104746/2009_H10N1	GenBank	CY183855	
A/Swine/Hong_Kong/644/1993_H1N1	GenBank	CY085009		A/Mallard/Sweden/105186/2009_H10N1	GenBank	CY183863	
A/Tufted_duck/Mongolia/1409/2010_H1N1	GenBank	KF501056		A/Mallard/Sweden/105323/2009_H10N1	GenBank	CY183887	CY183889

Viral Name	Source	Accession number HA segment	Accession number NA segment	Viral Name	Source	Accession number HA segment	Accession number NA segment
A/WDK/JX/12416/2005_H1N1	GenBank	FN436023		A/Mallard/Sweden/105402/2009_H10N1	GenBank	CY183911	
A/Wild_duck/Guangdong/520/2001_H1N9	GenBank	KF258943		A/Mallard/Sweden/105404/2009_H10N1	GenBank	CY183919	
A/Wild_duck/Korea/CSM38/2004a_H1N1	GenBank	HQ014744		A/Mallard/Sweden/105465/2009_H10N1	GenBank	CY183927	CY183929
A/Wild_duck/Korea/CW09/2005_H1N1	GenBank	HQ014776		A/Mallard/Sweden/105501/2009_H10N1	GenBank	CY183951	CY183953
A/Wild_duck/Korea/ESD48/2006_H1N1	GenBank	HQ014816		A/Mallard/Sweden/105522/2009_H10N1	GenBank	JX566079	JX566263
A/Wild_duck/Korea/HDR02/2005_H1N1	GenBank	HQ014768		A/Mallard/Sweden/105527/2009_H10N1	GenBank	CY183959	CY183961
A/Wild_duck/Korea/PJ25/2006_H1N3	GenBank	HQ014800		A/Mallard/Sweden/105536/2009_H10N1	GenBank	CY183967	
A/Wild_duck/Korea/PSC30-20/2010_H1N1	GenBank	KJ764764		A/Mallard/Sweden/133546/2011_H10N4	GenBank	CY183991	CY183993
A/Wild_duck/Korea/SH13/2006_H1N1	GenBank	HQ014784		A/Mallard/Sweden/1417/2002_H10N7	GenBank	CY183637	CY183639
A/Wild_duck/Korea/SH14/2006_H1N3	GenBank	HQ014792		A/Mallard/Sweden/223/2002_H10N2	GenBank	CY183580	
A/Wild_duck/Korea/SH29/2006_H1N3	GenBank	HQ014808		A/Mallard/Sweden/3151/2003_H10N7	GenBank	CY183645	CY183647
A/Wild_duck/Korea/SH60/2004_H1N1	GenBank	HQ014760		A/Mallard/Sweden/4258/2004_H10N4	GenBank	CY183653	CY183655
A/Wild_duck/Korea/UP122/2007_H1N1	GenBank	HQ014824		A/Mallard/Sweden/4401/2004_H10N4	GenBank	CY183661	CY183663
A/Wild_duck/Korea/YS44/2004_H1N2	GenBank	HQ014752		A/Mallard/Sweden/4411/2004_H10N4	GenBank	CY183669	
A/Wild_waterfowl/Dongting/C2383/2012_H1N2	GenBank	KF874481		A/Mallard/Sweden/51/2002_H10N2	GenBank	HM136575	
A/Avian/Germany-BB/R2859/2009_H6	GISAID	EPI339183		A/Mallard/Sweden/51548/2006_H10N4	GenBank	CY183718	CY183720
A/Goose/Germany-BB/R1625/2008_H6	GISAID	EPI279941		A/Mallard/Sweden/51619/2006_H10N4	GenBank	CY183734	CY183736
A/Ringed_teal/Germany-NRW/R641/2008_H6	GISAID	EPI279938		A/Mallard/Sweden/51933/2006_H10N9	GenBank	CY183758	
A/Wild_bird/Germany-HH/R1501/2008_H6	GISAID	EPI279939		A/Mallard/Sweden/52903/2006_H10N9	GenBank	CY183783	
A/Wild_bird/Germany-MV/R1511/2008_H6	GISAID	EPI279940		A/Mallard/Sweden/5812/2005_H10N9	GenBank	CY183677	
A/Environment/California/NWRC182841-09/2006_H6N1	GISAID	EPI406091		A/Mallard/Sweden/5824/2005_H10N7	GenBank	CY183685	
A/Environment/California/NWRC183200-14/2006_H6N1	GISAID	EPI406099		A/Mallard/Sweden/5932/2005_H10N7	GenBank	CY183401	CY183403
A/Environment/California/NWRC183274-04/2006_H6N1	GISAID	EPI406103		A/Mallard/Sweden/59463/2007_H10N7	GenBank	CY183791	CY183793
A/Green-winged_teal/Nova_Scotia/14917/2005_H6N1	GISAID	EPI327397		A/Mallard/Sweden/60065/2007_mixed	GenBank	CY184665	
A/Larus_argentatus/Belgium/02936pcs3/2010_H6N1	GISAID	EPI345428	EPI345427	A/Mallard/Sweden/6039/2005_H10N4	GenBank	CY184640	CY184642
A/Northern_shoveler/California/HKWF115/2007_H6N1	GISAID	EPI154816		A/Mallard/Sweden/64476/2007_H10N4	GenBank	CY183799	CY183801
A/Duck/Germany-MV/R871/2010_H6N2	GISAID	EPI339182		A/Mallard/Sweden/69773/2007_H10N5	GenBank	CY183807	CY183809
A/Green-winged_teal/Minnesota/Sg-00199/2007_H6N2	GISAID	EPI298290		A/Mallard/Sweden/69777/2007_H10N6	GenBank	CY183815	
A/Green-winged_teal/Minnesota/Sg-00222/2007_H6N2	GISAID	EPI298322		A/Mallard/Sweden/69792/2007_H10N5	GenBank	CY183823	
A/Mule_duck/Bulgaria/156/2010_H6N2	GISAID	EPI574180		A/Mallard/Sweden/737/2002_H10N2	GenBank	CY183588	CY183590
A/Mule_duck/Bulgaria/173/2009_H6N2	GISAID	EPI574206		A/Mallard/Sweden/766/2002_mixed	GenBank	CY183596	
A/Shoveler/Egypt/13251-NAMRU3/2006_H6N2	GISAID	EPI372371	EPI372370	A/Mallard/Sweden/8004/2005_H10N4	GenBank	CY184657	CY184659
A/Teal/Egypt/13203-NAMRU3/2006_H6N2	GISAID	EPI372386	EPI372385	A/Mallard/Sweden/8023/2005_H10N4	GenBank	CY183710	CY183712
A/Turkey/Germany/R617/2007_H6N2	GISAID	EPI317612		A/Mallard/Sweden/93475/2009_H10N6	GenBank	CY183831	
A/Wild_goose/Germany-BB/R2329/2008_H6N2	GISAID	EPI397608	EPI397607	A/Mallard/Sweden/948/2002_H10N9	GenBank	CY183629	
A/Environment/North_Carolina/NWRC183941-06/2006_H6N5	GISAID	EPI406114		A/Mallard/Switzerland/WV1090023/2009_H10	GenBank	HM179251	
A/Sentinel_mallard/Germany/Sum156/2007_H6N5	GISAID	EPI397610	EPI397609	A/Migratory_duck/Jiangxi/10857/2005_H10N2	GenBank	KP287998	KP288000
A/Duck/Germany-NW/R2185/2006_H6N8	GISAID	EPI397606		A/Migratory_duck/Jiangxi/10974/2005_H10N6	GenBank	KP287822	
A/Environment/California/NWRC183200-06/2006_H6N8	GISAID	EPI406098		A/Migratory_duck/Jiangxi/21248/2009_H10N8	GenBank	KP287958	
A/Goose/Germany/R1767/2007_H6N8	GISAID	EPI416259		A/Migratory_duck/Jiangxi/30246/2013_H10N5	GenBank	KP284877	KP284879
A/Mallard/Germany-BY/R1353/2010_H6N8	GISAID	EPI339180		A/Migratory_duck/Jiangxi/33038/2013_H10N7	GenBank	KP285893	KP285895
A/Mule_duck/Bulgaria/365/2010_H6N8	GISAID	EPI574207		A/Migratory_duck/Jiangxi/33158/2013_H10N7	GenBank	KP285901	

Viral Name	Source	Accession number HA segment	Accession number NA segment	Viral Name	Source	Accession number HA segment	Accession number NA segment
A/Mute_swan/Germany/R2927/2007_H6N8	GISAID	EPI185339		A/Migratory_duck/Jiangxi/33238/2013_H10N7	GenBank	KP285917	
A/Pink-footed_goose/Iceland/0987/2011_H6N8	GISAID	EPI476116		A/Migratory_duck/Jiangxi/593/2005_H10N8	GenBank	KP287934	
A/Mule_duck/Bulgaria/175/2009_H6	GISAID	EPI574252		A/Migratory_duck/Jiangxi/6847/2003_H10N5	GenBank	KP288014	KP288016
A/Mule_duck/Bulgaria/181/2010_H6	GISAID	EPI574247		A/Migratory_duck/Jiangxi/9334/2005_H10N6	GenBank	KP287814	
A/Mallard/Netherlands/78/2006_H6N8	GenBank	KR862436		A/Migratory_duck/Jiangxi/9492/2005_H10N6	GenBank	KP288006	
A/Eurasian_wigeon/Netherlands/1/2006_H6N2	GenBank	KR862437	KR862610	A/Ostrich/SouthAfrica/2001_H10N1	GenBank	GQ247860	
A/Mallard/Netherlands/40/2006_H6N8	GenBank	KR862438		A/Pekin_duck/South_Africa/AI1642/2009_H10N7	GenBank	GQ404728	
A/White-fronted_goose/Netherlands/3/2007_H6N5	GenBank	KR862439	KR862694	A/Ruddy_shelduck/Mongolia/1602/2010_H10N8	GenBank	KF501079	
A/Mallard/Netherlands/5/2008_H6N8	GenBank	KR862440		A/Ruddy_shelduck/Mongolia/974/2010_H10N7	GenBank	KF501093	KF667728
A/Bewicks_swan/Netherlands/8/2009_H6N8	GenBank	KR862441		A/Surf_scooter/Mongolia/878V/2009_H10N8	GenBank	KF501083	
A/White-fronted_goose/Netherlands/11/2009_H6N1	GenBank	KR862443	KR862556	A/Velvet_scoter/Mongolia/879V/2009_H10N8	GenBank	KF501096	
A/White-fronted_goose/Netherlands/4/2010_H6N8	GenBank	KR862447		A/Wild_bird/Korea/A01/2011_H10N4	GenBank	JN817570	JN817551
A/White-fronted_goose/Netherlands/9/2009_H6N2	GenBank	KR862444	KR862636	A/Wild_bird/Korea/A02/2011_H10N4	GenBank	JN817571	JN817552
A/Barnacle_goose/Netherlands/1/2010_H6N8	GenBank	KR862445		A/Wild_bird/Korea/A12/2010_H10N1	GenBank	JN817574	
A/White-fronted_goose/Netherlands/1/2010_H6N1	GenBank	KR862446	KR862557	A/Wild_bird/Korea/A13/2010_H10N1	GenBank	JN817575	
A/Mallard/Netherlands/2/2004_H6N8	GenBank	KR862428		A/Wild_bird/Korea/L110-2/2008_H10N4	GenBank	JN817580	JN817549
A/Common_teal/Netherlands/4/2005_H6N2	GenBank	KR862430	KR862604	A/Mallard/Netherlands/24/2006_H1N1	GenBank		KR862544
A/White-fronted_goose/Netherlands/1/2006_H6N2	GenBank	KR862431	KR862606	A/Mallard/Netherlands/25/2006_H1N1	GenBank		KR862545
A/Eurasian_wigeon/Netherlands/6/2005_H6N2	GenBank	KR862432	KR862607	A/Mallard/Netherlands/45/2006_H1N1	GenBank		KR862547
A/White-fronted_goose/Netherlands/1/2005_H6N8	GenBank	KR862433		A/Mallard/Netherlands/46/2006_H1N1	GenBank		KR862548
A/White-fronted_goose/Netherlands/3/2006_H6N8	GenBank	KR862434		A/Lesser_black-backed_gull/Netherlands/1/2007_H11N1	GenBank		KR862553
A/White-fronted_goose/Netherlands/2/2008_H6N8	GenBank	KR862455		A/Mallard/Netherlands/24/2009_H6N1	GenBank		KR862555
A/White-fronted_goose/Netherlands/2/2007_H6N8	GenBank	KR862456		A/Mallard/Netherlands/12/2005_H1N1	GenBank		KR862535
A/Mallard/Netherlands/8/2008_H6N1	GenBank	KR862457	KR862563	A/Mallard/Netherlands/14/2005_H1N1	GenBank		KR862536
A/Mallard/Netherlands/42/2008_H6N1	GenBank	KR862459	KR862569	A/Mallard/Netherlands/15/2005_H1N1	GenBank		KR862537
A/Eurasian_wigeon/Netherlands/1/2008_H6N1	GenBank	KR862461	KR862573	A/Brent_goose/Netherlands/1/2006_H1N1	GenBank		KR862541
A/Common_teal/Netherlands/3/2008_H6N1	GenBank	KR862462	KR862575	A/Mallard/Netherlands/83/2008_H7N1	GenBank		KR862561
A/Mallard/Netherlands/31/2008_H6N1	GenBank	KR862460	KR862572	A/Mallard/Netherlands/32/2008_H6N1	GenBank		KR862562
A/Mallard/Netherlands/17/2009_H6N8	GenBank	KR862463		A/Mallard/Netherlands/54/2008_H10N1	GenBank		KR862564
A/Mallard/Netherlands/15/2011_H6N8	GenBank	KR862465		A/Mallard/Netherlands/46/2008_H6N1	GenBank		KR862570
A/Mallard/Netherlands/20/2011_H6N8	GenBank	KR862466		A/Mallard/Netherlands/77/2008_H7N1	GenBank		KR862571
A/White-fronted_goose/Netherlands/6/2009_H6N8	GenBank	KR862470		A/Common_teal/Netherlands/1/2008_H6N1	GenBank		KR862574
A/Mallard/Netherlands/18/2010_H6N8	GenBank	KR862471		A/Mallard/Netherlands/28/2009_H1N1	GenBank		KR862580
A/Chicken/Netherlands/10010413/2010_H6N1	GenBank	pending	pending	A/Mallard/Netherlands/39/2011_N1	GenBank		KR862583
A/Turkey/Netherlands/06001571/2006_H6N5	GenBank	pending	pending	A/Mallard/Netherlands/40/2011_H7N1	GenBank		KR862584
A/American_wigeon/California/8910/2008_H6N1	GenBank	CY094405		A/Mallard/Netherlands/10/2012_H6N1	GenBank		KR862587
A/American_wigeon/California/HKWF371/2007_H6N5	GenBank	CY032704		A/Mallard/Netherlands/24/2012_N1	GenBank		KR862588
A/American_wigeon/California/HKWF42/2007_H6N1	GenBank	CY033420		A/Mallard/Netherlands/8/2012_H1N1	GenBank		KR862589
A/American_wigeon/California/HKWF541/2007_H6N5	GenBank	CY033436		A/Mallard/Netherlands/78/2008_H7N1	GenBank		KR862592
A/Anas_discors/New_Mexico/A00629390/2008_H6N1	GenBank	KF636135		A/Mallard/Netherlands/12/2012_H6N1	GenBank		KR862594
A/Anas_discors/New_Mexico/A00706363/2008_H6N1	GenBank	KF569944		A/Mallard/Netherlands/14/2012_N1	GenBank		KR862599
A/Aquatic_bird/Korea/W69/2005_H6N5	GenBank	CY098532	CY098534	A/White-fronted_goose/Germany-NI/R482/09_H1N1	GISAID	EPI248525	EPI248524
A/Avian/Japan/8KI0135/2008_H6N5	GenBank	CY079243	CY079245	A/White-fronted_goose/Netherlands/1/1999_H6N1	GenBank		CY060433
A/Avian/Japan/8KI0195/2008_H6N8	GenBank	CY079211		A/Domestic_goose/Germany-MV/R3298/2009_H6N1	GISAID		EPI339177
A/Barnacle_goose/Netherlands/1/2005_H6N2	GenBank	CY041386	CY041388	A/Mallard/Italy/4518/2007_H10N1	GISAID		EPI511812

Viral Name	Source	Accession number HA segment	Accession number NA segment	Viral Name	Source	Accession number HA segment	Accession number NA segment
A/Bewicks_swan/Netherlands/1/2005_H6N2	GenBank	DQ822190	DQ822192	A/Duck/Hokkaido/Vac-1/04_H5N1	GenBank		AB259714
A/Bewicks_swan/Netherlands/2/2005_H6N8	GenBank	DQ822198		A/Chicken/Hubei/Wf/2002_H5N1	GenBank		DQ997088
A/Black-headed_gull/Netherlands/1/2005_H6N8	GenBank	CY041378		A/Duck/Guangxi/xa/2001_H5N1	GenBank		DQ997514
A/Blue-winged_teal/Ohio/1386/2005_H6N2	GenBank	CY081380		A/Duck/Zhejiang/bj/2002_H5N1	GenBank		DQ997411
A/Blue-winged_teal/Ohio/1387/2005_H6N2	GenBank	CY021861		A/Chicken/Jilin/hk/2004_H5N1	GenBank		DQ997326
A/Blue-winged_teal/Ohio/1918/2006_H6N5	GenBank	CY095616		A/Teal/Hong_Kong/2978/03_H5N1	GenBank		EF467813
A/Common_gull/Norway/10_1602/2009_H6N8	GenBank	HE802707		A/Goose/Guangdong/3/97_H5N1	GenBank		AF364335
A/Common_teal/Netherlands/2/2005_H6N8	GenBank	CY041370		A/Duck/France/05066b/2005_H5N1	GenBank		AJ972921
A/Duck/Eastern_China/1/2008_H6N1	GenBank	JF965144		A/Duck/Viet_Nam/Ncvd1/2002_H5N1	GenBank		EF541465
A/Duck/Eastern_China/34/2005_H6N1	GenBank	JF965160	JF965335	A/Duck/Hokkaido/Vac-3/2007_H5N1	GenBank		AB355931
A/Duck/Eastern_China/50/2002_H6N8	GenBank	JF965168		A/Duck/Eastern_China/01/2002_H3N1	GenBank		EU429718
A/Duck/Eastern_China/52/2002_H6N2	GenBank	JF965170		A/Duck/Eastern_China/341/2003_H3N1	GenBank		EU429719
A/Duck/Eastern_China/53/2002_H6N2	GenBank	JF965171		A/Duck/Eastern_China/213/2003_H3N1	GenBank		EU429723
A/Duck/Eastern_China/54/2002_H6N2	GenBank	JF965172		A/Duck/Eastern_China/233/2003_H3N1	GenBank		EU429733
A/Duck/Eastern_China/55/2003_H6N2	GenBank	JF965173		A/Duck/Eastern_China/262/2003_H3N1	GenBank		EU429734
A/Duck/Eastern_China/57/2003_H6N2	GenBank	JF965174		A/Duck/Eastern_China/103/2003_H1N1	GenBank		EU429749
A/Duck/Eastern_China/58/2003_H6N2	GenBank	JF965175		A/Duck/Eastern_China/152/2003_H1N1	GenBank		EU429751
A/Duck/Eastern_China/59/2003_H6N2	GenBank	JF965176		A/Duck/Eastern_China/243/2003_H3N1	GenBank		EU429752
A/Duck/Eastern_China/60/2003_H6N2	GenBank	JF965177		A/Duck/Eastern_China/252/2003_H3N1	GenBank		EU429753
A/Duck/Guangxi/1074/2006_H6N2	GenBank	CY109226		A/Duck/Eastern_China/253/2003_H3N1	GenBank		EU429754
A/Duck/Guangxi/1157/2006_H6N2	GenBank	CY109234		A/Duck/Eastern_China/267/2003_H3N1	GenBank		EU429755
A/Duck/Guangxi/1248/2006_H6N2	GenBank	CY109242		A/Duck/Eastern_China/231/2003_H3N1	GenBank		EU429766
A/Duck/Guangxi/141/2005_H6N2	GenBank	HM144510	HM144680	A/Chicken/Shantou/904/2001_H5N1	GenBank		CY029008
A/Duck/Guangxi/1455/2004_H6N5	GenBank	HM144533	HM144703	A/Quail/Shantou/3846/2002_H5N1	GenBank		CY029169
A/Duck/Guangxi/1533/2007_H6N2	GenBank	CY109618		A/Chicken/Hong_Kong/715.5/01_H5N1	GenBank		AF509100
A/Duck/Guangxi/1598/2005_H6N2	GenBank	HM144511	HM144681	A/Duck/Hong_Kong/3461/99_H6N1	GenBank		AJ410564
A/Duck/Guangxi/2140/2006_H6N2	GenBank	CY109250		A/Goose/Hong_Kong/385.3/2000_H5N1	GenBank		AF398421
A/Duck/Guangxi/2736/2006_H6N8	GenBank	CY109258		A/Goose/Hong_Kong/385.5/2000_H5N1	GenBank		AF398422
A/Duck/Guangxi/3333/2006_H6N1	GenBank	CY109266	CY109268	A/Duck/Hokkaido/143/2003_H7N1	GenBank		AB451873
A/Duck/Guangxi/3459/2005_H6N2	GenBank	HM144512		A/Goose/Hong_Kong/Ww26/2000_H5N1	GenBank		AY059483
A/Duck/Guangxi/585/2005_H6N5	GenBank	HM144536	HM144706	A/Goose/Hong_Kong/Ww28/2000_H5N1	GenBank		AY059484
A/Duck/Guizhou/1084/2006_H6N2	GenBank	CY109290	CY109292	A/Duck/Hong_Kong/Ww381/2000_H5N1	GenBank		AY059485
A/Duck/Guizhou/1426/2006_H6N1	GenBank	CY109298	CY109300	A/Duck/Hong_Kong/Ww382/2000_H5N1	GenBank		AY059486
A/Duck/Guizhou/2492/2007_H6N1	GenBank	CY109658	CY109660	A/Duck/Hong_Kong/Ww461/2000_H5N1	GenBank		AY059487
A/Duck/Guizhou/879/2006_H6N1	GenBank	CY109921	CY109922	A/Goose/Hong_Kong/Ww491/2000_H5N1	GenBank		AY059489
A/Duck/Guizhou/888/2006_H6N5	GenBank	CY109282	CY109284	A/Duck/Hong_Kong/2986.1/2000_H5N1	GenBank		AY059490
A/Duck/Hokkaido/120/2001_H6N2	GenBank	AB286875		A/Goose/Hong_Kong/3014.8/2000_H5N1	GenBank		AY059491
A/Duck/Hokkaido/228/2003_H6N8	GenBank	AB294219		A/Mallard/France/2525/2001_H7N1	GenBank		AM157356
A/Duck/Hokkaido/W2/2004_H6N8	GenBank	AB450441		A/Mallard/France/2526/2001_H7N1	GenBank		AM157357
A/Duck/Hunan/1469/2002_H6N8	GenBank	HM144552		A/Mallard/France/691/2002_H1N1	GenBank		AM157358
A/Duck/Hunan/177/2005_H6N1	GenBank	HM144392	HM144562	A/Swan/Mangystau/3/2006_H5N1	GenBank		FJ436943
A/Duck/Hunan/2110/2006_H6N2	GenBank	CY109314		A/Chicken/Hong_Kong/317.5/2001b_H5N1	GenBank		AY075028
A/Duck/Hunan/5613/2003_H6N5	GenBank	HM144532	HM144702	A/Goose/Hong_Kong/3014.5/2000b_H5N1	GenBank		AY075031
A/Duck/Hunan/573/2002_H6N2	GenBank	HM144461		A/Duck/Hong_Kong/380.5/2001_H5N1	GenBank		AY075034
A/Duck/Hunan/748/2005_H6N5	GenBank	HM144537	HM144707	A/Mute_swan/Aktau/1460/2006_H5N1	GenBank		FJ434374
A/Duck/Shantou/7904/2006_H6N2	GenBank	CY109426		A/Duck/Hokkaido/83/2004_H1N1	GenBank		AB470661
A/Duck/Spain/539/2006_H6N8	GenBank	AM706353		A/Duck/Hokkaido/W73/2007_H1N1	GenBank		AB470663
A/Duck/Spain/543/2006_H6N8	GenBank	AM706355		A/Duck/Mongolia/116/2002_H1N1	GenBank		AB470667

Viral Name	Source	Accession number HA segment	Accession number NA segment	Viral Name	Source	Accession number HA segment	Accession number NA segment
A/Duck/Suphanburi/AI157/2005_H6N1	GenBank	JQ711490	JQ711488	A/Duck/Mongolia/253/2003_H1N1	GenBank		AB470668
A/Duck/Taiwan/WB459/04_H6N5	GenBank	DQ376651	DQ376723	A/Duck/Mongolia/540/2001_H1N1	GenBank		AB470669
A/Duck/Tsukuba/561/2006_H6N1	GenBank	AB669136		A/Duck/Mongolia/610/2002_H1N1	GenBank		AB470670
A/Duck/Yunnan/3136/2006_H6N2	GenBank	CY109346	CY109348	A/Duck/Mongolia/867/2002_H7N1	GenBank		AB473545
A/Dunlin/Barrow/65/2005_H6N1	GenBank	EF655836		A/Teal/Viet_Nam/MBP5/2006_H5N1	GenBank		FJ811998
A/Environment/California/NWRC184193-25/2006_H6	GenBank	CY122504		A/Duck/Tsukuba/67/2005_H1N1	GenBank		AB472015
A/Environment/Colorado/NWRC183938-30/2006_H6	GenBank	CY122496		A/Duck/Chiba/884/2004_H3N1	GenBank		AB472013
A/Environment/Delaware/NWRC186237-18/2007_H6	GenBank	CY122532		A/Anas_plathyrhynchos/Spain/1266/2007_H1N1	GenBank		FN386469
A/Environment/Illinois/NWRC183983-24/2006_H6N2	GenBank	CY122500		A/Anas_crecca/Spain/1402/2007_H1N1	GenBank		FN386473
A/Environment/Lousiana/NWRC186275-18/2007_H6	GenBank	CY122535		A/Duck/Korea/334-15/2008_H6N1	GenBank		GQ414900
A/Environment/New_York/23857/2005_H6N8	GenBank	CY095204		A/Duck/Korea/112-25/2008_H6N1	GenBank		GQ414901
A/Environment/New_York/32072-2/2006_H6N8	GenBank	CY095196		A/Spot-billed_duck/Korea/625/2008_H6N1	GenBank		GQ414902
A/Environment/New_York/NWRC183209-12/2006_H6N1	GenBank	CY122484		A/Spot-billed_duck/Korea/545/2008_H6N1	GenBank		GQ414903
A/Eurasian_wigeon/Netherlands/4/2000_H6N2	GenBank	KF695262		A/Spot-billed_duck/Korea/527/2008_H6N1	GenBank		GQ414905
A/Eurasian_wigeon/Sweden/1/2004_H6N2	GenBank	CY041362		A/Spot-billed_duck/Korea/534/2008_H6N1	GenBank		GQ414906
A/Glaucous_gull/Alaska/44198-119/2006_H6N1	GenBank	HM060001		A/Spot-billed_duck/Korea/546/2008_H6N1	GenBank		GQ414907
A/Greylag_goose/Iceland/0911/2011_H6N8	GenBank	CY149468		A/Spot-billed_duck/Korea/536/2008_H6N1	GenBank		GQ414908
A/Greylag_goose/Iceland/0921/2011_H6N8	GenBank	CY149476		A/Spot-billed_duck/Korea/540/2008_H6N1	GenBank		GQ414910
A/Greylag_goose/Iceland/0948/2011_H6N5	GenBank	CY149500	CY149502	A/Mallard/Korea/L08-8/2008_H6N1	GenBank		GQ414904
A/Greylag_goose/Iceland/0961/2011_H6N8	GenBank	CY149516		A/Mallard/Bavaria/47/2006_N1	GenBank		GU046750
A/Greylag_goose/Iceland/0976/2011_H6N8	GenBank	CY149524		A/Mallard/Bavaria/48/2006_N1	GenBank		GU046751
A/Greylag_goose/Iceland/0980/2011_H6N8	GenBank	CY149532		A/Duck/Bavaria/49/2006_H11N1	GenBank		GU046753
A/Greylag_goose/Iceland/1459/2011_H6N5	GenBank	CY149444	CY149446	A/Waterfowl/Hong_Kong/378.5/2001_H5N1	GenBank		GU186694
A/Greylag_goose/Iceland/1474/2011_H6N8	GenBank	CY149452		A/Goose/Hong_Kong/437-6/1999_H5N1	GenBank		GU052021
A/Greylag_goose/Iceland/1482/2011_H6N8	GenBank	CY149460		A/Goose/Hong_Kong/437-8/1999_H5N1	GenBank		GU052029
A/Greylag_goose/Netherlands/4/1999_H6N1	GenBank	CY060198	CY060200	A/Goose/Hong_Kong/485.3/2000_H5N1	GenBank		GU052044
A/Gull/Moscow/3100/2006_H6N2	GenBank	EU152237	EU152239	A/Goose/Hong_Kong/1032.6/2000_H5N1	GenBank		GU052052
A/Mallard_duck/Minnesota/Sg-00107/2007_H6N2	GenBank	CY034673		A/Goose/Hong_Kong/3014.5/2000a_H5N1	GenBank		GU052075
A/Mallard/California/8212/2008_H6N1	GenBank	CY094165		A/Chicken/Hong_Kong/317.5/2001a_H5N1	GenBank		GU052083
A/Mallard/California/8293/2008_H6N1	GenBank	CY094173		A/Hong_Kong/378.1/2001_H5N1	GenBank		GU052091
A/Mallard/Czech_Republic/14924-1/2007_H6N5	GenBank	JF789626	JF789628	A/Goose/Hong_Kong/668.1/2001_H5N1	GenBank		GU052467
A/Mallard/Czech_Republic/15902-17K/2009_H6N2	GenBank	HQ244430		A/Chicken/Hong_Kong/NT873.3/01-MB_H5N1	GenBank		AY221539
A/Mallard/Czech_Republic/15962-1T/2010_H6N9	GenBank	JQ737237		A/Pheasant/Hong_Kong/FY155/01-MB_H5N1	GenBank		AY221543
A/Mallard/Finland/13792/2007_H6N8	GenBank	KF183620		A/Pheasant/Hong_Kong/FY155/01_H5N1	GenBank		AY221544
A/Mallard/Hei_Longjiang/131/2006_H6N2	GenBank	EF634340		A/Chicken/Hong_Kong/FY150/01_H5N1	GenBank		AF509095
A/Mallard/Jiangxi/10071/2005_H6N1	GenBank	HM144393	HM144563	A/Mallard/Norway/10_1671/2007_H1N1	GenBank		FN773074
A/Mallard/Jiangxi/10668/2005_H6N1	GenBank	HM144394	HM144564	A/Mallard/Jiangxi/6845/2003_H6N1	GenBank		HM144559
A/Mallard/Jiangxi/12147/2005_H6N2	GenBank	HM144516		A/Aquatic_bird/India/NIV-17095/2007_H11N1	GenBank		CY055177
A/Mallard/Jiangxi/13228/2005_H6N1	GenBank	HM144395	HM144565	A/Mallard/PT/23059/2007_H1N1	GenBank		HM849011
A/Mallard/Jiangxi/227/2003_H6N1	GenBank	HM144388	HM144558	A/Chicken/Pakistan/NARC-16945/2010_H3N1	GenBank		HQ165998
A/Mallard/Jiangxi/7787/2003_H6N1	GenBank	HM144390	HM144560	A/Mallard/Bavaria/185-26/2008_H1N1	GenBank		HQ259234
A/Mallard/Jiangxi/8264/2004_H6N2	GenBank	HM144489	HM144659	A/Mallard/Netherlands/28/2006_H3N1	GenBank		CY076907
A/Mallard/Jiangxi/8341/2004_H6N5	GenBank	HM144534	HM144704	A/Duck/Korea/GJ74/2007_H3N1	GenBank		JN087234
A/Mallard/Jiangxi/8346/2004_H6N5	GenBank	HM144535	HM144705	A/Duck/Zhejiang/2245/2011_H5N1	GenBank		JN646732

Viral Name	Source	Accession number HA segment	Accession number NA segment	Viral Name	Source	Accession number HA segment	Accession number NA segment
A/Mallard/Maryland/06OS196/2006_H6N2	GenBank	CY190307		A/Lesser_whistling-duck/Thailand/CU-W3947/2010_H12N1	GenBank		JN982529
A/Mallard/Maryland/504/2006_mixed	GenBank	CY081404		A/Lesser_whistling-duck/Thailand/CU-W3946/2010_H12N1	GenBank		JN982521
A/Mallard/Minnesota/Sg-00104/2007_H6N1	GenBank	CY078034		A/Lesser_whistling-duck/Thailand/CU-W3941/2010_H12N1	GenBank		JN982513
A/Mallard/Minnesota/Sg-00105/2007_H6N1	GenBank	CY078042		A/Watercock/Thailand/CU-W3699/2009_H12N1	GenBank		JN982505
A/Mallard/Minnesota/Sg-00106/2007_H6N2	GenBank	CY078273		A/Chicken/England/1415-51184/2010_H9N1	GenBank		JQ609665
A/Mallard/Minnesota/Sg-00167/2007_H6N1	GenBank	CY078114		A/Mallard/Netherlands/10-Nmkt/1999_N1	GenBank		KC209509
A/Mallard/Minnesota/Sg-00214/2007_H6N1	GenBank	CY035387		A/Duck/Hokkaido/W26/2012_H12N1	GenBank		AB780370
A/Mallard/Minnesota/Sg-00223/2007_H6N1	GenBank	CY078249		A/Duck/Fujian/17/2001_H5N1	GenBank		AY585401
A/Mallard/Netherlands/11/2007_H6N5	GenBank	CY041402	CY041404	A/Duck/Guangdong/01/2001_H5N1	GenBank		AY585403
A/Mallard/Netherlands/16/99_H6N5	GenBank	AY684892		A/Duck/Guangdong/07/2000_H5N1	GenBank		AY585404
A/Mallard/Netherlands/71/2006_H6N2	GenBank	CY041394	CY041396	A/Duck/Guangdong/40/2000_H5N1	GenBank		AY585407
A/Mallard/Ontario/15915/2005_H6N5	GenBank	CY095323		A/Duck/Guangxi/35/2001_H5N1	GenBank		AY585410
A/Mallard/Republic_of_Georgia/13/2011_H6N2	GenBank	CY185577	CY185579	A/Duck/Shanghai/13/2001_H5N1	GenBank		AY585414
A/Mallard/SanJiang/113/2006_H6N2	GenBank	EU094473	EU094475	A/Duck/Zhejiang/52/2000_H5N1	GenBank		AY585419
A/Mallard/SanJiang/151/2006_H6N2	GenBank	EF592495		A/Turkey/Italy/604/2000_H7N1	GenBank		KF493262
A/Mallard/Sweden/3463/2003_H6N2	GenBank	CY186267	CY186269	A/European_teal/Novosibirsk/203/2011_H5N1	GenBank		KF462363
A/Mallard/Sweden/51984/2006_mixed	GenBank	CY164804	CY164807	A/European_teal/Novosibirsk/239/2011_H5N1	GenBank		KF462365
A/Mallard/Sweden/52/2003_H6N2	GenBank	CY060316	CY060318	A/European_teal/Novosibirsk/261/2011_H5N1	GenBank		KF462367
A/Mallard/Sweden/52593/2006_mixed	GenBank	CY164993	CY164996	A/Swine/Fujian/F1/2001_H5N1	GenBank		AY747618
A/Mallard/Sweden/54/2003_H6N1	GenBank	KF695223	KF695225	A/Common_goldeneye/Mongolia/1271/2010_H7N1	GenBank		KF667689
A/Mallard/Sweden/99817/2009_H6N2	GenBank	JX565991	JX566175	A/Mallard/Mongolia/1551/2010_H3N1	GenBank		KF667697
A/Mallard/Sweden/99847/2009_H6N2	GenBank	JX566005		A/Ruddy_shelduck/Mongolia/921C2/2009_H7N1	GenBank		KF667727
A/Mallard/Sweden/99850/2009_H6N2	GenBank	JX566006		A/Swine/Fujian/1/2003_H5N1	GenBank		AY747610
A/Mallard/Sweden/99854/2009_H6N2	GenBank	JX566010		A/Goose/Guangdong/1/96_H5N1	GenBank		AF144304
A/Mallard/Sweden/99934/2009_H6N2	GenBank	JX566024		A/Mallard/Sweden/52405/2006_N1	GenBank		CY164158
A/Mallard/Sweden/99966/2009_H6N2	GenBank	JX566028		A/Duck/Thailand/CU-13011C/2013_N1	GenBank		KJ525983
A/Mallard/Sweden/99983/2009_H6N2	GenBank	JX566029		A/Aquatic_Bird/Hong_Kong/m603/98_H11N1	GenBank		AF098551
A/Mallard/Sweden/99985/2009_H6N2	GenBank	JX566030		A/Mallard/Sweden/4737/2004_N1	GenBank		CY183476
A/Mallard/Sweden/99999/2009_H6N2	GenBank	JX566032	JX566216	A/Black-headed_gull/Republic_of_Georgia/1/2010_H11N1	GenBank		CY185411
A/Mallard/Wisconsin/1534/2009_H6N8	GenBank	CY097277		A/Black-headed_gull/Republic_of_Georgia/2/2011_H9N1	GenBank		CY185523
A/Muscovy_duck/France/09010/2009_H6N1	GenBank	JN860172	JN860174	A/Ruddy_turnstone/Iceland/2899/2013_H5N1	GenBank		KM213392
A/Northern_pintail/Alaska/44202-143/2006_H6N1	GenBank	EU557517		A/Chicken/Taiwan/0824/97_H6N1	GenBank		DQ376693
A/Northern_pintail/Alaska/44203-078/2006_H6N8	GenBank	EU557519		A/Chicken/Taiwan/na3/98_H6N1	GenBank		DQ376694
A/Northern_pintail/Alaska/44204-158/2006_H6N4	GenBank	EU557520		A/Chicken/Taiwan/165/99_H6N1	GenBank		DQ376698
A/Northern_shoveler/California/9187/2008_H6N2	GenBank	CY094309		A/Duck/Taiwan/WB29/99_H6N1	GenBank		DQ376699
A/Northern_shoveler/California/K138/2005_H6N2	GenBank	CY045343		A/Duck/Taiwan/A68/03_H6N1	GenBank		DQ376718
A/Pintail/Alberta/87/1993_H6N8	GenBank	CY127024		A/Swine/Fujian/2001_H5N1	GenBank		DQ432038
A/Ring-necked_duck/California/K90/2005_H6N8	GenBank	CY043808		A/Pekin_Duck/France/M-2060/01_H1N1	GenBank		AJ697876
A/Shorebird/Delaware_Bay/13/2004_H6N8	GenBank	CY127726		A/Environment/Hong_Kong/437-4/99_H5N1	GenBank		AF216714
A/Shorebird/Delaware_Bay/604/2008_H6N2	GenBank	CY127831		A/Environment/Hong_Kong/437-10/99_H5N1	GenBank		AF216738
A/Shorebird/Delaware_Bay/65/2004_H6N8	GenBank	CY127734		A/Mallard/Netherlands/85/2006_N2	GenBank		KR862609
A/Shorebird/Delaware_Bay/707/2009_H6N2	GenBank	CY127895		A/Mallard/Netherlands/12/2006_H5N2	GenBank		KR862611
A/Teal/Norway/10_476/2005_H6N2	GenBank	FM179757		A/Mallard/Netherlands/43/2006_H5N2	GenBank		KR862612
A/Turkey/France/09010-1/2009_H6N1	GenBank	JN860180		A/Mallard/Netherlands/58/2006_H4N2	GenBank		KR862614
A/Turkey/France/10-040/2010_H6N1	GenBank	JQ990779	JQ990781	A/Mallard/Netherlands/63/2006_H5N2	GenBank		KR862615

Viral Name	Source	Accession number HA segment	Accession number NA segment	Viral Name	Source	Accession number HA segment	Accession number NA segment
A/Whitefronted_goose/Netherlands/1/1999_H6N1	GenBank	CY060431		A/Bewicks_swan/Netherlands/4/2006_H9N2	GenBank		KR862616
A/Whitefronted_goose/Netherlands/2/1999_H6N2	GenBank	CY060439		A/Mallard/Netherlands/85/2008_N2	GenBank		KR862617
A/Wild_duck/Jiangxi/8462/2006_H6N1	GenBank	CY109338	CY109340	A/White-fronted_goose/Netherlands/8/2009_H6N2	GenBank		KR862635
A/Chicken/Netherlands/11004875/2011_H7N1	GISAID	pending	EPI316304	A/Bewicks_swan/Netherlands/1/2010_H5N2	GenBank		KR862637
A/Duck/Turkey/55/Cetinkaya/49/2006_H7N1	GISAID	EPI346007		A/White-fronted_goose/Netherlands/6/2010_H6N2	GenBank		KR862639
A/Guinea_fowl/Italy/407/2008_H7N1	GISAID	EPI210104		A/White-fronted_goose/Netherlands/7/2011_N2	GenBank		KR862642
A/Mallard/Denmark/58-62-KLSV-119/09_H7N1	GISAID	EPI492308		A/Mallard/Netherlands/13/2005_H6N2	GenBank		KR862600
A/Mallard/Italy/3397-65/2008_H7N1	GISAID	EPI167297		A/Eurasian_wigeon/Netherlands/2/2005_H1N2	GenBank		KR862601
A/Mallard/Italy/6103-5/2007_H7N1	GISAID	EPI167296		A/Eurasian_wigeon/Netherlands/5/2005_N2	GenBank		KR862603
A/Mallard/Italy/731/09_H7N1	GISAID	EPI492522		A/Mallard/Netherlands/19/2005_H3N2	GenBank		KR862605
A/Mallard/Italy/794-18/2008_H7N1	GISAID	EPI167299		A/Mallard/Netherlands/19/2007_H6N2	GenBank		KR862643
A/Shoveler/Egypt/00597-NAMRU3/2004_H7N1	GISAID	EPI372283	EPI372282	A/Mallard/Netherlands/3/2008_H6N2	GenBank		KR862644
A/Shoveler/Egypt/14879-NAMRU3/2006_H7N1	GISAID	EPI372363	EPI372362	A/Mallard/Netherlands/5/2009_H5N2	GenBank		KR862645
A/Tadorna_tadorna/Belgium/3441-P3/2009_H7N1	GISAID	EPI360900	EPI360901	A/Mallard/Netherlands/43/2008_H3N2	GenBank		KR862646
A/Teal/Italy/794-3/2008_H7N1	GISAID	EPI167298		A/Mallard/Netherlands/44/2008_H3N2	GenBank		KR862647
A/Chicken/Italy/2240/2003_H7N3	GISAID	EPI154960		A/Mallard/Netherlands/45/2008_H4N2	GenBank		KR862648
A/Chicken/Italy/2837-54/2007_H7N3	GISAID	EPI154980		A/Bewicks_swan/Netherlands/5/2008_H5N2	GenBank		KR862651
A/Chicken/Italy/2837-58/2007_H7N3	GISAID	EPI154981		A/White-fronted_goose/Netherlands/5/2008_H5N2	GenBank		KR862652
A/Chicken/Italy/8093/2002_H7N3	GISAID	EPI154966		A/White-fronted_goose/Netherlands/4/2009_H5N2	GenBank		KR862653
A/Guinea_fowl/Italy/1613/2003_H7N3	GISAID	EPI154959		A/Mallard/Netherlands/18/2009_H6N2	GenBank		KR862654
A/Mallard/Italy/1336/07_H7N3	GISAID	EPI167295		A/Eurasian_wigeon/Netherlands/1/2009_H5N2	GenBank		KR862655
A/Mallard/Italy/6103-12/2007_H7N3	GISAID	EPI154982		A/Black-headed_gull/Netherlands/8/2010_H13N2	GenBank		KR862656
A/Mallard/Italy/6104-14/2007_H7N3	GISAID	EPI167300		A/Mallard/Netherlands/28/2010_H5N2	GenBank		KR862657
A/Shoveler/Egypt/00017-NAMRU3/2007_H7N3	GISAID	EPI372450		A/Mallard/Netherlands/34/2010_H3N2	GenBank		KR862658
A/Shoveler/Egypt/00241-NAMRU3/2007_H7N3	GISAID	EPI372418		A/Mallard/Netherlands/13/2011_H3N2	GenBank		KR862659
A/Turkey/Italy/2963/2003_H7N3	GISAID	EPI243279		A/Eurasian_wigeon/Netherlands/1/2011_N2	GenBank		KR862660
A/Turkey/Italy/8307/2002_H7N3	GISAID	EPI154967		A/Mallard/Netherlands/17/2012_H3N2	GenBank		KR862663
A/Swan/Germany/R736/06_H7N4	GISAID	EPI492517		A/Mallard/Netherlands/27/2012_N2	GenBank		KR862665
A/Teal/Italy/11VIR-792/11_H7N6	GISAID	EPI492520		A/Mallard/Netherlands/15/2012_H3N2	GenBank		KR862664
A/Branta_canadensis/Belgium/13000-9-2/2010_H7N7	GISAID	EPI360902		A/Mallard/Netherlands/27/2008_H3N2	GenBank		KR862667
A/Chicken/Germany-NI/R874/2010_H7N7	GISAID	EPI302178		A/Mallard/Netherlands/50/2008_H3N2	GenBank		KR862668
A/Chicken/Germany/R1362/11_H7N7	GISAID	EPI492511		A/Mallard/Netherlands/52/2008_H5N2	GenBank		KR862674
A/Chicken/Netherlands/11008327/2011_H7N7	GenBank	pending	pending	A/White-fronted_goose/Netherlands/6/2008_H5N2	GenBank		KR862675
A/Chicken/Netherlands/11011326/2011_H7N7	GISAID	pending	EPI325342	A/Eurasian_wigeon/Netherlands/1/2010_H5N2	GenBank		KR862676
A/Chicken/Netherlands/12014794/2012_H7N7	GISAID	EPI390921	EPI390922	A/Mallard/Netherlands/55/2010_H3N2	GenBank		KR862677
A/Egyptian_goose/Egypt/05588-NAMRU3/2006_H7N7	GISAID	EPI372394	EPI372393	A/Mallard/Netherlands/18/2012_H4N2	GenBank		KR862680
A/Mallard/Denmark/303878-1S/13_H7N7	GISAID	EPI492307		A/Wild_duck/Germany/WV2555/2006_H3N2	GISAID		EPI185342
A/Mallard/Italy/11VIR-540/11_H7N7	GISAID	EPI492519		A/Pheasant/Ireland/PV12-010728/12_H5N2	GISAID		EPI375596
A/Mallard/Poland/01/08_H7N7	GISAID	EPI169422	EPI169423	A/Chicken/Italy/11VIR-7548/2011_H5N2	GISAID		EPI464929
A/Mallard/Poland/41/09_H7N7	GISAID	EPI211188		A/Turkey/Italy/12VIR-6607-5/2012_H5N2	GISAID		EPI464937
A/Mallard/Poland/446/09_H7N7	GISAID	EPI254381	EPI254382	A/Chicken/Italy/12VIR-7785-67/2012_H5N2	GISAID		EPI464945
A/Pochard/Germany/R916/06_H7N7	GISAID	EPI492516		A/Turkey/Italy/12VIR-8036-2/2012_H5N2	GISAID		EPI464953
A/Shoveler/Egypt/09864-NAMRU3/2004_H7N7	GISAID	EPI372323	EPI372322	A/Mule_duck/Bulgaria/61/2010_mixed	GISAID		EPI574173
A/Swan/Germany/R57/06_H7N7	GISAID	EPI492518		A/Mule_duck/Bulgaria/64/2010_mixed	GISAID		EPI574211
A/Teal/Egypt/00835-NAMRU3/2004_H7N7	GISAID	EPI372307		A/Mule_duck/Bulgaria/369/2009_H4N2	GISAID		EPI574213
A/Turkey/Germany-NW/R655/2009_H7N7	GISAID	EPI356351		A/Mule_duck/Bulgaria/105/2008_mixed	GISAID		EPI574258

Viral Name	Source	Accession number HA segment	Accession number NA segment	Viral Name	Source	Accession number HA segment	Accession number NA segment
A/Shoveler/Egypt/00215-NAMRU3/2007_H7N9	GISAID	EPI372410		A/Mule_duck/Bulgaria/174/2009_H6N2	GISAID		EPI574265
A/Mallard/Netherlands/82/2008_H7N7	GenBank	KR862473	KR862709	A/Mule_duck/Bulgaria/596/2010_H4N2	GISAID		EPI574276
A/Bewicks_swan/Netherlands/7/2008_H7N1	GenBank	KR862474	KR862554	A/Duck/Hong_Kong/301/1978_H7N2	GenBank		AB302790
A/Mallard/Lithuania/EMC-2/2010_H7N2	GenBank	KR862374		A/Korea/KBNP-0028/2000_H9N2	GenBank		EF620902
A/Mallard/Netherlands/60/2008_H7N1	GenBank	KR862477	KR862566	A/Garganey/SanJiang/160/2006_H5N2	GenBank		EF634334
A/Mallard/Netherlands/61/2008_H7N1	GenBank		KR862567	A/Duck/Jiang_Xi/1286/2005_H5N2	GenBank		EF597303
A/Mallard/Netherlands/2/2009_H7N7	GenBank	KR862479	KR862717	A/Duck/Jiang_Xi/3345/2005_H5N2	GenBank		EF597310
A/Mallard/Netherlands/4/2009_H7N7	GenBank	KR862481	KR862719	A/Duck/Eastern_China/164/2002_H6N2	GenBank		EU429762
A/Mallard/Netherlands/6/2009_H7N7	GenBank	KR862482	KR862720	A/Swine/Korea/C13/2008_H5N2	GenBank		FJ461597
A/Eurasian_wigeon/Netherlands/2/2008_H7N4	GenBank	KR862485	KR862692	A/Duck/Primorie/2621/2001_H5N2	GenBank		GQ162788
A/Bean_goose/Netherlands/1/2009_H7N1	GenBank	KR862487	KR862576	A/Spotbill_duck/Xuyi/18/2005_H5N2	GenBank		GQ184331
A/Mallard/Netherlands/2/2010_H7N3	GenBank	KR862488		A/Spotbill_duck/Xuyi/6/2005_H11N2	GenBank		GQ184332
A/Mallard/Netherlands/3/2010_H7N3	GenBank	KR862489		A/Mallard/Xuyi/10/2005_H5N2	GenBank		GQ184334
A/Mallard/Netherlands/1/2011_H7N7	GenBank	KR862490	KR862726	A/Duck/Tsukuba/9/2005_H2N2	GenBank		AB472017
A/Mallard/Netherlands/43/2011_H7N1	GenBank	KR862491		A/Mallard/Netherlands/2/2005_H4N2	GenBank		CY041252
A/Northern_shoveler/Egypt/EMC-1/2012_H7N1	GenBank	KR862376	KR862391	A/Mallard/Netherlands/26/2005_H11N2	GenBank		CY041420
A/Mallard/Netherlands/5/2010_H7N3	GenBank	KR862500		A/Duck/Shimane/19/2006_H5N2	GenBank		AB472053
A/Turkey/Netherlands/11011530/2011_H7N7	GenBank	pending	pending	A/Duck/Niigata/477/2007_H5N2	GenBank		AB472055
A/Chicken/Netherlands/10007882/2010_H7N4	GenBank	pending	pending	A/Wild_bird/Korea/A81/2009_H5N2	GenBank		GU086246
A/Anas_crecca/Spain/1460/2008_H7N9	GenBank	HQ244407		A/Duck/Korea/A14/2008_H5N2	GenBank		GU086248
A/Anas_platyrhynchos/Spain/1877/2009_H7N2	GenBank	KP636486		A/Duck/Korea/A93/2008_H5N2	GenBank		GU086249
A/Chicken/England/1158-11406/2008_H7N7	GenBank	FJ476173		A/Gadwall/Altai/1202/2007_H5N2	GenBank		CY049758
A/Chicken/England/4054/2006_H7N3	GenBank	EF467826		A/Mallard/Netherlands/3/1999_H5N2	GenBank		GU052558
A/Chicken/England/4266/2006_H7N3	GenBank	EF467825		A/Mallard/Sweden/7/2002_H5N2	GenBank		GU052566
A/Chicken/Italy/1067/99_H7N1	GenBank	AJ584647		A/Chicken/France/03426/2003_H5N2	GenBank		CY046126
A/Chicken/Italy/1082/1999_H7N1	GenBank	CY022677	CY022679	A/Duck/France/080032/2008_H5N2	GenBank		CY046176
A/Chicken/Italy/1279/1999_H7N1	GenBank	CY099597		A/Aquatic_bird/Korea/W96/2005_H5N2	GenBank		GU361236
A/Chicken/Italy/12rs206-19/1999_H7N1	GenBank	KF493039		A/Aquatic_bird/Korea/W113/2006_H5N2	GenBank		GU361237
A/Chicken/Italy/12rs206-3/1999_H7N1	GenBank	KF492993		A/Aquatic_bird/Korea/W114/2006_H5N2	GenBank		GU361238
A/Chicken/Italy/12rs206-4/1999_H7N1	GenBank	KF493027		A/Aquatic_bird/Korea/W121/2006_H5N2	GenBank		GU361240
A/Chicken/Italy/12rs206-5/1999_H7N1	GenBank	KF493029		A/Aquatic_bird/Korea/W125/2006_H5N2	GenBank		GU361241
A/Chicken/Italy/1391/1999_H7N1	GenBank	CY095514		A/Aquatic_bird/Korea/W163/2007_H5N2	GenBank		GU361244
A/Chicken/Italy/13VIR4527-11/2013_H7N7	GISAID	EPI677999	EPI677998	A/Aquatic_bird/Korea/W216/2007_H5N2	GenBank		GU361256
A/Chicken/Italy/270638/02_H7N3	GenBank	EU158111		A/Aquatic_bird/Korea/W230/2007_H5N2	GenBank		GU361262
A/Chicken/Italy/4616/2003_H7N3	GenBank	CY095522		A/Aquatic_bird/Korea/W234/2007_H5N2	GenBank		GU361264
A/Chicken/Italy/682/2003_H7N3	GenBank	CY034750		A/Aquatic_bird/Korea/W344/2008_H5N2	GenBank		GU361267
A/Chicken/Netherlands/06022003/2006_H7N7	GenBank	pending	pending	A/Mallard/Sweden/4/2002_H10N2	GenBank		CY060302
A/Chicken/Wales/1306/2007_H7N2	GenBank	EF675618		A/Teal/Norway/10_1360/2007_H4N2	GenBank		FN773069
A/Duck/Denmark/53-147-8/2008_H7N1	GenBank	GQ401157	GQ401158	A/Duck/Fujian/11339/2005_H6N2	GenBank		HM144700
A/Duck/Italy/4609/2003_H7N2	GenBank	CY031028		A/Duck/Fujian/8719/2005_H6N2	GenBank		HM144699
A/Duck/Italy/4692-9/2004_H7	GenBank	CY095570		A/Duck/Fujian/7033/2005_H6N2	GenBank		HM144698
A/Duck/Mongolia/47/2001_H7N1	GenBank	AB268557	AB302788	A/Duck/Fujian/5643/2005_H6N2	GenBank		HM144697
A/Duck/Mongolia/720/2007_H7N6	GenBank	AB450448		A/Duck/Fujian/5426/2005_H6N2	GenBank		HM144696
A/Duck/Yunnan/87/2007_H7N6	GenBank	KF258991		A/Duck/Fujian/5117/2005_H6N2	GenBank		HM144695
A/Garganey/Crimea/2027/2008_H7N8	GenBank	GU228596		A/Duck/Fujian/4125/2005_H6N2	GenBank		HM144694
A/Goose/Czech_Republic/1848-K9/2009_H7N9	GenBank	GU060482		A/Duck/Fujian/3937/2005_H6N2	GenBank		HM144693
A/Guinea_fowl/Italy/266184/02_H7N3	GenBank	EU158110		A/Duck/Fujian/3701/2005_H6N2	GenBank		HM144692
A/Italy/3/2013_H7N7	GenBank	KF918337	KF918339	A/Duck/Fujian/3193/2005_H6N2	GenBank		HM144691

Viral Name	Source	Accession number HA segment	Accession number NA segment	Viral Name	Source	Accession number HA segment	Accession number NA segment
A/Mallard/Italy/199/01_H7N3	GenBank	EU158109		A/Duck/Fujian/1695/2005_H6N2	GenBank		HM144689
A/Mallard/Italy/250/02_H7N1	GenBank	EU158105		A/Duck/Fujian/629/2005_H6N2	GenBank		HM144688
A/Mallard/Italy/299/05_H7N7	GenBank	EU158104		A/Duck/Fujian/420/2005_H6N2	GenBank		HM144687
A/Mallard/Italy/43/01_H7N3	GenBank	AY586410		A/Duck/Shantou/22596/2005_H6N2	GenBank		HM144679
A/Mallard/Netherlands/12/2000_H7N3	GenBank	KF695239		A/Mallard/Shantou/198/2005_H6N2	GenBank		HM144661
A/Mallard/Netherlands/22/2007_H7N1	GenBank	CY043840	CY043842	A/Wild_duck/Shantou/180/2005_H6N2	GenBank		HM144660
A/Mallard/Netherlands/29/2006_H7N2	GenBank	CY043832	CY043834	A/Wild_duck/Shantou/7900/2004_H6N2	GenBank		HM144658
A/Mallard/Netherlands/33/2006_H7N8	GenBank	CY041410		A/Wild_duck/Shantou/7307/2004_H6N2	GenBank		HM144656
A/Mallard/Netherlands/9/2005_H7N7	GenBank	CY077008	CY077010	A/Wild_duck/Shantou/3433/2003_H6N2	GenBank		HM144645
A/Mallard/Republic_of_Georgia/2/2010_H7N7	GenBank	CY185425	CY185427	A/Wild_duck/Shantou/2853/2003_H6N2	GenBank		HM144642
A/Mallard/Republic_of_Georgia/3/2010_H7N3	GenBank	CY185433		A/Mallard/Netherlands/7/2007_H4N2	GenBank		CY076923
A/Mallard/Sweden/100993/2008_H7N7	GenBank	FJ803198	FJ803196	A/Mallard/Sweden/74/2003_H5N2	GenBank		CY076931
A/Mallard/Sweden/1985/2003_H7N7	GenBank	CY183336	CY183338	A/Herring_gull/Atyrau/2186/2007_H11N2	GenBank		HQ541743
A/Mallard/Sweden/2051/2003_H7N7	GenBank	CY184568		A/Swine/KU/16/2001_H7N2	GenBank		CY067688
A/Mallard/Sweden/3269/2003_H7N7	GenBank	CY184576	CY184578	A/Duck/France/05057a/2005_H6N2	GenBank		AM489442
A/Mallard/Sweden/5994/2005_H7N7	GenBank	CY183409	CY183411	A/Spur-winged_goose/Nigeria/226/2008_H5N2	GenBank		FR771826
A/Mallard/Sweden/S90597/2005_H7N7	GenBank	FJ803182	FJ803175	A/Spur-winged_goose/Nigeria/210/2008_H5N2	GenBank		FR771827
A/Mute_swan/Hungary/5973/2007_H7N7	GenBank	GQ240813	GQ240815	A/Spur-winged_goose/Nigeria/2/2008_H5N2	GenBank		FR771828
A/Quail/Italy/3347/2004_H7N3	GenBank	CY020613		A/Avian/Japan/8KI0148/2008_H4N2	GenBank		CY088723
A/Quail/Italy/4610/2003_H7N2	GenBank	CY021509		A/Ostrich/South_Africa/9508103/95_H9N2	GenBank		AF508575
A/Shoveler/Italy/2698-27/2006_H7N7	GenBank	CY095600		A/Chicken/Korea/99029/99_H9N2	GenBank		AF508582
A/Shoveler/Italy/2698-3/2006_H7N7	GenBank	CY095592		A/Chicken/Eastern_China/43/2007_H6N2	GenBank		JF965302
A/Swan/Czech_Republic/5416/2011_H7N7	GenBank	JN966905	JN966907	A/Teal/Norway/10_1037/2010_H3N2	GenBank		FR873768
A/Swan/Slovenia/53/2009_H7N7	GenBank	HQ283357	HQ283359	A/Mallard/Norway/10_1368/2010_H6N2	GenBank		FR873773
A/Teal/Finland/9201/2010_H7N3	GenBank	KF183621		A/Avian/Israel/289/2001_H6N2	GenBank		JN575028
A/Tufted_duck/PT/13771/2006_H7N3	GenBank	HM849003		A/Chicken/Korea/KNUSWR09/2009_H9N2	GenBank		JN852797
A/Turkey/Germany/R11/2001_H7N7	GenBank	CY107856		A/Duck/Jiangxi/5748/2006_H6N2	GenBank		CY109332
A/Turkey/Italy/1010/2003_H7N3	GenBank	CY021365		A/Mallard/Netherlands/14/2007_H2N2	GenBank		CY121977
A/Turkey/Italy/1067/1999_H7N1	GenBank	CY095506		A/Mallard/Sweden/58112/2006_H2N2	GenBank		CY121929
A/Turkey/Italy/1083/1999_H7N1	GenBank	KF493003		A/Mallard/Sweden/58451/2006_H2N2	GenBank		CY121937
A/Turkey/Italy/1086/1999_H7N1	GenBank	KF493012		A/Mallard/Sweden/68735/2007_H2N2	GenBank		CY121945
A/Turkey/Italy/121964/03_H7N3	GenBank	EU158106		A/White-fronted_goose/Netherlands/22/1999_H2N2	GenBank		CY121961
A/Turkey/Italy/1265/1999_H7N1	GenBank	CY025189		A/Mallard/Sweden/99858/2009_H6N2	GenBank		JX566198
A/Turkey/Italy/12rs206-1/1999_H7N1	GenBank	KF492991		A/Mallard/Sweden/99843/2009_H6N2	GenBank		JX566185
A/Turkey/Italy/12rs206-11/1999_H7N1	GenBank	KF493032		A/Mallard/Sweden/99820/2009_H11N2	GenBank		JX566177
A/Turkey/Italy/12rs206-13/1999_H7N1	GenBank	KF493033		A/Mallard/Sweden/100537/2009_H6N2	GenBank		JX566222
A/Turkey/Italy/12rs206-14/1999_H7N1	GenBank	KF493037		A/Duck/Japan/9UO036/2009_H5N2	GenBank		JX673923
A/Turkey/Italy/12rs206-17/1999_H7N1	GenBank	KF493038		A/Duck/Japan/9UO139/2009_H5N2	GenBank		JX673929
A/Turkey/Italy/12rs206-18/1999_H7N1	GenBank	KF492992		A/Duck/Japan/9UO025/2009_H5N2	GenBank		JX673936
A/Turkey/Italy/12rs206-20/1999_H7N1	GenBank	KF493040		A/Duck/Jiangsu/26/2004_H3N2	GenBank		KC261671
A/Turkey/Italy/12rs206-6/1999_H7N1	GenBank	KF493031		A/Mallard/Finland/13748/2007_H5N2	GenBank		KF183617
A/Turkey/Italy/1555/1999_H7N1	GenBank	KF493042		A/Mallard/Sweden/21/2002_H5N2	GenBank		KF695272
A/Turkey/Italy/1744/1999_H7N1	GenBank	KF493043		A/Chicken/New_Jersey/251-4/2008_H5N2	GenBank		KJ018202
A/Turkey/Italy/2043/2003_H7N3	GenBank	CY022613		A/Mallard/Sweden/274/2002_H4N2	GenBank		CY164218
A/Turkey/Italy/214845/2002_H7N3	GenBank	AJ627491		A/Mallard/Sweden/906/2002_H4N2	GenBank		CY164258
A/Turkey/Italy/2379/2000_H7N1	GenBank	GU053007		A/Mallard/Sweden/1195/2002_H4N2	GenBank		CY164274
A/Turkey/Italy/251/2003_H7N3	GenBank	CY020589		A/Mallard/Sweden/58359/2006_mixed	GenBank		CY165021
A/Turkey/Italy/2684/2003_H7N3	GenBank	CY095554		A/Mallard/Sweden/58463/2006_mixed	GenBank		CY165049

Viral Name	Source	Accession number HA segment	Accession number NA segment
A/Turkey/Italy/2715/1999_H7N1	GenBank	CY025173	
A/Turkey/Italy/2716/1999_H7N1	GenBank	KF493058	
A/Turkey/Italy/2732/1999_H7N1	GenBank	GU052969	
A/Turkey/Italy/2962/2003_H7N3	GenBank	JX515663	
A/Turkey/Italy/2987/2003_H7N3	GenBank	CY021493	
A/Turkey/Italy/3283/1999_H7N1	GenBank	GU052976	
A/Turkey/Italy/3337/2004_H7N3	GenBank	CY021501	
A/Turkey/Italy/3620/2003_H7N3	GenBank	CY021357	
A/Turkey/Italy/3807/2004_H7N3	GenBank	CY020597	
A/Turkey/Italy/3829/2004_H7N3	GenBank	CY028676	
A/Turkey/Italy/3889/99_H7N1	GenBank	AJ493466	
A/Turkey/Italy/4130/2004_H7N3	GenBank	CY029913	
A/Turkey/Italy/4169/1999_H7N1	GenBank	CY006037	
A/Turkey/Italy/4372/2004_H7N3	GenBank	CY095538	
A/Turkey/Italy/4479/2004_H7N3	GenBank	CY020581	
A/Turkey/Italy/4603/99_H7N1	GenBank	AJ493471	
A/Turkey/Italy/4608/2003_H7N3	GenBank	CY021485	
A/Turkey/Italy/68819/03_H7N3	GenBank	EU158100	
A/Turkey/Italy/8000/2002_H7N3	GenBank	CY024738	
A/Turkey/Italy/8458/2002_H7N3	GenBank	CY095562	
A/Turkey/Italy/8534/2002_H7N3	GenBank	CY095530	
A/Turkey/Italy/8912/2002_H7N3	GenBank	CY020605	
A/Turkey/Italy/9739/2002_H7N3	GenBank	CY031611	
A/Turkey/Italy/977/1999_H7N1	GenBank	GU052999	
A/Turkey/Italy12rs206-2/1999_H7N1	GenBank	KF492994	
A/Wild_duck/Mongolia/1-241/2008_H7N9	GenBank	JN029686	
A/Yellow-legged_gull/Republic_of_Georgia/1/2012_mixed	GenBank	CY185372	
A/Northern_shoveler/Mississippi/11OS5900/2011_H8N1	GISAID	EPI512596	
A/American_black_duck/Nova_Scotia/02043/2007_H8N4	GISAID	EPI404484	
A/American_green-winged_teal/California/44287-373/2007_H8N4	GISAID	EPI292438	
A/American_green-winged_teal/Interior_Alaska/9BM5045R0/2009_H8N4	GISAID	EPI433069	EPI433100
A/Chicken/Netherlands/10009401/2010_H8N4	GenBank	pending	pending
A/Environment/Pennsylvania/NWRC182092-24/2006_H8N4	GISAID	EPI406002	
A/Garganey/Ukraine/05835-NAMRU3/2006_H8N4	GISAID	EPI372512	EPI372511
A/Mallard/Interior_Alaska/8BM1966R1/2008_H8N4	GISAID	EPI299970	
A/Mallard/Interior_Alaska/8MP0547/2008_H8N4	GISAID	EPI299411	
A/Mallard/Interior_Alaska/9BM10537R0/2009_H8N4	GISAID	EPI436880	
A/Mallard/Interior_Alaska/9BM8389R0/2009_H8N4	GISAID	EPI452280	EPI452282
A/Mallard/Minnesota/Sg-00675/2008_H8N4	GISAID	EPI188649	
A/Northern_pintail/Interior_Alaska/8BM2011R1/2008_H8N4	GISAID	EPI299978	
A/Northern_pintail/Interior_Alaska/8BM2046R1/2008_H8N4	GISAID	EPI299419	
A/Northern_pintail/Interior_Alaska/8BM2621R1/2008_H8N4	GISAID	EPI299686	
A/Mallard/Sweden/68504/2007_H4N2	GenBank		CY165100
A/Mallard/Netherlands/1/2007_H3N2	GenBank		CY043818
A/Eurasian_wigeon/Netherlands/3/2005_H9N2	GenBank		CY043858
A/Mallard/Sweden/58705/2006_H5N2	GenBank		CY184143
A/Mallard/Sweden/79196/2008_mixed	GenBank		CY184415
A/Migratory_duck/Jiangxi/10861/2005_H10N2	GenBank		KP287808
A/Duck/Nanchang/1749/1992_H11N2	GenBank		CY005532
A/Chicken/Korea/MS96/96_H9N2	GenBank		AF203786
A/Muskrat/Russia/63/2014_H2N2	GenBank		KR052706
A/Duck/Denmark/65047/04_H5N2	GenBank		DQ251448
A/Dk/Hong_Kong/293/1978_H7N2	GenBank		CY005620
A/Duck/Kingmen/E322/04_H6N2	GenBank		DQ376721
A/Ruddy_turnstone/Netherlands/1/2008_H10N4	GenBank		KR862686
A/Ruddy_turnstone/Netherlands/5/2008_H10N4	GenBank		KR862688
A/Ruddy_turnstone/Netherlands/1/2009_H10N4	GenBank		KR862689
A/Mallard/Netherlands/11/2006_H8N4	GenBank		KR862681
A/Mallard/Netherlands/13/2006_H8N4	GenBank		KR862682
A/Mallard/Netherlands/30/2011_H6N4	GenBank		KR862693
A/Ruddy_Turnstone/Delaware/67/98_H12N4	GISAID		EPI16616
A/Pintail/Alaska/314/2005_H12N4	GISAID		EPI307548
A/Blue-winged_teal/Guatemala/CIP049-04/2010_H8N4	GenBank		CY096650
A/Red_knot/Delaware_Bay/227/1994_mixed	GISAID		EPI345275
A/Shorebird/Delaware_Bay/215/1994_mixed	GISAID		EPI437174
A/Ruddy_turnstone/Delaware_Bay/124/1994_mixed	GISAID		EPI437442
A/Ruddy_turnstone/Delaware_Bay/150/1994_H1N4	GISAID		EPI437470
A/Mallard/Sweden/100546/2009_H8N4	GenBank		JX566224
A/Blue-winged_teal/ALB/685/1982_H6N4	GISAID		EPI85929
A/Mallard_duck/Alberta/299/1977_H4N4	GISAID		EPI87231
A/Mallard/Alberta/194/1992_H8N4	GISAID		EPI87925
A/Duck/Hokkaido/18/00_H10N4	GenBank		AB274042
A/Chicken/New_South_Wales/2/1997_H7N4	GenBank		CY022695
A/Chicken/New_South_Wales/327/1997_H7N4	GenBank		CY022703
A/Emu/New_South_Wales/775/1997_H7N4	GenBank		CY022711
A/Duck/Eastern_China/01/2005_H8N4	GenBank		EU429780
A/Mallard/Iran/V16/04_H8N4	GenBank		AM933239
A/Duck/Hubei/137/1985_H10N4	GenBank		EU559265
A/Mink/Sweden/3900/1984_H10N4	GenBank		GQ176142
A/Mallard/Gloucestershire/PD374/1985_H10N4	GenBank		GQ176126
A/Fowl/Hampshire/PD378/1985_H10N4	GenBank		GQ176118
A/Whistling_swan/Shimane/468/1988_H10N4	GenBank		GQ176110
A/Anas_plathyrhynchos/Spain/1495/2008_H10N4	GenBank		FN386476
A/Anas_plathyrhynchos/Spain/1502/2008_H8N4	GenBank		FN386477

Viral Name	Source	Accession number HA segment	Accession number NA segment	Viral Name	Source	Accession number HA segment	Accession number NA segment
A/Northern_pintail/Interior_Alaska/8BM2987/2008_H8N4	GISAID	EPI299122		A/Gray_teal/Western_Australia/1840/1979_H4N4	GenBank		CY045265
A/Northern_pintail/Interior_Alaska/8BM3088/2008_H8N4	GISAID	EPI299146		A/Chicken/NSW/1651/1997_H7N4	GenBank		GU053096
A/Northern_pintail/Interior_Alaska/8BM3091/2008_H8N4	GISAID	EPI299694		A/Mallard/Sweden/8/2003_H8N4	GenBank		CY060406
A/Northern_pintail/Interior_Alaska/8BM3137/2008_H8N4	GISAID	EPI299702		A/Mallard/PT/9408-3/2006_H9N4	GenBank		HM849002
A/Northern_pintail/Interior_Alaska/8MP0689/2008_H8N4	GISAID	EPI299090		A/Duck/Victoria/24/1981_H8N4	GenBank		CY094945
A/Northern_pintail/Interior_Alaska/9BM11556R0/2009_H8N4	GISAID	EPI452128		A/Teal/Chany/7119/2008_H15N4	GenBank		CY098542
A/Northern_pintail/Interior_Alaska/9BM11643R0/2009_H8N4	GISAID	EPI452142	EPI452144	A/Duck/Tsukuba/20/2007_H8N4	GenBank		AB669141
A/Northern_pintail/Interior_Alaska/9BM6510R0/2009_H8N4	GISAID	EPI452001	EPI452003	A/Duck/Thailand/CU-9754C/2010_H7N4	GenBank		JX307164
A/Northern_pintail/Interior_Alaska/9BM7152R2/2009_H8N4	GISAID	EPI452015	EPI452017	A/Duck/Thailand/CU-10524C/2011_H7N4	GenBank		JX307193
A/Northern_pintail/Interior_Alaska/9BM7240R0/2009_H8N4	GISAID	EPI452050		A/Duck/Thailand/CU-9744C_/2010_H7N4	GenBank		JX307210
A/Northern_pintail/Interior_Alaska/9BM7882R0/2009_H8N4	GISAID	EPI452219		A/Environment/Korea/PSC13-43/2008_H8N4	GenBank		JX679162
A/Northern_pintail/Interior_Alaska/9BM8109R0/2009_H8N4	GISAID	EPI436940		A/Mallard/Alberta/58/1989_H6N4	GenBank		CY126443
A/Northern_pintail/Interior_Alaska/9BM8237R0/2009_H8N4	GISAID	EPI452226		A/Ruddy_turnstone/Delaware/AI03-378/2003_H12N4	GenBank		CY144383
A/Northern_pintail/Interior_Alaska/9BM8967R0/2009_H8N4	GISAID	EPI436976		A/Duck/Thailand/CU-10510C/2011_H7N4	GenBank		KF591867
A/Northern_shoveler/California/AKS273/2007_H8N4	GISAID	EPI178742		A/Mallard/Sweden/17/2002_H10N4	GenBank		KF695363
A/Northern_shoveler/California/HKWF1203/2007_H8N4	GISAID	EPI222411		A/Mallard/Wisconsin/772/1982_H6N4	GenBank		CY178136
A/Northern_shoveler/California/HKWF1204/2007_H8N4	GISAID	EPI178782		A/Duck/Wisconsin/2366/1985_N4	GenBank		CY177458
A/Northern_shoveler/California/HKWF1325/2007_H8N4	GISAID	EPI160322		A/Mallard/Wisconsin/1360/1983_H7N4	GenBank		CY179525
A/Northern_shoveler/Interior_Alaska/9BM2925R0/2009_H8N4	GISAID	EPI432837		A/Mallard/Sweden/396/2002_H10N4	GenBank		CY184618
A/Northern_shoveler/Minnesota/Sg-00648/2008_H8N4	GISAID	EPI449391		A/Mallard_duck/ALB/7/1987_H8N4	GenBank		CY004998
A/Mallard/Netherlands/14/2006_H8N4	GenBank	KR862502		A/Mallard_duck/ALB/581/1983_H4N4	GenBank		CY004813
A/Chicken/Netherlands/11004004/2011_H8N4	GenBank	pending	pending	A/Mallard/Netherlands/83/2008_H12N5	GenBank		KR862695
A/American_black_duck/Illinois/4119/2009_H8N4	GenBank	CY097534		A/Eurasian_wigeon/Netherlands/2/2009_H12N5	GenBank		KR862696
A/Anas_crecca/Spain/1459/2008_H8N4	GenBank	FN386466	FN386475	A/Mallard/Netherlands/4/2011_H12N5	GenBank		KR862698
A/Common_teal/Netherlands/1/2005_H8N4	GenBank	CY041258	CY041260	A/Mallard/Netherlands/13/2008_H4N5	GenBank		KR862699
A/Duck/Alaska/702/1991_H8N2	GenBank	CY015173		A/Duck/Hokkaido/24/04_H10N5	GISAID		EPI160652
A/Duck/Hokkaido/207/2014_H8N2	GenBank	LC029898		A/Mallard/Denmark/77-64590-5/2005_H7N5	GISAID		EPI174859
A/Duck/Hokkaido/95/1981_H8N4	GenBank	AB450454	AB450455	A/Mallard/California/6524/2008_H12N5	GISAID		EPI328292
A/Duck/LA/B174/1986_H8N4	GenBank	GU186458		A/Mallard/Alberta/220/2006_	GISAID		EPI343416
A/Duck/Thailand/SP-355/2007_H8N4	GenBank	FJ802406	FJ802407	A/Mallard/Alberta/12/1993_	GISAID		EPI344668
A/Duck/Tsukuba/255/2005_H8N5	GenBank	AB669137	AB472028	A/Arenaria_interpres/Belgium/02936pcs1/2010_H12N5	GISAID		EPI345387
A/Duck/Yangzhou/02/2005_H8N4	GenBank	EF061122	EF061126	A/Mallard/Ohio/170/1999_H6N5	GISAID		EPI44086
A/Mallard_duck/Alberta/7/1987_H8N4	GenBank	CY014583		A/Emperor_goose/Alaska/44064-075/2006_H2N5	GISAID		EPI442443
A/Mallard/Alaska/708/2005_H8N4	GenBank	CY017749	CY017751	A/Mallard/Minnesota/182729/1998_H6N5	GISAID		EPI448392
A/Mallard/ALB/194/1992_H8N4	GenBank	CY005972		A/Green-winged_teal/Minnesota/Sg-00820/2008_H4N5	GISAID		EPI449492
A/Mallard/Alberta/283/1977_H8N4	GenBank	CY005970	AY207531	A/Ruddy_turnstone/New_Jersey/AI07-697/2007_H12N5	GISAID		EPI454987
A/Mallard/Interior_Alaska/8BM3061/2008_H8N4	GenBank	CY079099		A/Ruddy_turnstone/New_Jersey/AI07-803/2007_H12N5	GISAID		EPI455001

Viral Name	Source	Accession number HA segment	Accession number NA segment	Viral Name	Source	Accession number HA segment	Accession number NA segment
A/Mallard/Interior_Alaska/8BM3327/2008_H8N4	GenBank	CY080025		A/Mule_duck/Bulgaria/674/2010_H6N5	GISAID		EPI574266
A/Mallard/Interior_Alaska/8BM3584R1/2008_H8N4	GenBank	CY079677		A/Mallard/Alberta/202/1996_H2N5	GISAID		EPI85385
A/Mallard/Interior_Alaska/8MP0457R1/2008_H8N4	GenBank	CY079963		A/Pintail/Alberta/49/2003_H9N5	GISAID		EPI86317
A/Mallard/Minnesota/AI09-1833/2009_H8N4	GenBank	CY140663	CY140665	A/Mallard/Alberta/52/1997_H12N5	GISAID		EPI86417
A/Mallard/Minnesota/AI09-1854/2009_H8N4	GenBank	CY140671		A/Green-winged_teal/ALB/199/1991_H12N5	GISAID		EPI88775
A/Mallard/Minnesota/AI09-1867/2009_H8N4	GenBank	CY140679		A/Duck/Hokkaido/66/01_H12N5	GenBank		AB270747
A/Mallard/Minnesota/Sg-00062/2007_H8N4	GenBank	CY064121		A/Duck/Hokkaido/1058/2001_H4N5	GenBank		AB270594
A/Mallard/Minnesota/Sg-00570/2008_H8N4	GenBank	CY139985		A/Chicken/Hubei/119/1983_H10N5	GenBank		EU559271
A/Mallard/Minnesota/Sg-00571/2008_mixed	GenBank	CY139994		A/Duck/Tsukuba/11/2004_H6N5	GenBank		AB472026
A/Mallard/Minnesota/Sg-00678/2008_H8N4	GenBank	CY042859		A/Duck/Shiga/69/2006_H6N5	GenBank		AB472027
A/Mallard/Minnesota/SG-00680/2008_mixed	GenBank	CY140079		A/Anas_plathyrhynchos/Spain/1252/2007_H6N4	GenBank		FN386470
A/Mallard/Minnesota/SG-00684/2008_H8N4	GenBank	CY140088		A/Mallard/Switzerland/WV4060167/2006_H3N5	GenBank		GQ415323
A/Mallard/Minnesota/Sg-00686/2008_H8N4	GenBank	CY140096		A/Duck/Yangzhou/013/2008_H6N5	GenBank		GU220601
A/Mallard/Minnesota/Sg-00688/2008_H8N4	GenBank	CY042898		A/Duck/Eastern_China/031/2009_H5N5	GenBank		GU727663
A/Mallard/Minnesota/Sg-00690/2008_H8N4	GenBank	CY042906		A/Duck/Eastern_China/008/2008_H5N5	GenBank		GU727655
A/Mallard/Minnesota/Sg-00701/2008_H8N4	GenBank	CY140342		A/Mallard/Netherlands/2/1999_H3N5	GenBank		CY060263
A/Mallard/Netherlands/1/2006_H8N4	GenBank	CY043848	CY043850	A/Black-headed_gull/Netherlands/1/2006_H4N5	GenBank		CY076994
A/Mallard/Sweden/101165/2009_H8N4	GenBank	CY183572	CY183574	A/Avian/Japan/8KI0040/2008_H3N5	GenBank		CY079277
A/Mallard/Sweden/24/2002_H8N4	GenBank	CY060249	CY064796	A/Duck/Vietnam/G18/2009_H12N5	GenBank		AB593481
A/Mallard/Sweden/2834/2003_H8N4	GenBank	CY183368	CY183370	A/Duck/Mongolia/OIE-7457/2011_H3N5	GenBank		AB701297
A/Mallard/Sweden/2990/2003_H8N4	GenBank	CY183441	CY183443	A/Goose/Guangdong/K0103/2010_H5N5	GenBank		JQ973688
A/Mallard/Sweden/3240/2003_H8N4	GenBank	CY183449	CY183451	A/Quail/Jiangsu/K0104/2010_H5N5	GenBank		JQ973680
A/Mallard/Sweden/3244/2003_H8N4	GenBank	CY183457	CY183459	A/Mallard/Sweden/30/2002_H2N5	GenBank		CY122069
A/Mallard/Sweden/4486/2004_H8N4	GenBank	CY183465	CY183467	A/Duck/Guangxi/GXd-1/2009_H6N5	GenBank		JX293561
A/Mallard/Sweden/4737/2004_mixed	GenBank	CY183474		A/Aquatic_bird/Korea/CN5/2009_H6N5	GenBank		JX465642
A/Mallard/Sweden/50055/2006_H8N4	GenBank	CY183515	CY183517	A/Swine/Hubei/10/2008_H10N5	GenBank		JX500445
A/Mallard/Sweden/51156/2006_H8N4	GenBank	CY183523	CY183525	A/Mallard/Sweden/100127/2009_H12N5	GenBank		JX566221
A/Mallard/Sweden/51671/2006_H8N4	GenBank	CY184600	CY184602	A/Wild_duck/Korea/SH12-7/2008_H10N5	GenBank		JX679163
A/Mallard/Sweden/5389/2005_H8N4	GenBank	CY183483	CY183485	A/Duck/HuBei/03/2010_H5N5	GenBank		JX878685
A/Mallard/Sweden/541/2002_H8N4	GenBank	CY184592		A/Ruddy_turnstone/Delaware_Bay/118/2007_mixed	GenBank		CY127777
A/Mallard/Sweden/58256/2006_H8N4	GenBank	CY183531	CY183533	A/Duck/Vietnam/OIE-707/2011_H11N5	GenBank		AB781683
A/Mallard/Sweden/59475/2007_H8N4	GenBank	CY183539	CY183541	A/Mallard/Astrakhan/263/1982_H14N5	GenBank		AB289336
A/Mallard/Sweden/60041/2007_H8N2	GenBank	CY183425		A/Ruddy_turnstone/New_Jersey/AI07-796/2007_H12N5	GenBank		CY144725
A/Mallard/Sweden/68537/2007_mixed	GenBank	CY183555		A/Mallard/Finland/10952/2008_H4N5	GenBank		KF183615
A/Mallard/Sweden/7242/2004_H8N4	GenBank	CY183491	CY183493	A/Black-headed_gull/Iceland/1298/2011_H10N5	GenBank		CY149486
A/Mallard/Sweden/7996/2005_H8N4	GenBank	CY183499	CY183501	A/Duck/Guangdong/Wy11/2008_H5N5	GenBank		CY091629
A/Mallard/Sweden/8005/2005_H8N4	GenBank	CY183507	CY183509	A/Duck/Guangdong/Wy19/2008_H5N5	GenBank		CY091637
A/Mallard/Sweden/99377/2009_H8N4	GenBank	CY183564	CY183566	A/Duck/Guangdong/Wy24/2008_H5N5	GenBank		CY091645
A/Mallard/Wisconsin/11OS4489/2011_H8N4	GenBank	CY166162		A/Mallard/Sweden/50709/2006_H4N5	GenBank		CY164570
A/Mallard/Wisconsin/2080/1984_H8N4	GenBank	CY178863		A/Mallard/Sweden/343/2002_H12N5	GenBank		CY184001
A/Mallard/Wisconsin/2086/1984_H8N4	GenBank	CY178214	CY178216	A/Mallard/Sweden/2213/2003_H12N5	GenBank		CY184025
A/Mallard/Wisconsin/426/1979_H8N4	GenBank	CY180660	CY180662	A/Mallard/Sweden/3328/2003_H12N5	GenBank		CY184033
A/Northern_pintail/Alaska/44204-073/2006_H8N4	GenBank	EU557521		A/Mallard/Sweden/50968/2006_H12N5	GenBank		CY184041
A/Northern_pintail/Alaska/44340-503/2007_H8N4	GenBank	GU168306		A/Mallard/Sweden/60069/2007_H12N5	GenBank		CY184049
A/Northern_pintail/Alaska/44420-106/2008_H8	GenBank	GU168307		A/Mallard/Sweden/68529/2007_H12N5	GenBank		CY184057
A/Northern_pintail/Alaska/44500-066/2009_H8N4	GenBank	JX080768		A/Black-headed_gull/Republic_of_Georgia/9/2012_ H2N5	GenBank		CY185619
A/Northern_shoveler/Netherlands/1/2006_H8N4	GenBank	CY077024	CY077026	A/Mallard/Sweden/79389/2008_mixed	GenBank		CY186278

Viral Name	Source	Accession number HA segment	Accession number NA segment	Viral Name	Source	Accession number HA segment	Accession number NA segment
A/Pintail_duck/Alberta/114/1979_H8N4	GenBank	CY005971		A/Mallard/Sweden/80329/2008_H11N5	GenBank		CY186398
A/Pintail/Alaska/246/2005_mixed	GenBank	CY096764	CY096768	A/Mallard/Alberta/26/2001_mixed	GenBank		CY185798
A/Pintail/Barrow/140/2005_H8N4	GenBank	EF655844		A/Migratory_duck/Jiangxi/31454/2013_H10N5	GenBank		KP284895
A/Pintail/Barrow/38/2005_H8N4	GenBank	EF655828		A/Migratory_duck/Jiangxi/31577/2013_H10N5	GenBank		KP285887
A/Ruddy_shelduck/Mongolia/592/2010_H8N6	GenBank	KF501097		A/Migratory_duck/Jiangxi/7231/2003_H10N5	GenBank		KP288024
A/Ruddy_shelduck/Mongolia/593/2010_H8N4	GenBank	KF501064		A/Black_duck/AUS/4045/1980_H6N5	GenBank		CY005693
A/Teal/Chany/444/2009_H8N8	GenBank	CY098524		A/Mallard/Netherlands/2/2007_H10N7	GenBank		KR862705
A/Turkey/Colorado/235497/2003_H8N4	GenBank	GU051909		A/Mallard/Netherlands/3/2007_H10N7	GenBank		KR862706
A/Turkey/Ontario/6118/1968_H8N4	GenBank	CY130046	EU429793	A/Northern_shoveler/Egypt/EMC-4/2012_H10N7	GenBank		KR862401
A/Teal/Northern_Ireland/14567-10-5257/2007_H9N1	GISAID	EPI383878		A/Mallard/Netherlands/68/2008_H10N7	GenBank		KR862712
A/Common_coot/Poland/88/13_H9N2	GISAID	EPI505111		A/Mallard/Netherlands/3/2009_H7N7	GenBank		KR862718
A/Mallard/Iran/C364/2007_H9N2	GISAID	EPI302559		A/Mallard/Netherlands/7/2009_H7N7	GenBank		KR862721
A/Turkey/England/13437/2013_H9N2	GISAID	EPI585514		A/Mallard/Netherlands/21/2010_H10N7	GenBank		KR862723
A/Turkey/England/13538/2013_H9N2	GISAID	EPI585519		A/Mallard/Netherlands/22/2010_H10N7	GenBank		KR862724
A/Turkey/Poland/14/13_H9N2	GISAID	EPI500817		A/Mallard/Netherlands/23/2010_H10N7	GenBank		KR862725
A/Turkey/Poland/20/13_H9N2	GISAID	EPI505105		A/Northern_shoveler/Egypt/EMC-2/2012_H10N7	GenBank		KR862402
A/Ruddy_turnstone/New_Jersey/AI03-128/2003_H9N7	GISAID	EPI454831		A/Mallard/Netherlands/73/2008_H10N7	GenBank		KR862733
A/Ruddy_turnstone/New_Jersey/AI03-444/2003_H9N9	GISAID	EPI454810		A/Mallard/Netherlands/74/2008_H10N7	GenBank		KR862734
A/Mallard/Netherlands/1/2005_H9N2	GenBank	KR862503		A/Mallard/Netherlands/15/2013_H7N7	GenBank		KR862742
A/Gadwall/Netherlands/2/2006_H9N2	GenBank	KR862504		A/Mallard/Poland/16/09_H7N7	GISAID		EPI254380
A/Chicken/Netherlands/10020245/2010_H9N2	GenBank	pending	pending	A/Turkey/Netherlands/03003568/03_H7N7	GISAID		EPI290239
A/Baikal_teal/Xianghai/421/2011_H9N2	GenBank	KC162234		A/Chicken/Germany/R1801/2011_H7N7	GISAID		EPI356304
A/Bewicks_swan/Netherlands/5/2007_H9N2	GenBank	CY041274		A/Turkey/Germany/R1775/2011_H7N7	GISAID		EPI356305
A/Chicken/Korea/25232-96006/1996_H9N2	GenBank	KF188387	KF188388	A/Turkey/Germany-NI/R534/2013_H7N7	GISAID		EPI470367
A/Chicken/Korea/25232-MS96CE6/1996_H9N2	GenBank	KF188345		A/Duck/Mongolia/583/02_H4N7	GenBank		AB289334
A/Chicken/Korea/AI-96004/1996_H9N2	GenBank	GU053194		A/Anser_anser/Germany/R752/06_H7N7	GenBank		AM933236
A/Chicken/Korea/GH2/2007_H9N2	GenBank	HQ871933	HQ871935	A/Anas_crecca/Germany/Wv177/05_H7N7	GenBank		AM933237
A/Chicken/Korea/MS96-CE6/1996_H9N2	GenBank	GU053186	GU053188	A/Whooper_swan/Norway/10_438/2006_H7N7	GenBank		FM179762
A/Common_murre/Oregon/19497-004/2005_H9N5	GenBank	CY075925	CY075927	A/Duck/Hokkaido/W90/2007_H10N7	GenBank		AB450445
A/Duck/Chiba/1/2007_H9N2	GenBank	AB874675		A/Duck/Taiwan/4201/99_H7N7	GenBank		AB450450
A/Duck/Germany/113/1995_H9N2	GenBank	HE802066		A/Mallard/Korea/GH170/2007_H7N7	GenBank		FJ750866
A/Duck/Henan/03/2009_H9N2	GenBank	KJ162122		A/Magpie/Korea/YJD174/2007_H7N7	GenBank		FJ750856
A/Duck/Hokkaido/13/00_H9N2	GenBank	AB276111	AB276112	A/Mallard/Sweden/S90735/2003_H7N7	GenBank		FJ803183
A/Duck/Hokkaido/238/2008_H9N2	GenBank	AB485600		A/Mallard/Korea/GH171/2007_H7N7	GenBank		FJ959088
A/Duck/Hokkaido/49/98_H9N2	GenBank	AB125928		A/Duck/Shimane/18/2006_H7N7	GenBank		AB472030
A/Duck/Hokkaido/9/99_H9N2	GenBank	AB262463	AB262465	A/Duck/Shiga/B149/2007_H7N7	GenBank		AB472031
A/Duck/Hokkaido/HY57/2005_H9N4	GenBank	AB455035	AB455036	A/Duck/Tsukuba/664/2007_H7N7	GenBank		AB472059
A/Duck/Hokkaido/K04/2014_H9N2	GenBank	LC042043		A/Duck/Tsukuba/922/2008_H7N7	GenBank		AB472060
A/Duck/Hong_Kong/Y439/1997_H9N2	GenBank	KF188265		A/Duck/Chiba/13/2008_H7N7	GenBank		AB472061
A/Duck/Italy/260/2004_H9N8	GenBank	JX273564		A/Duck/Tsukuba/30/2007_H7N7	GenBank		AB472063
A/Duck/Shantou/1588/00_H9N1	GenBank	AF523389		A/Northern_pintail/Aomori/372/2008_H7N7	GenBank		AB516423
A/Duck/Shantou/2030/00_H9N1	GenBank	AF523390		A/Northern_pintail/Aomori/1001/2008_H7N7	GenBank		AB517633
A/Duck/Thailand/CU-8319T/2010_H9N7	GenBank	KF591855	KF591857	A/Northern_pintail/Akita/1366/2008_H7N7	GenBank		AB517634
A/Duck/Viet_Nam/340/2001_H9N3	GenBank	EF541420		A/Northern_pintail/Akita/1367/2008_H7N7	GenBank		AB517636
A/Duck/Viet_Nam/68/2001_H9N3	GenBank	EF541419		A/Mallard/PT/14683/2006_H6N7	GenBank		HM849014
A/Environment/Bangladesh/1041/2009_H9N2	GenBank	KC757809		A/Chicken/Netherlands/1/2003_H7N7	GenBank		AY340077

Viral Name	Source	Accession number HA segment	Accession number NA segment	Viral Name	Source	Accession number HA segment	Accession number NA segment
A/Eurasian_wigeon/Netherlands/4/2005_H9N2	GenBank	HM136574	KR862602	A/Netherlands/219/03_H7N7	GenBank		AY340079
A/Gadwall/Netherlands/1/2006_H9N2	GenBank	CY043864	CY043866	A/King_eider/Alaska/44068-067/2006_H4N7	GenBank		JX081153
A/Knot/England/497/2002_H9N9	GenBank	JX273565		A/Mallard/Sweden/109/2002_H2N7	GenBank		CY122033
A/Laughing_gull/Delaware_Bay/5/2003_H9N1	GenBank	CY102720		A/Mallard/Sweden/112/2002_H2N7	GenBank		CY122049
A/Mallard/Austria/WV1090234/2007_H9N2	GenBank	GU194485		A/Mallard/Sweden/9/2003_H2N7	GenBank		CY122141
A/Mallard/England/7798-6499/2006_H9N2	GenBank	JX273566		A/Chicken/Germany/R28/03_H7N7	GenBank		AJ620349
A/Mallard/Finland/10940/2009_H9N2	GenBank	KF183626		A/Mallard/Portugal/79905/2009_H10N7	GenBank		CY116607
A/Mallard/Finland/13353/2010_H9N2	GenBank	KF183630		A/Mallard/Korea/GG2/2007_H7N7	GenBank		KC609788
A/Mallard/Finland/13384/2010_H9N2	GenBank	KF183632		A/Wild_bird_feces/Korea/HDR22/2006_H7N7	GenBank		KC609785
A/Mallard/Finland/13977/2010_H9N2	GenBank	KF183634		A/Wild_duck/Korea/MHC40-28/2010_H7N7	GenBank		KC609802
A/Mallard/France/090360/2009_H9N2	GenBank	CY080415	CY080417	A/Mallard/Korea/NHG187/2008_H7N7	GenBank		KC609794
A/Mallard/Iran/T366/2007_H9N2	GenBank	FN600117		A/Wild_bird_feces/Korea/HDR23/2006_H7N7	GenBank		KC609786
A/Mallard/Ireland/PV46B/1993_H9N3	GenBank	AB303077		A/Mallard/64650/03_H5N7	GenBank		AY531030
A/Mallard/Norway/10_1537/2009_H9N2	GenBank	HE802723		A/Wild_goose/Dongting/PC0360/2012_H7N7	GenBank		KC876685
A/Mallard/Portugal/83660/2009_H9N2	GenBank	CY116614	CY184159	A/Duck/Fukui/1/2004_H7N7	GenBank		AB824727
A/Mallard/Portugal/83695/2009_H9N2	GenBank	CY184165	CY184167	A/Turkey/Ireland/PV8/1995_H7N7	GenBank		KF160885
A/Mallard/Portugal/99002/2009_H9N2	GenBank	CY116616		A/Environment/Hunan/S4484/2011_H12N7	GenBank		CY146766
A/Mallard/Portugal/99005/2009_H9N2	GenBank	CY116618		A/Duck/Fujian/5408/2008_H7N7	GenBank		KF259621
A/Mallard/Portugal/99006/2009_H9N2	GenBank	CY116620		A/Duck/Fujian/5476/2008_H7N7	GenBank		KF259622
A/Mallard/PT/27972-B139/2007_H9N2	GenBank	JF745931		A/Duck/Jiangxi/16309/2010_H7N7	GenBank		KF259628
A/Mallard/Sweden/4932/2004_H9N2	GenBank	CY184117	CY184119	A/Duck/Jiangxi/16326/2010_H7N7	GenBank		KF259629
A/Mallard/Sweden/67860/2007_H9N2	GenBank	CY184149		A/Duck/Jiangxi/16769/2010_H7N7	GenBank		KF259630
A/Mallard/Sweden/7146/2004_H9N2	GenBank	CY184125	CY184127	A/Duck/Jiangxi/21980/2010_H7N7	GenBank		KF259631
A/Mallard/Sweden/99668/2009_H9N2	GenBank	CY184173		A/Duck/Jiangxi/1717/2003_H7N7	GenBank		KF259633
A/Mallard/Sweden/99785/2009_H9N2	GenBank	CY184181		A/Duck/Jiangxi/1748/2003_H7N7	GenBank		KF259634
A/Mallard/Switzerland/WV1070800/2007_H9N2	GenBank	GU194480		A/Common_teal/Hong_Kong/MPM1670/2011_H7N7	GenBank		KF259636
A/Mallard/Switzerland/WV1070805/2007_H9N2	GenBank	GU194481		A/Common_teal/Hong_Kong/MPL634/2011_H7N7	GenBank		KF259637
A/Mallard/Switzerland/WV1080875/2008_H9N2	GenBank	GU194482		A/Common_teal/Hong_Kong/MPM1740/2011_H7N7	GenBank		KF259638
A/Mallard/Switzerland/WV3080008/2007_H9N2	GenBank	GU194486		A/Wild_waterfowl/Hong_Kong/MPL705/2011_H7N7	GenBank		KF259639
A/Mallard/Switzerland/WV3080036/2008_H9N2	GenBank	GU194487		A/Wild_waterfowl/Hong_Kong/MPM2121/2011_H7N7	GenBank		KF259640
A/Mandarin_duck/Korea/K12-256/2012_H9N2	GenBank	KR234076		A/Wild_waterfowl/Hong_Kong/MPL1006/2011_H7N7	GenBank		KF259641
A/Ostrich/South_Africa/AI1586/2008_H9N2	GenBank	GQ404721		A/Mallard/Sweden/105/2002_H7N7	GenBank		KF695338
A/Pelican/Zambia/13/2009_H9N1	GenBank	AB569567		A/Mallard/Sweden/7206/2004_H7N7	GenBank		CY183419
A/Pink-footed_goose/Netherlands/1/2006_H9N2	GenBank	CY041266	CY041268	A/Mallard/Sweden/124987/2010_H7N7	GenBank		CY183435
A/Ruddy_turnstone/Delaware_Bay/261/1999_H9N7	GenBank	CY102532		A/Mallard/Sweden/6148/2005_H10N7	GenBank		CY183704
A/Ruddy_turnstone/Delaware/AI03-114/2003_H9N2	GenBank	CY144483		A/Mallard/Sweden/885/2002_H7N7	GenBank		CY184506
A/Ruddy_turnstone/Delaware/AI03-123/2003_ mixed	GenBank	CY144473		A/Mallard/Sweden/1337/2002_H7N7	GenBank		CY184514
A/Ruddy_turnstone/Delaware/AI03-162/2003_ mixed	GenBank	CY144464	CY144466	A/Mallard/Sweden/1645/2002_H7N7	GenBank		CY184522
A/Ruddy_turnstone/Delaware/AI03-163/2003_H9N8	GenBank	CY144547		A/Mallard/Sweden/5944/2005_H7N7	GenBank		CY184586
A/Ruddy_turnstone/Delaware/AI03-165/2003_H9N5	GenBank	CY144456	CY144458	A/Mallard/Republic_of_Georgia/1/2010_H10N7	GenBank		CY185419
A/Ruddy_turnstone/Delaware/AI03-180/2003_H9N9	GenBank	CY144563		A/Domestic_duck/Republic_of_Georgia/1/2010_H10N7	GenBank		CY185451
A/Ruddy_turnstone/Delaware/AI03-193/2003_H9N5	GenBank	CY144373	CY144375	A/Domestic_duck/Republic_of_Georgia/2/2010_H10N7	GenBank		CY185459
A/Ruddy_turnstone/Delaware/AI03-200/2003_H9N8	GenBank	CY144555		A/Black-headed_gull/Republic_of_Georgia/7/2012_ H2N7	GenBank		CY185651
A/Ruddy_turnstone/Delaware/AI03-224/2003_H9N9	GenBank	CY144523		A/Black-headed_gull/Republic_of_Georgia/8/2012_ H2N7	GenBank		CY185699
A/Shorebird/DE/261/2003_H9N5	GenBank	CY005992		A/Mallard/Sweden/1628/2002_H7N7	GenBank		CY186302

Viral Name	Source	Accession number HA segment	Accession number NA segment	Viral Name	Source	Accession number HA segment	Accession number NA segment
A/Shorebird/Delaware_Bay/127/2003_H9N2	GenBank	CY102728		A/Mallard/Sweden/1671/2002_H7N7	GenBank		CY186310
A/Shorebird/Delaware_Bay/163/2003_H9N2	GenBank	KF188256		A/Mallard/Sweden/1678/2002_H7N7	GenBank		CY186318
A/Shorebird/Delaware_Bay/246/2003_H9N5	GenBank	CY102736	CY102738	A/Mallard/Sweden/1682/2002_H7N7	GenBank		CY186334
A/Shorebird/Delaware_Bay/276/1999_H9N2	GenBank	KF188373		A/Mallard/Sweden/1448/2002_H7N7	GenBank		CY186416
A/Shorebird/Delaware_Bay/283/2003_H9N1	GenBank	CY102744		A/Ruddy_turnstone/Iceland/1946/2012_H2N7	GenBank		KM213382
A/Shorebird/Delaware_Bay/286/2003_H9N2	GenBank	KF188278		A/Duck/Jiangxi/5879/2008_mixed	GenBank		KP287687
A/Shorebird/Delaware_Bay/293/2003_H9N2	GenBank	KF188333		A/Duck/Jiangxi/1410/2008_H10N7	GenBank		KP287896
A/Shorebird/Delaware_Bay/73/2003_H9N2	GenBank	CY101323		A/Duck/Jiangxi/1591/2008_H10N7	GenBank		KP287912
A/Teal/Finland/10529/2010_H9N2	GenBank	KF183628		A/Chicken/Jiangxi/10784/2014_H7N7	GenBank		KP414901
A/Teal/Primorie/3628/02_H9N2	GenBank	DQ787797		A/Wild_bird/Jiangxi/34458/2013_H7N7	GenBank		KP417105
A/Teal/Primorie/3631/02_H9N2	GenBank	DQ787802		A/Wild_bird/Jiangxi/35982/2013_H7N7	GenBank		KP417121

Table S3. Details of the low pathogenic avian influenza virus (LPAIV) sequences downloaded from the GISAID EpiFlu Database (160). We gratefully acknowledge the authors, originating and submitting laboratories of the sequences from the GISAID EpiFlu Database on which this research is based. All submitters may be contacted directly via the GISAID website.

Isolate name	Accession number HA segment	Accession number NA segment	Country	Collection Date	Originating Laboratory	Submitting Laboratory	Authors
A/Teal/Egypt/20431-NAMRU3/2003_H1N2	EPI372275		Egypt	22-Dec-2003	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/Shoveler/Egypt/00134-NAMRU3/2005_H1N1	EPI372331		Egypt	13-Jan-2005	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/Shoveler/Egypt/14029-NAMRU3/2006_H1N1	EPI372378	EPI372377	Egypt	8-Dec-2006	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/Teal/Egypt/01351-NAMRU3/2007_H1N1	EPI372466	EPI372465	Egypt	26-Jan-2007	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/Teal/Egypt/00677-NAMRU3/2004_H1N1	EPI372528		Egypt	28-Jan-2004	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/Goose/Italy/6117/2004_H1N1	EPI178520	EPI178522	Italy	2004*	-	Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emi	-
A/Mallard/Germany/R2843/06_H1N1	EPI222781		Germany	2006*	-	Friedrich-Loeffler-Institut	Fereidouni,SR.
A/Mallard/Germany-RP/R193/09_H1N1	EPI248501	EPI248500	Germany	2009*	-	Friedrich-Loeffler-Institut	-
A/Wild_duck/Germany/WV30/06_H1N1	EPI248514	EPI248512	Germany	2006*	-	Friedrich-Loeffler-Institut	-
A/Mallard/Germany/WV355/07_H1N1	EPI248519	EPI248517	Germany	2007*	-	Friedrich-Loeffler-Institut	-
A/Wild_duck/Germany-NW/R04/08_H1N1	EPI248521		Germany	2008*	-	Friedrich-Loeffler-Institut	-
A/Anas_platyrhynchos/Belgium/09-762/2008_H1N1	EPI257212	EPI257214	Belgium	Nov-2008*	Veterinary and Agrochemical Research Institute	Veterinary and Agrochemical Research Institute	Van Borm,Steven
A/Wild_duck/Korea/CSM38/2004b_H1N1	EPI296244		Korea	Nov-2004*	-	Other database import	Jeong,O.-M.; Kim,Y.-J.; Choi,J.-G.; Kang,H.-M.; Kim,M.-C.; Kwon,J.-H.; Lee,Y.-J.
A/Duck/Italy/7686-11/10_H1N1	EPI301849		Italy	15-Dec-2010	Istituto Zooprofilattico Sperimentale Delle Venezie	Istituto Zooprofilattico Sperimentale Delle Venezie	Monne,I.; Fusaro,A.; Valastro,V.; Schivo,A.; Buratin,A.; Terregino,C.; Capua,I.; Cattoli,G.
A/Pintail/Italy/2703-25/06_H1N1	EPI301856		Italy	5-Sep-2006	Istituto Zooprofilattico Sperimentale Delle Venezie	Istituto Zooprofilattico Sperimentale Delle Venezie	Monne,I.; Fusaro,A.; Valastro,V.; Schivo,A.; Buratin,A.; Terregino,C.; Capua,I.; Cattoli,G.
A/Mallard/Italy/378-49/06_H1N1	EPI301857		Italy	4-May-2006	Istituto Zooprofilattico Sperimentale Delle Venezie	Istituto Zooprofilattico Sperimentale Delle Venezie	Monne,I.; Fusaro,A.; Valastro,V.; Schivo,A.; Buratin,A.; Terregino,C.; Capua,I.; Cattoli,G.
A/Teal/Italy/6323-5/07_H1N1	EPI301858		Italy	30-Nov-2007	Istituto Zooprofilattico Sperimentale Delle Venezie	Istituto Zooprofilattico Sperimentale Delle Venezie	Monne,I.; Fusaro,A.; Valastro,V.; Schivo,A.; Buratin,A.; Terregino,C.; Capua,I.; Cattoli,G.
A/Mallard/Italy/432-21/08_H1N1	EPI301859		Italy	15-Feb-2008	Istituto Zooprofilattico Sperimentale Delle Venezie	Istituto Zooprofilattico Sperimentale Delle Venezie	Monne,I.; Fusaro,A.; Valastro,V.; Schivo,A.; Buratin,A.; Terregino,C.; Capua,I.; Cattoli,G.
A/Shoveler/Italy/6965-6/07_H1N3	EPI301860		Italy	1-Feb-2008	Istituto Zooprofilattico Sperimentale Delle Venezie	Istituto Zooprofilattico Sperimentale Delle Venezie	Monne,I.; Fusaro,A.; Valastro,V.; Schivo,A.; Buratin,A.; Terregino,C.; Capua,I.; Cattoli,G.
A/Avian/Germany-BB/R2859/2009_H6	EPI339183		Germany	2009*	-	Friedrich-Loeffler-Institut	-
A/Goose/Germany-BB/R1625/2008_H6	EPI279941		Germany	2008*	-	Friedrich-Loeffler-Institut	-
A/Ringed_teal/Germany-NRW/R641/2008_H6	EPI279938		Germany	2008*	-	Friedrich-Loeffler-Institut	-
A/Wild_bird/Germany-HH/R1501/2008_H6	EPI279939		Germany	2008*	-	Friedrich-Loeffler-Institut	-
A/Wild_bird/Germany-MV/R1511/2008_H6	EPI279940		Germany	2008*	-	Friedrich-Loeffler-Institut	-
A/Environment/California/NWRC182841-09/2006_H6N1	EPI406091		USA	2006*	-	Other database import	Anderson,T.; Piaggio,T.
A/Environment/California/NWRC183200-14/2006_H6N1	EPI406099		USA	2006*	-	Other database import	Anderson,T.; Piaggio,T.
A/Environment/California/NWRC183274-04/2006_H6N1	EPI406103		USA	2006*	-	Other database import	Anderson,T.; Piaggio,T.
A/Green-winged_teal/Nova_Scotia/14917/2005_H6N1	EPI327397		Canada	14-Sep-2005	-	Other database import	The NIAID Influenza Genome Sequencing Consortium
A/Larus_argentatus/Belgium/02936pcs3/2010_H6N1	EPI345428	EPI345427	Belgium	27-Jan-2010	Veterinary and Agrochemical Research Institute	Veterinary and Agrochemical Research Institute	Van Borm,S.; Rosseel,T.;Lambrecht,B.; Vangeluwe,D.; Vandenbussche,F.; van den Berg,T.
A/Northern_shoveler/California/HKWF115/2007_H6N1	EPI154816		USA	24-Oct-2007	-	Other database import	-
A/Duck/Germany-MV/R871/2010_H6N2	EPI339182		Germany	2010*	-	Friedrich-Loeffler-Institut	-
A/Green-winged_teal/Minnesota/Sg-00199/2007_H6N2	EPI298290		USA	14-Sep-2007	-	Other database import	The NIAID Influenza Genome Sequencing Consortium
A/Green-winged_teal/Minnesota/Sg-00222/2007_H6N2	EPI298322		USA	16-Sep-2007	-	Other database import	The NIAID Influenza Genome Sequencing Consortium
A/Mule_duck/Bulgaria/156/2010_H6N2	EPI574180		Bulgaria	9-Feb-2010	-	Other database import	Marinove-Petkova,A.; Georgiev,G.; Petkov,T.; Darnell,D.; Franks,J.; Walker,D.; Seiler,P.; Danner,A.; Graham,A.; McKenzie,P.; Krauss,S.; Webby,R.J.; Webster,R.G.
A/Mule_duck/Bulgaria/173/2009_H6N2	EPI574206		Bulgaria	12-Jan-2009	-	Other database import	Marinove-Petkova,A.; Georgiev,G.; Petkov,T.; Darnell,D.; Franks,J.; Walker,D.; Seiler,P.; Danner,A.; Graham,A.; McKenzie,P.; Krauss,S.; Webby,R.J.; Webster,R.G.
A/Shoveler/Egypt/13251-NAMRU3/2006_H6N2	EPI372371	EPI372370	Egypt	2-Dec-2006	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/Teal/Egypt/13203-NAMRU3/2006_H6N2	EPI372386	EPI372385	Egypt	2-Dec-2006	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/Turkey/Germany/R617/2007_H6N2	EPI317612		Germany	2007*	Friedrich-Loeffler-Institut	Friedrich-Loeffler-Institut	Stech,O; Weber,S; Mettenleiter,TC; Stech,J
A/Wild_goose/Germany-BB/R2329/2008_H6N2	EPI397608	EPI397607	Germany	2008*	-	Friedrich-Loeffler-Institut	-
A/Environment/North_Carolina/NWRC183941-06/2006_H6N5	EPI406114		USA	2006*	-	Other database import	Anderson,T.; Piaggio,T.
A/Sentinel_mallard/Germany/Sum156/2007_H6N5	EPI397610	EPI397609	Germany	2007*	-	Friedrich-Loeffler-Institut	-
A/Duck/Germany-NW/R2185/2006_H6N8	EPI397606		Germany	2006*	-	Friedrich-Loeffler-Institut	-
A/Environment/California/NWRC183200-06/2006_H6N8	EPI406098		USA	2006*	-	Other database import	Anderson,T.; Piaggio,T.
A/Goose/Germany/R1767/2007_H6N8	EPI416259		Germany	2007*	-	Friedrich-Loeffler-Institut	-
A/Mallard/Germany-BY/R1353/2010_H6N8	EPI339180		Germany	2010*	-	Friedrich-Loeffler-Institut	-
A/Mule_duck/Bulgaria/365/2010_H6N8	EPI574207		Bulgaria	9-Mar-2010	-	Other database import	Marinove-Petkova,A.; Georgiev,G.; Petkov,T.; Darnell,D.; Franks,J.; Walker,D.; Seiler,P.; Danner,A.; Graham,A.; McKenzie,P.; Krauss,S.; Webby,R.J.; Webster,R.G.
A/Mute_swan/Germany/R2927/2007_H6N8	EPI185339		Germany	2007*	-	Friedrich-Loeffler-Institut	-
A/Pink-footed_goose/Iceland/0987/2011_H6N8	EPI476116		Iceland	1-Nov-2011	-	Other database import	Wentworth,D.E.; Halpin,R.A.; Lin,X.; Bera,J.; Akopov,A.; Ransier,A.; Mohan,M.; Fedorova,N.; Tsitrin,T.; Stockwell,T.; Amedeo,P.; Appalla,L.; Bishop,B.; Edworthy,P.; Gupta,N.; Hoover,J.; Katzel,D.; Li,K.; Schobel,S.; Shrivastava,S.; Thovarai,V.; Wang,S.; Bao,Y.; Sanders,R.; Zhdanov,S.; Kiryutin,B.; Lipman,D.J.; Tatusova,T.; Hall,J.S.
A/Mule_duck/Bulgaria/175/2009_H6	EPI574252		Bulgaria	12-Jan-2009	-	Other database import	Marinove-Petkova,A.; Georgiev,G.; Petkov,T.; Darnell,D.; Franks,J.; Walker,D.; Seiler,P.; Danner,A.; Graham,A.; McKenzie,P.; Krauss,S.; Webby,R.J.; Webster,R.G.
A/Mule_duck/Bulgaria/181/2010_H6	EPI574247		Bulgaria	11-Feb-2010	-	Other database import	Marinove-Petkova,A.; Georgiev,G.; Petkov,T.; Darnell,D.; Franks,J.; Walker,D.; Seiler,P.; Danner,A.; Graham,A.; McKenzie,P.; Krauss,S.; Webby,R.J.; Webster,R.G.
A/Chicken/Netherlands/11004875/2011_H7N1	pending	EPI316304	Netherlands	22-Apr-2011	Central Veterinary Institute	Central Veterinary Institute	Heutink R., S. Pritz-Verschuren and G.Koch
A/Duck/Turkey/55/Cetinkaya/49/2006_H7N1	EPI346007		Turkey	2-Mar-2006	Pendik Veterinary Control and Research Institute	Animal and Plant Health Agency (APHA)	Collins,S; Russell,C; Focosi-Snyman,R; Essen,S; Shell,W; Akcadag,B; Iyisan,S; Unsal Baca,A; Reid,S; Manvell,R; Banks,J

Isolate name	Accession number HA segment	Accession number NA segment	Country	Collection Date	Originating Laboratory	Submitting Laboratory	Authors
A/Guinea_fowl/Italy/407/2008_H7N1	EPI210104		Italy	2008*	Istituto Zooprofilattico Sperimentale Delle Venezie	Istituto Zooprofilattico Sperimentale Delle Venezie	-
A/Mallard/Denmark/58-62-KLSV-119/09_H7N1	EPI492308		Denmark	21-Oct-2009	National Veterinary Institute	Animal and Plant Health Agency (APHA)	Barrass,L; Russell,C; Shell,W; Manvell,R; Jorgensen,P; Reid,S
A/Mallard/Italy/3397-65/2008_H7N1	EPI167297		Italy	2008*	-	Istituto Zooprofilattico Sperimentale Delle Venezie	-
A/Mallard/Italy/6103-5/2007_H7N1	EPI167296		Italy	2007*	-	Istituto Zooprofilattico Sperimentale Delle Venezie	-
A/Mallard/Italy/731/09_H7N1	EPI492522		Italy	7-Jan-2009	Istituto Zooprofilattico Sperimentale Delle Venezie	Animal and Plant Health Agency (APHA)	Agyeman-Dua,E; Russell,C; Shell,W; Manvell,R; Terregino,C; Reid,S
A/Mallard/Italy/794-18/2008_H7N1	EPI167299		Italy	2008*	-	Istituto Zooprofilattico Sperimentale Delle Venezie	-
A/Shoveler/Egypt/00597-NAMRU3/2004_H7N1	EPI372283	EPI372282	Egypt	27-Jan-2004	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/Shoveler/Egypt/14879-NAMRU3/2006_H7N1	EPI372363	EPI372362	Egypt	22-Dec-2006	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/Tadorna_tadorna/Belgium/3441-P3/2009_H7N1	EPI360900	EPI360901	Belgium	21-Mar-2009	Veterinary and Agrochemical Research Institute	Veterinary and Agrochemical Research Institute	-
A/Teal/Italy/794-3/2008_H7N1	EPI167298		Italy	2008*	-	Istituto Zooprofilattico Sperimentale Delle Venezie	-
A/Chicken/Italy/2240/2003_H7N3	EPI154960		Italy	2003*	-	Istituto Zooprofilattico Sperimentale Delle Venezie	Fusaro, A.; Tassoni, L.; Milani, A.; Salviato, A.; Schivo, A.; Monne, I.; Cattoli, G.
A/Chicken/Italy/2837-54/2007_H7N3	EPI154980		Italy	2007*	-	Istituto Zooprofilattico Sperimentale Delle Venezie	-
A/Chicken/Italy/2837-58/2007_H7N3	EPI154981		Italy	2007*	-	Istituto Zooprofilattico Sperimentale Delle Venezie	-
A/Chicken/Italy/8093/2002_H7N3	EPI154966		Italy	2002*	-	Istituto Zooprofilattico Sperimentale Delle Venezie	Fusaro, A.; Tassoni, L.; Milani, A.; Salviato, A.; Schivo, A.; Monne, I.; Cattoli, G.
A/Guinea_fowl/Italy/1613/2003_H7N3	EPI154959		Italy	2003*	-	Istituto Zooprofilattico Sperimentale Delle Venezie	Fusaro, A.; Tassoni, L.; Milani, A.; Salviato, A.; Schivo, A.; Monne, I.; Cattoli, G.
A/Mallard/Italy/1336/07_H7N3	EPI167295		Italy	2007*	-	Istituto Zooprofilattico Sperimentale Delle Venezie	-
A/Mallard/Italy/6103-12/2007_H7N3	EPI154982		Italy	2007*	-	Istituto Zooprofilattico Sperimentale Delle Venezie	-
A/Mallard/Italy/6104-14/2007_H7N3	EPI167300		Italy	2007*	-	Istituto Zooprofilattico Sperimentale Delle Venezie	-
A/Shoveler/Egypt/00017-NAMRU3/2007_H7N3	EPI372450		Egypt	29-Dec-2006	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/Shoveler/Egypt/00241-NAMRU3/2007_H7N3	EPI372418		Egypt	5-Jan-2007	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/Turkey/Italy/2963/2003_H7N3	EPI243279		Italy	23-May-2003	-	Istituto Zooprofilattico Sperimentale Delle Venezie	-
A/Turkey/Italy/8307/2002_H7N3	EPI154967		Italy	2002*	-	Istituto Zooprofilattico Sperimentale Delle Venezie	Fusaro, A.; Tassoni, L.; Milani, A.; Salviato, A.; Schivo, A.; Monne, I.; Cattoli, G.
A/Swan/Germany/R736/06_H7N4	EPI492517		Germany	Mar-2003*	Friedrich-Loeffler-Institut	Animal and Plant Health Agency (APHA)	Hanna,A; Russell,C; Shell,W; Harder,T; Grund,C; Starick,E; Manvell,R
A/Teal/Italy/11VIR-792/11_H7N6	EPI492520		Italy	31-Jan-2011	Istituto Zooprofilattico Sperimentale Delle Venezie	Animal and Plant Health Agency (APHA)	Collins,S; Hanna,A; Essen,S; Focosi-Snyman,R; Manvell, R; Terregino,C; Reid,S
A/Branta_canadensis/Belgium/13000-9-2/2010_H7N7	EPI360902		Belgium	2010*	Veterinary and Agrochemical Research Institute	Veterinary and Agrochemical Research Institute	-
A/Chicken/Germany-NI/R874/2010_H7N7	EPI302178		Germany	2010*	-	Friedrich-Loeffler-Institut	-
A/Chicken/Germany/R1362/11_H7N7	EPI492511		Germany	May-2011*	Friedrich-Loeffler-Institut	Animal and Plant Health Agency (APHA)	Agyeman-Dua,E; Russell,C; Shell,W; Manvell,R; Harder,T; Grund,C; Starick,E; Fereidouni,S; Reid,S
A/Chicken/Netherlands/11011326/2011_H7N7	pending	EPI325342	Netherlands	21-Jun-2006	Central Veterinary Institute	Central Veterinary Institute	Pritz-Vershuren, Sylvia J.; Heutink, Rene; Koch, Guus
A/Chicken/Netherlands/12014794/2012_H7N7	EPI390921	EPI390922	Netherlands	9-Aug-2012	Central Veterinary Institute	Central Veterinary Institute	Heutink,Rene; Pritz-Verschuren,Sylvia; Bouwstra,Ruth; Koch, Guus
A/Egyptian_goose/Egypt/05588-NAMRU3/2006_H7N7	EPI372394	EPI372393	Egypt	7-Apr-2006	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/Mallard/Denmark/303878-1S/13_H7N7	EPI492307		Denmark	26-May-2013	National Veterinary Institute	Animal and Plant Health Agency (APHA)	Puranik,A; Hanna,A; Essen,S; Focosi-Snyman,R; Manvell,R; Hjulsager,C; Trebbien,R; Breum,S; Larsen,L; Reid,S
A/Mallard/Italy/11VIR-540/11_H7N7	EPI492519		Italy	10-Jan-2011	Istituto Zooprofilattico Sperimentale Delle Venezie	Animal and Plant Health Agency (APHA)	Collins,S; Hanna,A; Essen,S; Focosi-Snyman,R; Manvell, R; Terregino,C; Reid,S
A/Mallard/Poland/01/08_H7N7	EPI169422	EPI169423	Poland	28-Dec-2007	National Veterinary Research Institute	National Veterinary Research Institute	-
A/Mallard/Poland/41/09_H7N7	EPI211188		Poland	16-Feb-2009	National Veterinary Research Institute	National Veterinary Research Institute	-
A/Mallard/Poland/446/09_H7N7	EPI254381	EPI254382	Poland	27-Dec-2009	National Veterinary Research Institute	National Veterinary Research Institute	Smietanka,K; Pikula,A; Minta,Z
A/Pochard/Germany/R916/06_H7N7	EPI492516		Germany	Mar-2006*	Friedrich-Loeffler-Institut	Animal and Plant Health Agency (APHA)	Hanna,A; Russell,C; Shell,W; Harder,T; Grund,C; Starick,E; Manvell,R
A/Shoveler/Egypt/09864-NAMRU3/2004_H7N7	EPI372323	EPI372322	Egypt	22-Dec-2004	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/Swan/Germany/R57/06_H7N7	EPI492518		Germany	Jan-2006*	Friedrich-Loeffler-Institut	Animal and Plant Health Agency (APHA)	Hanna,A; Russell,C; Shell,W; Harder,T; Grund,C; Starick,E; Manvell,R
A/Teal/Egypt/00835-NAMRU3/2004_H7N7	EPI372307		Egypt	18-Feb-2004	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/Turkey/Germany-NW/R655/2009_H7N7	EPI356351		Germany	2009*	-	Friedrich-Loeffler-Institut	-
A/Shoveler/Egypt/00215-NAMRU3/2007_H7N9	EPI372410		Egypt	5-Jan-2007	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/Chicken/Italy/13VIR4527-11/2013_H7N7	EPI677999	EPI677998	Italy	13-Aug-2013	-	Istituto Zooprofilattico Sperimentale Delle Venezie	Fusaro, A.; Tassoni, L.; Milani, A.; Hughes, J.; Salviato, A.; Murcia, P.; Massi, P.; Bonfanti, L.; Marangon, S.; Cattoli, G.; Monne, I.
A/Northern_shoveler/Mississippi/11OS5900/2011_H8N1	EPI512596		USA	8-Dec-2011	-	Other database import	Wentworth,D.E.; Halpin,R.A.; Lin,X.; Bera,J.; Akopov,A.; Ransier,A.; Mohan,M.; Fedorova,N.; Tsitrin,T.; Puri,V.; Stockwell,T.; Amedeo,P.; Appalla,L.; Bishop,B.; Edworthy,P.; Gupta,N.; Hoover,J.; Katzel,D.; Li,K.; Schobel,S.; Shrivastava,S.; Thovarai,V.; Wang,S.; Nolting,J.M.; Fries,A.C.; Bowman,A.S.; Bao,Y.; Sanders,R.; Zhdanov,S.; Kiryutin,B.; Lipman,D.J.; Tatusova,T.; Slemons,R.
A/American_black_duck/Nova_Scotia/02043/2007_H8N4	EPI404484		Canada	8-Aug-2007	-	Other database import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; Ghedin,E.; Fedorova,N.; Overton,L.; Tsitrin,T.; Stockwell,T.; Amedeo,P.; Bishop,B.; Chen,H.;
A/American_green-winged_teal/California/44287-373/2007_H8N4	EPI292438		USA	27-Jan-2007	-	Other database import	The NIAID Influenza Genome Sequencing Consortium

Isolate name	Accession number HA segment	Accession number NA segment	Country	Collection Date	Originating Laboratory	Submitting Laboratory	Authors
A/American_green-winged_teal/Interior_Alaska/9BM5045R0/2009_H8N4	EPI433069	EPI433100	USA	29-Jul-2009	-	Other database import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; Ghedin,E.; Fedorova,N.; Overton,L.; Tsitrin,T.; Stockwell,T.; Amedeo,P.; Bishop,B.; Chen,H.; Edworthy,P.; Gupta,N.; Katzel,D.; Li,K.; Schobel,S.; Shrivastava,S.; Thovarai,V.; Wang,S.; Runstadler,J.; Lindberg,M.; Huettmann,F.; Petrula,M.; Meixell,B.; Gingrich,J.P.; Gildehaus,L.A.; Vick,L.; Kokx,K.; Dillon,D.; Aldehoff,F.; Felker,E.P.; Marcotte,R.W.; Schmidt,J.C.; Moore,J.R.; Gerdes,K.E.; Bao,Y.; Sanders,R.; Dernovoy,D.; Kiryutin,B.; Lipman,D.J.; Tatusova,T.
A/Environment/Pennsylvania/NWRC182092-24/2006_H8N4	EPI406002		USA	2006*	-	Other database import	Anderson,T.; Piaggio,T.
A/Garganey/Ukraine/05835-NAMRU3/2006_H8N4	EPI372512	EPI372511	Ukraine	13-Aug-2006	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/Mallard/Interior_Alaska/8BM1966R1/2008_H8N4	EPI299970		USA	8-Aug-2008	-	Other database import	The NIAID Influenza Genome Sequencing Consortium
A/Mallard/Interior_Alaska/8MP0547/2008_H8N4	EPI299411		USA	11-Aug-2008	-	Other database import	The NIAID Influenza Genome Sequencing Consortium
A/Mallard/Interior_Alaska/9BM10537R0/2009_H8N4	EPI436880		USA	2009*	-	Other database import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; Ghedin,E.; Fedorova,N.; Overton,L.; Tsitrin,T.; Stockwell,T.; Amedeo,P.; Bishop,B.; Chen,H.;
A/Mallard/Interior_Alaska/9BM8389R0/2009_H8N4	EPI452280	EPI452282	USA	2009*	-	Other database import	-
A/Mallard/Minnesota/Sg-00675/2008_H8N4	EPI188649		USA	3-Aug-2008	-	Other database import	Sreevatsan,S.; Ramakrishnan,M.A.; Wang,P.; Anderson,T.L.; Jindal,N.; Chander,Y.; Goyal,S.M.; Osterholm,M.T.
A/Northern_pintail/Interior_Alaska/8BM2011R1/2008_H8N4	EPI299978		USA	9-Aug-2008	-	Other database import	The NIAID Influenza Genome Sequencing Consortium
A/Northern_pintail/Interior_Alaska/8BM2046R1/2008_H8N4	EPI299419		USA	9-Aug-2008	-	Other database import	The NIAID Influenza Genome Sequencing Consortium
A/Northern_pintail/Interior_Alaska/8BM2621R1/2008_H8N4	EPI299686		USA	14-Aug-2008	-	Other database import	The NIAID Influenza Genome Sequencing Consortium
A/Northern_pintail/Interior_Alaska/8BM2987/2008_H8N4	EPI299122		USA	17-Aug-2008	-	Other database import	The NIAID Influenza Genome Sequencing Consortium
A/Northern_pintail/Interior_Alaska/8BM3088/2008_H8N4	EPI299146		USA	18-Aug-2008	-	Other database import	The NIAID Influenza Genome Sequencing Consortium
A/Northern_pintail/Interior_Alaska/8BM3091/2008_H8N4	EPI299694		USA	18-Aug-2008	-	Other database import	The NIAID Influenza Genome Sequencing Consortium
A/Northern_pintail/Interior_Alaska/8BM3137/2008_H8N4	EPI299702		USA	18-Aug-2008	-	Other database import	The NIAID Influenza Genome Sequencing Consortium
A/Northern_pintail/Interior_Alaska/8MP0689/2008_H8N4	EPI299090		USA	13-Aug-2008	-	Other database import	The NIAID Influenza Genome Sequencing Consortium
A/Northern_pintail/Interior_Alaska/9BM11556R0/2009_H8N4	EPI452128		USA	2009*	-	Other database import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; Ghedin,E.; Fedorova,N.; Overton,L.; Tsitrin,T.; Stockwell,T.; Amedeo,P.; Bishop,B.; Chen,H.;
A/Northern_pintail/Interior_Alaska/9BM11643R0/2009_H8N4	EPI452142	EPI452144	USA	2009*	-	Other database import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; Ghedin,E.; Fedorova,N.; Overton,L.; Tsitrin,T.; Stockwell,T.; Amedeo,P.; Bishop,B.; Chen,H.;
A/Northern_pintail/Interior_Alaska/9BM6510R0/2009_H8N4	EPI452001	EPI452003	USA	2009*	-	Other database import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; Ghedin,E.; Fedorova,N.; Overton,L.; Tsitrin,T.; Stockwell,T.; Amedeo,P.; Bishop,B.; Chen,H.; Edworthy,P.; Gupta,N.; Katzel,D.; Li,K.; Schobel,S.; Shrivastava,S.; Thovarai,V.; Wang,S.; Runstadler,J.; Lindberg,M.; Huettmann,F.; Petrula,M.; Meixell,B.; Gingrich,J.P.; Gildehaus,L.A.; Vick,L.; Kokx,K.; Dillon,D.; Aldehoff,F.; Felker,E.P.; Marcotte,R.W.; Schmidt,J.C.; Moore,J.R.; Gerdes,K.E.; Bao,Y.; Sanders,R.; Dernovoy,D.; Kiryutin,B.; Lipman,D.J.; Tatusova,T.
A/Northern_pintail/Interior_Alaska/9BM7152R2/2009_H8N4	EPI452015	EPI452017	USA	2009*	-	Other database import	-
A/Northern_pintail/Interior_Alaska/9BM7240R0/2009_H8N4	EPI452050		USA	2009*	-	Other database import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; Ghedin,E.; Fedorova,N.; Overton,L.; Tsitrin,T.; Stockwell,T.; Amedeo,P.; Bishop,B.; Chen,H.;
A/Northern_pintail/Interior_Alaska/9BM7882R0/2009_H8N4	EPI452219		USA	2009*	-	Other database import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; Ghedin,E.; Fedorova,N.; Overton,L.; Tsitrin,T.; Stockwell,T.; Amedeo,P.; Bishop,B.; Chen,H.;
A/Northern_pintail/Interior_Alaska/9BM8109R0/2009_H8N4	EPI436940		USA	2009*	-	Other database import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; Ghedin,E.; Fedorova,N.; Overton,L.; Tsitrin,T.; Stockwell,T.; Amedeo,P.; Bishop,B.; Chen,H.;
A/Northern_pintail/Interior_Alaska/9BM8237R0/2009_H8N4	EPI452226		USA	2009*	-	Other database import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; Ghedin,E.; Fedorova,N.; Overton,L.; Tsitrin,T.; Stockwell,T.; Amedeo,P.; Bishop,B.; Chen,H.;
A/Northern_pintail/Interior_Alaska/9BM8967R0/2009_H8N4	EPI436976		USA	2009*	-	Other database import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; Ghedin,E.; Fedorova,N.; Overton,L.; Tsitrin,T.; Stockwell,T.; Amedeo,P.; Bishop,B.; Chen,H.;
A/Northern_shoveler/California/AKS273/2007_H8N4	EPI178742		USA	1-Dec-2007	-	Other database import	-
A/Northern_shoveler/California/HKWF1203/2007_H8N4	EPI222411		USA	5-Dec-2007	-	Other database import	Cardona,C.; Boyce,W.M.; Nelson,J.; Anchell,N.; Dao,N.; Anunciacion,J.; Cai,H.; Green,L.; Detter,C.; Kiss,H.; Dannen,M.
A/Northern_shoveler/California/HKWF1204/2007_H8N4	EPI178782		USA	5-Dec-2007	-	Other database import	-
A/Northern_shoveler/California/HKWF1325/2007_H8N4	EPI160322		USA	9-Dec-2007	-	Other database import	-
A/Northern_shoveler/Interior_Alaska/9BM2925R0/2009_H8N4	EPI432837		USA	16-Jul-2009	-	Other database import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; Ghedin,E.; Fedorova,N.; Overton,L.; Tsitrin,T.; Stockwell,T.; Amedeo,P.; Bishop,B.; Chen,H.;
A/Northern_shoveler/Minnesota/Sg-00648/2008_H8N4	EPI449391		USA	1-Aug-2008	-	Other database import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; Wester,E.; Ghedin,E.; Fedorova,N.; Tsitrin,T.; Stockwell,T.; Amedeo,P.; Bishop,B.; Edworthy,P.; Gupta,N.; Katzel,D.; Li,K.; Schobel,S.; Shrivastava,S.; Thovarai,V.; Wang,S.; Lebarbenchon,C.; Sreevatsan,S.; Poulson,B.; Yang,M.; Stallknecht,D.; Bao,Y.; Sanders,R.; Dernovoy,D.; Kiryutin,B.; Lipman,D.J.; Tatusova,T.
A/Teal/Northern_Ireland/14567-10-5257/2007_H9N1	EPI383878		Ireland	2007*	-	Other database import	Slomka,M.J.; Hanna,A.; Mahmood,S.; Govil,J.; Krill,D.; Manvell,R.J.; Shell,W.; Arnold,M.E.; Banks,J.; Brown,I.H.
A/Common_coot/Poland/88/13_H9N2	EPI505111		Poland	22-Sep-2013	-	National Veterinary Research Institut Poland, PIWet-PIB	-
A/Mallard/Iran/C364/2007_H9N2	EPI302559		Iran	2007*	-	Other database import	Fereidouni,SR.
A/Turkey/England/13437/2013_H9N2	EPI585514		England	14-Apr-2013	Animal and Plant Health Agency (APHA)	Animal and Plant Health Agency (APHA)	Reid, SM; Banks, J; Ceeraz, V; Cox, WJ; Howard, WA; Puranik, A; Collins, S; Seekings, A; Manvell, R; Irvine, RM; Brown, IH
A/Turkey/England/13538/2013_H9N2	EPI585519		England	14-Apr-2013	Animal and Plant Health Agency (APHA)	Animal and Plant Health Agency (APHA)	Reid, SM; Banks, J; Ceeraz, V; Cox, WJ; Howard, WA; Puranik, A; Collins, S; Seekings, A; Manvell, R; Irvine, RM; Brown, IH
A/Turkey/Poland/14/13_H9N2	EPI500817		Poland	23-Apr-2013	-	National Veterinary Research Institut Poland, PIWet-PIB	-
A/Turkey/Poland/20/13_H9N2	EPI505105		Poland	15-May-2013	-	National Veterinary Research Institut Poland, PIWet-PIB	-
A/Ruddy_turnstone/New_Jersey/AI03-128/2003_H9N7	EPI454831		USA	21-May-2003	-	Other database import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; Wester,E.; Ghedin,E.; Fedorova,N.; Tsitrin,T.; Stockwell,T.; Amedeo,P.; Bishop,B.; Edworthy,P.; Gupta,N.; Katzel,D.; Li,K.; Schobel,S.; Shrivastava,S.; Thovarai,V.; Wang,S.; Lebarbenchon,C.; Sreevatsan,S.; Poulson,B.; Yang,M.; Stallknecht,D.; Bao,Y.; Sanders,R.; Dernovoy,D.; Kiryutin,B.; Lipman,D.J.; Tatusova,T.
A/Ruddy_turnstone/New_Jersey/AI03-444/2003_H9N9	EPI454810		USA	20-May-2003	-	Other database import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; Wester,E.; Ghedin,E.; Fedorova,N.; Tsitrin,T.; Stockwell,T.; Amedeo,P.; Bishop,B.; Edworthy,P.; Gupta,N.; Katzel,D.; Li,K.; Schobel,S.; Shrivastava,S.; Thovarai,V.; Wang,S.; Lebarbenchon,C.; Sreevatsan,S.; Poulson,B.; Yang,M.; Stallknecht,D.; Bao,Y.; Sanders,R.; Dernovoy,D.; Kiryutin,B.; Lipman,D.J.; Tatusova,T.

Isolate name	Accession number HA segment	Accession number NA segment	Country	Collection Date	Originating Laboratory	Submitting Laboratory	Authors
A/Duck/Tsukuba/574/2006_H10N1	EPI356629		Japan	2006*	-	Other database import	Tsukamoto,K.; Javier,P.; Shishido,M.; Noguchi,D.; Pearce,J.; Kang,H.; Jeong,O.; Lee,Y.; Nakanishi,K.; Ashizawa,T.
A/Shoveler/Egypt/00006-NAMRU3/2007_H10N1	EPI372442		Egypt	29-Dec-2006	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/Teal/Egypt/12908-NAMRU3/2005_H10N1	EPI372481	EPI372480	Egypt	21-Nov-2005	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/Wild_bird/Korea/A323/2009_H10N1	EPI387876		Korea	Nov-2009*	-	Other database import	Kim,H.R.; Lee,Y.J.; Oem,J.K.; Bae,Y.C.; Kang,M.S.; Kang,H.M.; Choi,J.G.; Park,C.K.; Kwon,Y.K.
A/Duck/Hokkaido/W87/2007_H10N2	EPI161527		Japan	2007*	-	Other database import	-
A/Duck/Hunan/S11205/2012_H10N3	EPI461563		China	25-Mar-2012	-	Other database import	Deng,G.; Tan,D.; Shi,J.; Cui,P.; Jiang,Y.; Liu,L.; Tian,G.; Kawaoka,Y.; Li,C.; Chen,H.
A/Duck/Thailand/LM-CU4747/2009_H10N3	EPI314742		Thailand	11-2009*	-	Other database import	Amonsin,A.; Lapkuntod,J.; Suradhat,S.; Tantilertcharoen,R.; Bunpapong,N.; Boonyapisitsopa,S.; Wongphatcharachai,M.; Wisedchanwet,T.; Poovorawan,Y.; Sasipreeyajan,J.; Thanawongnuwech,R.
A/Duck/Thailand/LM-CU4753/2009_H10N3	EPI314746		Thailand	11-2009*	-	Other database import	Amonsin,A.; Lapkuntod,J.; Suradhat,S.; Tantilertcharoen,R.; Bunpapong,N.; Boonyapisitsopa,S.; Wongphatcharachai,M.; Wisedchanwet,T.; Poovorawan,Y.; Sasipreeyajan,J.; Thanawongnuwech,R.
A/Muscovy_duck/Thailand/CU-LM4754/2009_H10N3	EPI256770		Thailand	11-2009*	-	Other database import	Amonsin,A.
A/Duck/Italy/268302/2004_H10N4	EPI178493	EPI178495	Italy	2004*	-	Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emi	-
A/Pied_avocet/Ukraine/05848-NAMRU3/2006_H10N4	EPI372496	EPI372495	Ukraine	1-May-2006	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/Shoveler/Egypt/01574-NAMRU3/2007_H10N4	EPI372458	EPI372457	Egypt	9-Feb-2007	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/Long-tailed_duck/Wisconsin/10OS3919/2010_H10N6	EPI419336		USA	16-Nov-2010	-	Other database import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; Wester,E.; Ghedin,E.; Fedorova,N.; Tsitrin,T.; Stockwell,T.; Amedeo,P.; Bishop,B.; Edworthy,P.; Gupta,N.; Katzel,D.; Li,K.; Schobel,S.; Shrivastava,S.; Thovarai,V.; Wang,S.; Lebarbenchon,C.; Sreevatsan,S.; Poulson,B.; Yang,M.; Stallknecht,D.; Bao,Y.; Sanders,R.; Dernovoy,D.; Kiryutin,B.; Lipman,D.J.; Tatusova,T.
A/Mallard/Denmark/16109-4/2011-11-14_H10N6	EPI541472		Denmark	14-Nov-2011	Technical University of Denmark	Technical University of Denmark	Hjulsager, Charlotte; Breum, Solvej; Trebbien, Ramona; Larsen, Lars E
A/Anas_platyrhynchos/Camargue/091863/09_H10N7	EPI332944	EPI332955	France	20-May-2009	Station Biologique "Tour du Valat"	Institut Pasteur	VITTECOQ Marion, GRANDHOMME Viviane
A/Avian/Israel/232/2001_H10N7	EPI456969		Israel	Feb-2001*	-	Other database import	Shkoda,I.; Panshin,A.; Shihmanter,E.; Lapin,K.; Lipkind,M.
A/Duck/Italy/62330/2006_H10N7	EPI178528		Italy	2006*	-	Istituto Zooprofilattico Sperimentale Delle Venezie	-
A/Duck/Italy/73383/2006_H10N7	EPI174777		Italy	2006*	-	Istituto Zooprofilattico Sperimentale Delle Venezie	-
A/Harbor_seal/Denmark/14-5061-1lu/2014-07_H10N7	EPI541474		Denmark	Jul-2014*	Technical University of Denmark	Technical University of Denmark	Krog, Jesper Schak; Hjulsager, Charlotte; Larsen, Lars E
A/Mallard/Egypt/EMC-4/2012_H10N7	EPI552755		Egypt	9-Mar-2012	Erasmus Medical Center	Erasmus Medical Center	Bodewes, Rogier; Bestebroer, Theo M.; Van der Vries, Erhard; Verhagen, Josanne H.; Koopmans, Marion P.; Fouchier, Ron A.M.; Wohlsein, Peter; Siebert, Ursula; Baumgärtner, Wolfgang; Osterhaus, Albert D.M.E.
A/Mallard/Netherlands/1/2012_H10N7	EPI552756		Netherlands	14-Jan-2012	Erasmus Medical Center	Erasmus Medical Center	Bodewes, Rogier; Bestebroer, Theo M.; Van der Vries, Erhard; Verhagen, Josanne H.; Koopmans, Marion P.; Fouchier, Ron A.M.; Wohlsein, Peter; Siebert, Ursula; Baumgärtner, Wolfgang; Osterhaus, Albert D.M.E.
A/Mallard/Netherlands/1/2014_H10N7	EPI552751		Netherlands	17-Feb-2014	Erasmus Medical Center	Erasmus Medical Center	Bodewes, Rogier; Bestebroer, Theo M.; Van der Vries, Erhard; Verhagen, Josanne H.; Koopmans, Marion P.; Fouchier, Ron A.M.; Wohlsein, Peter; Siebert, Ursula; Baumgärtner, Wolfgang; Osterhaus, Albert D.M.E.
A/Northern_pintail/Egypt/EMC-1/2012_H10N7	EPI552754		Egypt	20-Jan-2012	Erasmus Medical Center	Erasmus Medical Center	Bodewes, Rogier; Bestebroer, Theo M.; Van der Vries, Erhard; Verhagen, Josanne H.; Koopmans, Marion P.; Fouchier, Ron A.M.; Wohlsein, Peter; Siebert, Ursula; Baumgärtner, Wolfgang; Osterhaus, Albert D.M.E.
A/Seal/Sweden/SVA0546/2014_H10N7	EPI545212		Sweden	16-Apr-2014	Swedish Museum of Natural History	National Veterinary Institute	Siamak,Zohari; Aleksija,Neimanis; Tero,Härkönen; Charlotta,Moraeus; Jean-Francois,Vallachare
A/Shoveler/Egypt/00600-NAMRU3/2004_H10N7	EPI372291		Egypt	27-Jan-2004	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/Shoveler/Egypt/01198-NAMRU3/2007_H10N7	EPI372402		Egypt	19-Jan-2007	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/Shoveler/Egypt/09781-NAMRU3/2004_H10N7	EPI372339	EPI372338	Egypt	18-Dec-2004	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/Teal/Egypt/01207-NAMRU3/2007_H10N7	EPI372426	EPI372425	Egypt	19-Jan-2007	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/Seal/Sweden/SVA0824/2014_/H10N7_H10N7	EPI547696		Sweden	29-Aug-2014	Swedish Museum of Natural History	National Veterinary Institute	Siamak,Zohari; Aleksija,Neimanis; Tero,Härkönen; Charlotta,Moraeus; Jean-Francois,Vallachare
A/Chicken/77/Jiangxi/2014_H10N8	EPI537463		China	9-Jan-2014	-	Tsinghua University	-
A/Chicken/Jiangxi/102/2013_H10N8	EPI530542		China	8-Dec-2013	South China Agricultural University	South China Agricultural University	Wenbao,Qi; Xianfeng,Zhou; Lihong,Huang; Huanan,Li; Qian,Li; Ming,Liao; Mingbin,Liu
A/Environment/Dongting_Lake/Hunan/3-9/2007_H10N8	EPI221966		China	2007*	-	Other database import	Zhang,H.; Chen, Z.
A/Environment/Jiangxi/03366/2013_H10N8	EPI530386		China	8-Dec-2013	WHO Chinese National Influenza Center	WHO Chinese National Influenza Center	Wang,Dayan; Gao,Rongbao; Yang,Lei; Zhou,Shumei; Li,Xiyan; Zhou,Jianfang; Zhu,Wenfei; Guo,Junfeng; Li,Xiaodan; Dong,Jie; Huang,Weijuan; Zhang,Ye; Dong,Libo; Zhao,Xiang; Lu,Jian; Lan,Yu; Shu,Yuelong
A/Environment/Jiangxi/03367/2013_H10N8	EPI530394		China	8-Dec-2013	WHO Chinese National Influenza Center	WHO Chinese National Influenza Center	Wang,Dayan; Gao,Rongbao; Yang,Lei; Zhou,Shumei; Li,Xiyan; Zhou,Jianfang; Zhu,Wenfei; Guo,Junfeng; Li,Xiaodan; Dong,Jie; Huang,Weijuan; Zhang,Ye; Dong,Libo; Zhao,Xiang; Lu,Jian; Lan,Yu; Shu,Yuelong
A/Environment/Jiangxi/03413/2013_H10N8	EPI530402		China	11-Dec-2013	WHO Chinese National Influenza Center	WHO Chinese National Influenza Center	Wang,Dayan; Gao,Rongbao; Yang,Lei; Zhou,Shumei; Li,Xiyan; Zhou,Jianfang; Zhu,Wenfei; Guo,Junfeng; Li,Xiaodan; Dong,Jie; Huang,Weijuan; Zhang,Ye; Dong,Libo; Zhao,Xiang; Lu,Jian; Lan,Yu; Shu,Yuelong
A/Environment/Jiangxi/03489/2013_H10N8	EPI530410		China	27-Dec-2013	WHO Chinese National Influenza Center	WHO Chinese National Influenza Center	Wang,Dayan; Gao,Rongbao; Yang,Lei; Zhou,Shumei; Li,Xiyan; Zhou,Jianfang; Zhu,Wenfei; Guo,Junfeng; Li,Xiaodan; Dong,Jie; Huang,Weijuan; Zhang,Ye; Dong,Libo; Zhao,Xiang; Lu,Jian; Lan,Yu; Shu,Yuelong
A/Environment/Jiangxi/10615/2014_H10N8	EPI530418		China	10-Jan-2014	WHO Chinese National Influenza Center	WHO Chinese National Influenza Center	Wang,Dayan; Gao,Rongbao; Yang,Lei; Zhou,Shumei; Li,Xiyan; Zhou,Jianfang; Zhu,Wenfei; Guo,Junfeng; Li,Xiaodan; Dong,Jie; Huang,Weijuan; Zhang,Ye; Dong,Libo; Zhao,Xiang; Lu,Jian; Lan,Yu; Shu,Yuelong
A/Environment/Jiangxi/10721/2014_H10N8	EPI530426		China	15-Jan-2014	WHO Chinese National Influenza Center	WHO Chinese National Influenza Center	Wang,Dayan; Gao,Rongbao; Yang,Lei; Zhou,Shumei; Li,Xiyan; Zhou,Jianfang; Zhu,Wenfei; Guo,Junfeng; Li,Xiaodan; Dong,Jie; Huang,Weijuan; Zhang,Ye; Dong,Libo; Zhao,Xiang; Lu,Jian; Lan,Yu; Shu,Yuelong
A/Environment/Jiangxi/10738/2014_H10N8	EPI530434		China	14-Jan-2014	WHO Chinese National Influenza Center	WHO Chinese National Influenza Center	Wang,Dayan; Gao,Rongbao; Yang,Lei; Zhou,Shumei; Li,Xiyan; Zhou,Jianfang; Zhu,Wenfei; Guo,Junfeng; Li,Xiaodan; Dong,Jie; Huang,Weijuan; Zhang,Ye; Dong,Libo; Zhao,Xiang; Lu,Jian; Lan,Yu; Shu,Yuelong
A/Jiangxi-Donghu/346/2013_H10N8	EPI497477		China	4-Dec-2013	-	WHO Chinese National Influenza Center	Rongbao,Gao; Shumei,Zou; Xiang,Zhao; Lei,Yang; Dayan,Wang; Yuelong,Shu
A/Jiangxi/09037/2014_H10N8	EPI530450		China	8-Jan-2014	WHO Chinese National Influenza Center	WHO Chinese National Influenza Center	Wang,Dayan; Gao,Rongbao; Yang,Lei; Zhou,Shumei; Li,Xiyan; Zhou,Jianfang; Zhu,Wenfei; Guo,Junfeng; Li,Xiaodan; Dong,Jie; Huang,Weijuan; Zhang,Ye; Dong,Libo; Zhao,Xiang; Lu,Jian; Lan,Yu; Shu,Yuelong
A/Mallard/Sweden/7/2003_H10N8	EPI251793		Sweden	24-May-2003	-	Other database import	Fouchier, R.
A/Northern_shoveler/Hong_Kong/MPC657/2006_H10N9	EPI469806		Hong Kong (SAR)	2006*	-	Other database import	Lam,T.T.Y.; Wang,J.; Shen,Y.; Zhou,B.; Duan,L.; Cheung,C.L.; Ma,C.; Lycett,S.J.; Leung,C.Y.H.; Chen,X.; Li,L.; Hong,W.; Chai,Y.; Zhou,L.; Liang,H.; Ou,Z.; Liu,Y.; Farooqui,A.; Kelvin,D.J.; Poon,L.L.M.; Smith,D.K.; Pybus,O.G.; Leung,G.M.; Shu,Y.; Webster,R.G.; Webby,R.J.; Peiris,J.S.M.; Rambaut,A.; Zhu,H.; Guan,Y.
A/Northern_shoveler/Hong_Kong/MPE2531/2008_H10N9	EPI469805		Hong Kong (SAR)	2008*	-	Other database import	Lam,T.T.Y.; Wang,J.; Shen,Y.; Zhou,B.; Duan,L.; Cheung,C.L.; Ma,C.; Lycett,S.J.; Leung,C.Y.H.; Chen,X.; Li,L.; Hong,W.; Chai,Y.; Zhou,L.; Liang,H.; Ou,Z.; Liu,Y.; Farooqui,A.; Kelvin,D.J.; Poon,L.L.M.; Smith,D.K.; Pybus,O.G.; Leung,G.M.; Shu,Y.; Webster,R.G.; Webby,R.J.; Peiris,J.S.M.; Rambaut,A.; Zhu,H.; Guan,Y.
A/Northern_shoveler/Hong_Kong/MPE2984/2008_H10N9	EPI469808		Hong Kong (SAR)	2008*	-	Other database import	Lam,T.T.Y.; Wang,J.; Shen,Y.; Zhou,B.; Duan,L.; Cheung,C.L.; Ma,C.; Lycett,S.J.; Leung,C.Y.H.; Chen,X.; Li,L.; Hong,W.; Chai,Y.; Zhou,L.; Liang,H.; Ou,Z.; Liu,Y.; Farooqui,A.; Kelvin,D.J.; Poon,L.L.M.; Smith,D.K.; Pybus,O.G.; Leung,G.M.; Shu,Y.; Webster,R.G.; Webby,R.J.; Peiris,J.S.M.; Rambaut,A.; Zhu,H.; Guan,Y.

Isolate name	Accession number HA segment	Accession number NA segment	Country	Collection Date	Originating Laboratory	Submitting Laboratory	Authors
A/Shoveler/Egypt/00004-NAMRU3/2007_H10N9	EPI372434		Egypt	29-Dec-2006	U.S. Naval Medical Research Unit No.3	Centers for Disease Control and Prevention	Gerloff, Nancy; Simpson, Natosha; Jones, Joyce; Kis, Zoltan; Bahgat, Verina; Soliman,Atef; Elassal, Emad; Ahmed, Lu'ay; Gaynor, Anne; Cornelius, Claire; Davis,Todd
A/White-fronted_goose/Germany-NI/R482/09_H1N1	EPI248525	EPI248524	Germany	2009*	-	Friedrich-Loeffler-Institut	-
A/Domestic_goose/Germany-MV/R3298/2009_H6N1		EPI339177	Germany	2009*	-	Friedrich-Loeffler-Institut	-
A/Mallard/Italy/4518/2007_H10N1		EPI511812	Italy	2007*	Istituto Zooprofilattico Sperimentale Delle Venezie	Istituto Zooprofilattico Sperimentale Delle Venezie	-
A/Wild_duck/Germany/WV2555/2006_H3N2		EPI185342	Germany	2006*	-	Friedrich-Loeffler-Institut	-
A/Pheasant/Ireland/PV12-010728/12_H5N2		EPI375596	Ireland	24-Mar-2012	Central Veterinary Research Laboratory	Animal and Plant Health Agency (APHA)	Puranik, A; Thomas, S; Hanna, A; Essen, S; Focosi-Snyman, R; Manvell, R.J; Raleigh, P; Flynn, O; Reid, S
A/Chicken/Italy/11VIR-7548/2011_H5N2		EPI464929	Italy	28-Dec-2012	Istituto Zooprofilattico Sperimentale Delle Venezie	Istituto Zooprofilattico Sperimentale Delle Venezie	Monne, I.; Salviato, A.; Tassoni, L.; Cattoli, G.
A/Turkey/Italy/12VIR-6607-5/2012_H5N2		EPI464937	Italy	31-Aug-2012	Istituto Zooprofilattico Sperimentale Delle Venezie	Istituto Zooprofilattico Sperimentale Delle Venezie	Monne, I.; Salviato, A.; Tassoni, L.; Cattoli, G.
A/Chicken/Italy/12VIR-7785-67/2012_H5N2		EPI464945	Italy	28-Sep-2012	Istituto Zooprofilattico Sperimentale Delle Venezie	Istituto Zooprofilattico Sperimentale Delle Venezie	Monne, I.; Salviato, A.; Tassoni, L.; Cattoli, G.
A/Turkey/Italy/12VIR-8036-2/2012_H5N2		EPI464953	Italy	28-Jun-2012	Istituto Zooprofilattico Sperimentale Delle Venezie	Istituto Zooprofilattico Sperimentale Delle Venezie	Monne, I.; Salviato, A.; Tassoni, L.; Cattoli, G.
A/Mule_duck/Bulgaria/61/2010_mixed		EPI574173	Bulgaria	10-Jan-2010	-	Other database import	Marinove-Petkova,A.; Georgiev,G.; Petkov,T.; Darnell,D.; Franks,J.; Walker,D.; Seiler,P.; Danner,A.; Graham,A.; McKenzie,P.; Krauss,S.; Webby,R.J.; Webster,R.G.
A/Mule_duck/Bulgaria/64/2010_mixed		EPI574211	Bulgaria	9-Jan-2010	-	Other database import	Marinove-Petkova,A.; Georgiev,G.; Petkov,T.; Darnell,D.; Franks,J.; Walker,D.; Seiler,P.; Danner,A.; Graham,A.; McKenzie,P.; Krauss,S.; Webby,R.J.; Webster,R.G.
A/Mule_duck/Bulgaria/369/2009_H4N2		EPI574213	Bulgaria	16-Mar-2009	-	Other database import	Marinove-Petkova,A.; Georgiev,G.; Petkov,T.; Darnell,D.; Franks,J.; Walker,D.; Seiler,P.; Danner,A.; Graham,A.; McKenzie,P.; Krauss,S.; Webby,R.J.; Webster,R.G.
A/Mule_duck/Bulgaria/105/2008_mixed		EPI574258	Bulgaria	16-Dec-2008	-	Other database import	Marinove-Petkova,A.; Georgiev,G.; Petkov,T.; Darnell,D.; Franks,J.; Walker,D.; Seiler,P.; Danner,A.; Graham,A.; McKenzie,P.; Krauss,S.; Webby,R.J.; Webster,R.G.
A/Mule_duck/Bulgaria/174/2009_H6N2		EPI574265	Bulgaria	12-Jan-2009	-	Other database import	Marinove-Petkova,A.; Georgiev,G.; Petkov,T.; Darnell,D.; Franks,J.; Walker,D.; Seiler,P.; Danner,A.; Graham,A.; McKenzie,P.; Krauss,S.; Webby,R.J.; Webster,R.G.
A/Mule_duck/Bulgaria/596/2010_H4N2		EPI574276	Bulgaria	29-Mar-2010	-	Other database import	Marinove-Petkova,A.; Georgiev,G.; Petkov,T.; Darnell,D.; Franks,J.; Walker,D.; Seiler,P.; Danner,A.; Graham,A.; McKenzie,P.; Krauss,S.; Webby,R.J.; Webster,R.G.
A/Ruddy_Turnstone/Delaware/67/98_H12N4		EPI16616	USA	1998*	-	Other database import	-
A/Pintail/Alaska/314/2005_H12N4		EPI307548	USA	11-Aug-2005	-	Other database import	The NIAID Influenza Genome Sequencing Consortium
A/Red_knot/Delaware_Bay/227/1994_mixed		EPI345275	USA	23-May-1994	-	Other database import	The NIAID Influenza Genome Sequencing Consortium
A/Shorebird/Delaware_Bay/215/1994_mixed		EPI437174	USA	23-May-1994	-	Other database import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; Ghedin,E.; Fedorova,N.; Overton,L.; Tsitrin,T.; Stockwell,T.; Amedeo,P.; Bishop,B.; Chen,H.; Edworthy,P.; Gupta,N.; Katzel,D.; Li,K.; Schobel,S.; Shrivastava,S.; Thovarai,V.; Wang,S.; Runstadler,J.; Lindberg,M.; Huettmann,F.; Petrula,M.; Meixell,B.; Gingrich,J.P.; Gildehaus,L.A.; Vick,L.; Kokx,K.; Dillon,D.; Aldehoff,F.; Felker,E.P.; Marcotte,R.W.; Schmidt,J.C.; Moore,J.R.; Gerdes,K.E.; Bao,Y.; Sanders,R.; Dernovoy,D.; Kiryutin,B.; Lipman,D.J.; Tatusova,T.
A/Ruddy_turnstone/Delaware_Bay/124/1994_mixed		EPI437442	USA	22-May-1994	-	Other database import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; Ghedin,E.; Fedorova,N.; Overton,L.; Tsitrin,T.; Stockwell,T.; Amedeo,P.; Bishop,B.; Chen,H.; Edworthy,P.; Gupta,N.; Katzel,D.; Li,K.; Schobel,S.; Shrivastava,S.; Thovarai,V.; Wang,S.; Runstadler,J.; Lindberg,M.; Huettmann,F.; Petrula,M.; Meixell,B.; Gingrich,J.P.; Gildehaus,L.A.; Vick,L.; Kokx,K.; Dillon,D.; Aldehoff,F.; Felker,E.P.; Marcotte,R.W.; Schmidt,J.C.; Moore,J.R.; Gerdes,K.E.; Bao,Y.; Sanders,R.; Dernovoy,D.; Kiryutin,B.; Lipman,D.J.; Tatusova,T.
A/Ruddy_turnstone/Delaware_Bay/150/1994_H1N4		EPI437470	USA	22-May-1994	-	Other database import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; Ghedin,E.; Fedorova,N.; Overton,L.; Tsitrin,T.; Stockwell,T.; Amedeo,P.; Bishop,B.; Chen,H.; Edworthy,P.; Gupta,N.; Katzel,D.; Li,K.; Schobel,S.; Shrivastava,S.; Thovarai,V.; Wang,S.; Runstadler,J.; Lindberg,M.; Huettmann,F.; Petrula,M.; Meixell,B.; Gingrich,J.P.; Gildehaus,L.A.; Vick,L.; Kokx,K.; Dillon,D.; Aldehoff,F.; Felker,E.P.; Marcotte,R.W.; Schmidt,J.C.; Moore,J.R.; Gerdes,K.E.; Bao,Y.; Sanders,R.; Dernovoy,D.; Kiryutin,B.; Lipman,D.J.; Tatusova,T.
A/Blue-winged_teal/ALB/685/1982_H6N4		EPI85929	Canada	20-Aug-1982	-	Other database import	-
A/Mallard_duck/Alberta/299/1977_H4N4		EPI87231	Canada	10-Aug-1977	-	Other database import	-
A/Mallard/Alberta/194/1992_H8N4		EPI87925	Canada	1-Dec-1992	-	Other database import	-
A/Duck/Hokkaido/24/04_H10N5		EPI160652	Japan	2004*	-	Other database import	-
A/Mallard/Denmark/77-64590-5/2005_H7N5		EPI174859	Denmark	19-Sep-2005	Technical University of Denmark	National Veterinary Institute	-
A/Mallard/California/6524/2008_H12N5		EPI328292	USA	5-Nov-2008	-	Other database import	The NIAID Influenza Genome Sequencing Consortium
A/Mallard/Alberta/220/2006_		EPI343416	Canada	9-Aug-2006	-	Other database import	The NIAID Influenza Genome Sequencing Consortium
A/Mallard/Alberta/12/1993_		EPI344668	Canada	20-Aug-1993	-	Other database import	The NIAID Influenza Genome Sequencing Consortium
A/Arenaria_interpres/Belgium/02936pcs1/2010_H12N5		EPI345387	Belgium	27-Feb-2010	Veterinary and Agrochemical Research Institute	Veterinary and Agrochemical Research Institute	Van Borm,S.; Rosseel,T.;Lambrecht,B.; Vangeluwe,D.; Vandenbussche,F.; van den Berg,T.
A/Mallard/Ohio/170/1999_H6N5		EPI44086	USA	23-Oct-1999	-	Other database import	-
A/Emperor_goose/Alaska/44064-075/2006_H2N5		EPI442443	USA	25-May-2006	-	Other database import	Reeves,A.B.; Pearce,J.M.; Ramey,A.M.; Ely,C.R.; Schmutz,J.A.; Flint,P.L.; Derksen,D.V.; Ip,H.S.; Trust,K.A.
A/Mallard/Minnesota/182729/1998_H6N5		EPI448392	USA	1-Sep-1998	-	Other database import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; Wester,E.; Ghedin,E.; Fedorova,N.; Tsitrin,T.; Stockwell,T.; Amedeo,P.; Bishop,B.; Edworthy,P.; Gupta,N.; Katzel,D.; Li,K.; Schobel,S.; Shrivastava,S.; Thovarai,V.; Wang,S.; Lebarbenchon,C.; Sreevatsan,S.; Poulson,B.; Yang,M.; Stallknecht,D.; Bao,Y.; Sanders,R.; Dernovoy,D.; Kiryutin,B.; Lipman,D.J.; Tatusova,T.
A/Green-winged_teal/Minnesota/Sg-00820/2008_H4N5		EPI449492	USA	3-Sep-2008	-	Other database import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; Wester,E.; Ghedin,E.; Fedorova,N.; Tsitrin,T.; Stockwell,T.; Amedeo,P.; Bishop,B.; Edworthy,P.; Gupta,N.; Katzel,D.; Li,K.; Schobel,S.; Shrivastava,S.; Thovarai,V.; Wang,S.; Lebarbenchon,C.; Sreevatsan,S.; Poulson,B.; Yang,M.; Stallknecht,D.; Bao,Y.; Sanders,R.; Dernovoy,D.; Kiryutin,B.; Lipman,D.J.; Tatusova,T.
A/Ruddy_turnstone/New_Jersey/AI07-697/2007_H12N5		EPI454987	USA	10-Aug-2007	-	Other database import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; Wester,E.; Ghedin,E.; Fedorova,N.; Tsitrin,T.; Stockwell,T.; Amedeo,P.; Bishop,B.; Edworthy,P.; Gupta,N.; Katzel,D.; Li,K.; Schobel,S.; Shrivastava,S.; Thovarai,V.; Wang,S.; Lebarbenchon,C.; Sreevatsan,S.; Poulson,B.; Yang,M.; Stallknecht,D.; Bao,Y.; Sanders,R.; Dernovoy,D.; Kiryutin,B.; Lipman,D.J.; Tatusova,T.
A/Ruddy_turnstone/New_Jersey/AI07-803/2007_H12N5		EPI455001	USA	16-Aug-2007	-	Other database import	Wentworth,D.E.; Dugan,V.; Halpin,R.; Lin,X.; Bera,J.; Wester,E.; Ghedin,E.; Fedorova,N.; Tsitrin,T.; Stockwell,T.; Amedeo,P.; Bishop,B.; Edworthy,P.; Gupta,N.; Katzel,D.; Li,K.; Schobel,S.; Shrivastava,S.; Thovarai,V.; Wang,S.; Lebarbenchon,C.; Sreevatsan,S.; Poulson,B.; Yang,M.; Stallknecht,D.; Bao,Y.; Sanders,R.; Dernovoy,D.; Kiryutin,B.; Lipman,D.J.; Tatusova,T.
A/Mule_duck/Bulgaria/674/2010_H6N5		EPI574266	Bulgaria	12-Apr-2010	-	Other database import	Marinove-Petkova,A.; Georgiev,G.; Petkov,T.; Darnell,D.; Franks,J.; Walker,D.; Seiler,P.; Danner,A.; Graham,A.; McKenzie,P.; Krauss,S.; Webby,R.J.; Webster,R.G.

Isolate name	Accession number HA segment	Accession number NA segment	Country	Collection Date	Originating Laboratory	Submitting Laboratory	Authors
A/Mallard/Alberta/202/1996_H2N5		EPI85385	Canada	1996*	-	Other database import	-
A/Pintail/Alberta/49/2003_H9N5		EPI86317	Canada	2003*	-	Other database import	-
A/Mallard/Alberta/52/1997_H12N5		EPI86417	Canada	31-Oct-1997	-	Other database import	-
A/Green-winged_teal/ALB/199/1991_H12N5		EPI88775	Canada	26-Aug-1991	-	Other database import	-
A/Mallard/Poland/16/09_H7N7		EPI254380	Poland	12-Jan-2009	National Veterinary Research Institute	National Veterinary Research Institute	-
A/Turkey/Netherlands/03003568/03_H7N7		EPI290239	Netherlands	9-Mar-2003	-	Central Veterinary Institute	-
A/Chicken/Germany/R1801/2011_H7N7		EPI356304	Germany	2011*	-	Friedrich-Loeffler-Institut	-
A/Turkey/Germany/R1775/2011_H7N7		EPI356305	Germany	2011*	-	Friedrich-Loeffler-Institut	-
A/Turkey/Germany-NI/R534/2013_H7N7		EPI470367	Germany	11-Apr-2013	-	Friedrich-Loeffler-Institut	-

CHAPTER 3.3
Josanne H. Verhagen, Sander Herfst
Ron A.M. Fouchier

How a virus travels the world: Wild birds may spread the H5N8 virus

Perspective in *Science* (2015), Volume 347, Issue 6222: 623-625. DOI: 10.1126/science.aaa6724

In November and December 2014, highly pathogenic avian influenza (HPAI) viruses of the H5 subtype originating from China were detected in poultry and wild birds in various countries of Asia and Europe, and, for the first time, in North America. These incursions of newly emerging HPAI H5 viruses constitute a threat to animal and potentially human health and raise questions about the routes of transmission.

Wild birds of the orders Anseriformes (ducks, geese, swans) and Charadriiformes (gulls, terns, waders) are the natural reservoir for low pathogenic avian influenza (LPAI) viruses. On the basis of viral hemagglutinin (HA) and neuraminidase (NA) pro- teins, these viruses are classified into 16 HA subtypes and nine NA subtypes, found in numerous combinations such as H5N1 and H5N8. LPAI viruses generally do not cause substantial disease in wild birds and poultry. However, viruses of subtypes H5 and H7 can evolve into HPAI viruses upon introduction into poultry, causing up to 100% mortality in poultry species. Historically, HPAI outbreaks in poultry have been controlled rapidly by methods such as mass culling. However, since 1997, HPAI

H5N1 viruses that share a common ancestral virus (A/Goose/Guangdong/1/96, GsGd) have continued to cause outbreaks in poultry populations. These outbreaks were associated with the first recorded cases of human infections with H5 influenza viruses and with spillback of HPAI viruses to wild birds.

HPAI H5N1 viruses of the GsGd lineage were first detected in poultry in Hong Kong in 1997. They resurfaced in 2001 and 2002, with frequent outbreaks in poultry in numerous Asian countries since 2003. In 2005, the viruses were detected during mass die- offs of wild birds in Mongolia, followed by reports in poultry and wild birds in Russia and Kazakhstan; the virus then spread across Europe, the Middle East, Asia, and

Africa, in part associated with wild bird migrations. Since then, detections have continued to be reported in poultry and wild birds in Eurasia, with the most recent outbreaks occurring in Egypt and southeastern parts of Asia. Since 2003, 694 laboratory-confirmed human cases of H5N1 virus infection have been reported to the World Health Organization, including 402 fatalities (270).

During the initial circulation and spread of the H5N1 viruses, the HA genes diversified into multiple genetic lineages ("clades"), without evidence of gene exchange between the influenza viruses (271). However, this changed from 2009 onward, when HPAI viruses of subtypes H5N2, H5N5, H5N6, and H5N8 were found to contain the H5 gene of the GsGd lineage, together with NA and various other genes of LPAI virus origin (272-275). After numerous poultry outbreaks in eastern Asia and occasional detection in wild birds, these viruses spread into Europe and North America by December 2014 (276). H5 outbreaks were also reported from Africa and the Middle East, but whether these were also caused by H5 viruses of the GsGd lineage needs to be confirmed (277).

What explains the sudden global spread of this H5 lineage? The timing and direction of intercontinental spread coincided with fall bird migration out of Russia; the H5N8 virus was identified in a long-distance migrant bird in Russia in September 2014 and subsequently in Japan, Germany, and the Netherlands (278) and the western United States (277), which suggests that wild birds carried the virus out of Russia into other parts of the world. So far, HPAI H5N8 virus of the GsGd lineage has been isolated exclusively from wild birds of the orders Anseriformes and Gruiformes (coots and cranes). However, given the subclinical infections in some species, other wild birds may also be susceptible. H5-specific antibodies have been detected in 10 to 53% of common teals, mallards, spot-billed ducks, Eurasian wigeons, and Baikal teals (279); these findings suggest that the virus has circulated for some time in ducks that survived infection, and this may play a role in HPAI H5N8 virus epidemiology. The almost simultaneous detection of HPAI H5 viruses in wild birds and poultry in Asia, Europe, and North America suggests that the virus was potentially introduced from a relatively large region in Russia (Figure 1).

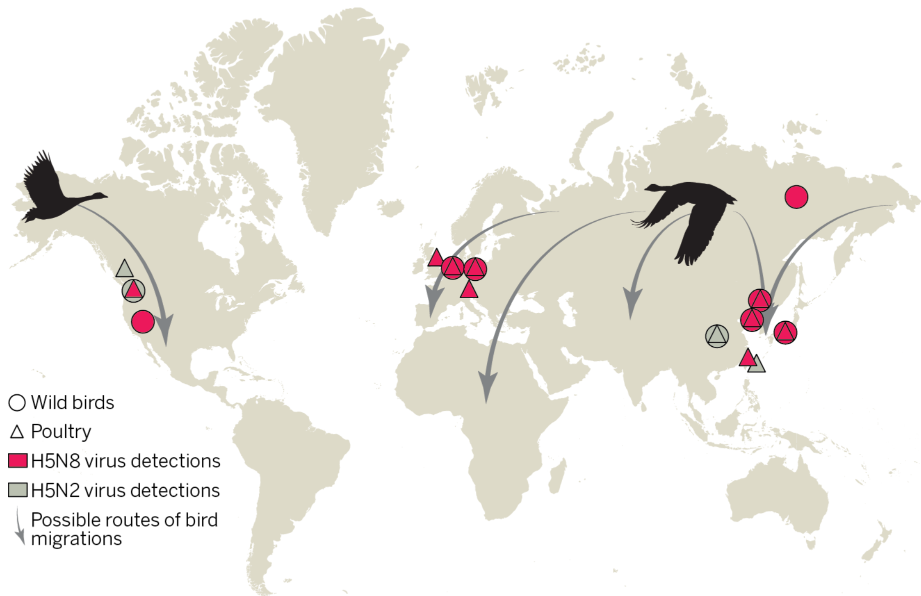


Figure 1. H5N2 and H5N8 virus detections in poultry and wild birds in 2014. The almost simultaneous detection of closely related viruses in Asia, Europe, and North America suggests linkage with wild bird migration via a large region in Russia. Data from (276).

The HPAI H5N8 viruses in domestic and wild birds share a common ancestor and contain HA and a polymerase gene (PB2) derived from viruses of the GsGd H5 lineage (280). The increased geographical spread of HPAI H5 viruses and the isolation of H5N8 from live wild birds suggest that this lineage may have evolved to be better adapted to wild birds than are other poultry influenza viruses, supporting its spread.

Ferrets are frequently used to study virus replication, pathogenesis, and transmissibility as a means of assessing potential public health risk upon human exposure to virus- infected poultry, wild birds, or other animals (281, 282). Upon inoculation with HPAI H5N8 virus A/Mallard/Korea/W452/2014, ferrets did not develop any remarkable signs of illness (280). The H5N8-inoculated animals did not lose weight, in contrast with animals inoculated with human seasonal influenza viruses or H5N1 virus (282). The HPAI H5N8 virus replicated mainly in the respiratory tract of ferrets (280); this is in contrast to HPAI H5N1 viruses, which replicate abundantly in extra-respiratory organs.

Influenza viruses are mainly transmitted between humans via respiratory droplets or aerosols. In the ferret model, low, short-term shedding of A/Mallard/Korea/W452/2014 was observed from the upper respiratory tract of inoculated animals, which did not transmit the virus via the airborne route or direct contact to naïve ferrets

(280). H5N8 viruses A/Duck/Shandong/Q1/2013 and A/Duck/Jiangsu/k1203/2010 did not transmit through direct contact between guinea pigs (283). Pathogenicity and transmission in animal models may not be directly extrapolated to humans, but these data suggest that the public health threat of the currently circulating HPAI H5N8 strains is low.

Vaccination and antiviral therapy are the main options for preventing human influenza virus infections. Several H5 candidate influenza vaccine strains are available but are unlikely to provide sufficient protection against H5N8 virus (284). HPAI H5N8 virus A/ Mallard/Korea/W452/2014 was found to be sensitive to oseltamivir, zanamivir, and peramivir (280), which suggests that drugs can be used prophylactically or therapeutically if the need arises.

The presence of HPAI H5 viruses in migrating birds and the dispersed spatial pattern of virus detections globally are worrisome; more poultry outbreaks could occur in the future, especially in countries that are ill-prepared. Despite the currently low public health risk, the outbreaks should be monitored closely, given that several animal species are susceptible (280) and that influenza viruses are generally unpredictable. Wild birds covering multiple migratory flyways should be monitored for virus presence and for H5-specific antibodies as a cost-effective alternative to measure circulation of viruses of the GsGd H5 lineage (285). Control measures and research priorities aimed at eradicating HPAI H5 viruses from poultry populations should be redefined, as current strategies appear to be insufficient.

Josanne H. Verhagen, Henk P. van der Jeugd, Bart A. Nolet, Roy Slaterus
Sergey P. Kharitonov, Peter P. de Vries, Oanh Vuong
Frank Majoor, Thijs Kuiken
Ron A.M. Fouchier

Wild bird surveillance around outbreaks of highly pathogenic avian influenza A(H5N8) virus in the Netherlands, 2014, within the context of global flyways

Eurosurveillance (2015), Volume 20, Issue 12: pii=21069.

Highly pathogenic avian influenza (HPAI) A(H5N8) viruses that emerged in poultry in east Asia since 2010 spread to Europe and North America by late 2014. Despite detections in migrating birds, the role of free-living wild birds in the global dispersal of H5N8 virus is unclear. Here, wild bird sampling activities in response to the H5N8 virus outbreaks in poultry in the Netherlands are summarised along with a review on ring recoveries. HPAI H5N8 virus was detected exclusively in two samples from ducks of the Eurasian wig- eon species, among 4,018 birds sampled within a three months period from mid-November 2014. The H5N8 viruses isolated from wild birds in the Netherlands were genetically closely related to and had the same gene constellation as H5N8 viruses detected elsewhere in Europe, in Asia and in North America, suggesting a common origin. Ring recoveries of migratory duck species from which H5N8 viruses have been isolated overall provide evidence for indirect migratory connections between East Asia and Western Europe and between East Asia and North America. This study is useful for better understanding the role of wild birds in

the global epidemiology of H5N8 viruses. The need for sampling large numbers of wild birds for the detection of H5N8 virus and H5N8-virus-specific antibodies in a variety of species globally is highlighted, with specific emphasis in north-eastern Europe, Russia and northern China.

INTRODUCTION

Wild aquatic birds are the natural reservoir for low pathogenic avian influenza A (LPAI) viruses, which are classified based on their surface proteins haemagglutinin (HA, H1–H16) and neuraminidase (NA, N1–N9) (12, 21). These viruses can be carried over long distances along migratory flyways (97, 145, 286). LPAI viruses of the H5 and H7 subtype can evolve into highly pathogenic avian influenza (HPAI) viruses upon introduction into poultry. HPAI H5N8 viruses, such as A/duck/Jiangsu/ k1203/2010, were first detected in birds on live bird markets in China in 2010 (274). These H5N8 viruses contain genes derived from HPAI H5N1 viruses of the so-called A/Goose/Guangdong/1/1996 (GsGd) lineage (280) that have caused outbreaks in numerous countries of the eastern hemisphere since 1997.

In January 2014, HPAI H5N8 viruses were detected in South Korea, where they infected birds of 161 poultry farms and resulted in the culling of 14 million poultry by September 2014 (279). In April 2014, HPAI H5N8 virus was detected on a chicken farm in Japan. Over the summer of 2014, no new cases were reported outside South Korea. In September, HPAI H5N8 virus was detected in China in a domestic duck and an environmental sample. During the same month, H5N8 virus was also detected in north-eastern Russia in a Eurasian wigeon (*Anas penelope*). From November 2014 to February 2015, HPAI H5N8 virus has been found in poultry and/or free-living wild birds in Asia (Japan and Taiwan), Europe (Germany, Hungary, Italy, the Netherlands and the United Kingdom (UK)), and North America (US) (270, 278). HPAI H5N8 virus was also detected in captive wild birds: dead gyrfalcons (*Falco rusticolus*) in the north west of the United States (US) and white storks (*Ciconia ciconia*) in a zoo in Germany (Table 1) (277). The HA of HPAI H5N8 viruses detected in domestic and wild birds in Asia, Europe and North America belonged to the GsGd H5 clade 2.3.4.4 (271). Genetic closely related H5N8 viruses belonging to the same GsGd H5 clade 2.3.4.4 were detected in China since 2010.

So far, HPAI H5N8 virus has been isolated from free-living wild birds of the orders Accipitriformes, Anseriformes, Charadriiformes, Falconiformes and Gruiformes in several countries including Germany, Japan, Russia, South Korea, Taiwan, the Netherlands, and the US (Table 1). In live wild birds, H5N8 virus detections were limited to ducks (order: Anseriformes) of the species common teal (*Anas crecca*), mallard (*Anas platyrhynchos*),

spot-billed duck (*Anas poecilorhyncha*), Eurasian wigeon, American wigeon (*Anas americana*) and gadwall (*Anas strepera*) (270, 279) (Table 1). In addition, H5N8-virus-specific antibodies were detected in 10 to 53% of ducks of the species Baikal teal (*Anas formosa*), common teal, mallard, Eurasian wigeon and spot-billed duck in South Korea (279), suggesting that this virus had been circulating in these species for some time and that these individual birds had survived infection and thus may have played a role in the dispersal of H5N8. Wild ducks of some species (e.g. *Anas* species) may be less likely to exhibit clinical signs when infected with HPAI H5N8 than e.g. geese, swans and cranes; alternatively, ducks are more intensively hunted and sampled, potentially explaining a higher detection rate of H5N8 in live wild ducks than in other wild bird species. Despite H5N8 virus detections in a range of wild bird species globally, it is unknown to what extent these viruses circulate in wild bird populations in Europe.

This study presents data on wild bird surveillance activities in the Netherlands that were intensified in the country, in response to the HPAI H5N8 virus outbreaks on poultry farms at the end of 2014. We present our findings in the perspective of the distribution and migratory flyways of H5N8-virus-positive bird species.

METHODS

Sampling wild birds

After detection of HPAI H5N8 virus on a chicken farm in the Netherlands on 14 November 2014, sampling of live wild birds of various species was intensified in the country in an attempt to detect H5N8 virus. Birds were captured using duck decoys, clap nets, mist nets, noose or by hand. Capturing of wild birds was approved by the Dutch Ministry of Economic Affairs based on the Flora and Fauna Act (permit number FF/75A/2009/067 and FF/75A/2014/054). Handling and sampling of wild birds were approved by the Animal Experiment Committee of the Erasmus MC (permit number 122–11–31). Sampling activities targeted long-distance migratory bird species and/or bird species that had been found infected with HPAI H5N8 virus earlier in 2014, e.g. Bewick's swan (Cygnus columbianus bewickii) in Japan. Sample locations were both within and outside a 10 km radius of Dutch poultry farms where H5N8-virus-infections had been detected and varied in function of the distribution of wild bird species of interest combined with capture opportunities. Disposable gloves and disinfectants for boots and equipment (Virkon S) were used. Birds were sampled for virus detection by collecting samples from cloaca, from both cloaca and oropharynx, or from fresh faeces as described by Munster et al. (151). For cloaca and oropharynx samples, the number of tested birds depended on the bird species, capture method and capture success. For fresh faeces, swab samples

Table 1. Global detection of highly pathogenic avian influenza A(H5N8) virus and other viruses belonging to the H5 clade 2.3.4.4 in wild birds and poultry, 2014. Data from (276, 279, 287).

Host type	Order	Family	Poultry type or bird species	AIV subtype	Country
Captive	Anseriformes	Ducks	Domestic duck	H5N2; H5N3 H5N8	Canada (H5N2); China (H5N2; H5N8); Hungary (H5N8); Netherlands (H5N8); South Korea (H5N8); Taiwan (H5N2; H5N3; H5N8); UK (H5N8); USA (H5N2; H5N8)
		Geese	Domestic goose	H5N2; H5N3 H5N8	Canada (H5N2); South Korea (H5N8); Taiwan (H5N2; H5N3; H5N8); USA (H5N2, H5N8)
	Galliformes	Chickens	Chicken	H5N1; H5N2 H5N3; H5N8	Canada (H5N1, H5N2); China (H5N2); Japan (H5N8); Netherlands (H5N8); South Korea (H5N8); Taiwan (H5N2; H5N3; H5N8); USA (H5N2, H5N8)
		Turkeys	Domestic turkey	H5N2; H5N3 H5N8	Canada (H5N2); Germany (H5N8); Italy (H5N8); Taiwan (H5N2; H5N8); USA (H5N2; H5N8); Canada (H5N2)
	Ciconiiformes	Storks	White stork (*Ciconia ciconia*)	H5N8	Germany
	Falconiformes	Falcons	Gyrfalcon (*Falco rusticolus*)	H5N8	USA
			Peregrine falcon (*Falco peregrinus*)	H5N2	USA
	Strigiformes	Owls	Great horned owl (*Bubo virginianus*)	H5N2	USA
Wild	Accipitriformes	Eagles	Bald eagle (*Haliaeetus leucocephalus*)	H5N8	USA
		Hawks	Cooper's hawk (*Accipiter cooperii*)	H5N2	USA
			Red-tailed hawk (*Buteo jamaicensis*)	H5N2	USA
	Anseriformes	Ducks	Baikal teal (*Anas formosa*)	H5N8	South Korea
			Mallard (*Anas platyrhynchos*)	H5N2; H5N8	Germany (H5N8); Japan (H5N8); South Korea (H5N8); USA (H5N2)
			Common teal (*Anas crecca*)	H5N8	Germany; South Korea
			Green-winged teal (*Anas carolinensis*)	H5N1; H5N8	USA
			Spot-billed duck (*Anas poecilorhyncha*)	H5N8	South Korea
			Eurasian wigeon (*Anas penelope*)	H5N8	Netherlands; Russia
			Northern pintail (*Anas acuta*)	H5N2	USA
			Mandarin duck (*Aix galericulata*)	H5N8	Japan

Host type	Order	Family	Poultry type or bird species	AIV subtype	Country
			Gadwall (*Anas strepera*)	H5N8	USA
			American wigeon (*Anas americana*)	H5N8	USA
			Wood duck (*Aix sponsa*)	H5N2	USA
			Northern shoveler (*Anas clypeata*)	H5N2	USA
		Geese	Bean goose (*Anser fabalis*)	H5N8	South Korea
			White-fronted goose (*Anser albifrons*)	H5N8	South Korea
		Swans	Bewick's swan (*Cygnus columbianus bewickii*)	H5N8	Japan; South Korea
	Charadriiformes	Gulls	Great black-backed gull (*Larus marinus*)	H5N8	Germany
	Falconiformes	Falcons	Peregrine falcon (*Falco peregrinus*)	H5N8	USA
	Gruiformes	Cranes	White-naped crane (*Grus vipio*)	H5N8	Japan
			Hooded crane (*Grus monacha*)	H5N8	Japan
		Coots	Common coot (*Fulica atra*)	H5N8	South Korea
	Passeriformes	Bulbuls	Light-vented bulbul (*Pycnonotus sinensis*)	H5N3	Taiwan
	Pelecaniformes	Herons	Black-crowned night-heron (*Nycticorax nycticorax*)	H5N2	Taiwan

Table 2. Information on influenza A virus sequences obtained from the Global Initiative on Sharing Avian Influenza Data used for the study. Seg., segment.

Segment ID	Seg.	Country	Collection date	Isolate name	Originating laboratory	Submitting laboratory	Authors
EPI552760	HA	Netherlands	2014-Nov-24	A/eurasian wigeon/Netherlands/emc-1/2014 (H5N8)	Erasmus MC	Erasmus MC	Fouchier *et al.*
EPI552762	NA	Netherlands	2014-Nov-24	A/eurasian wigeon/Netherlands/emc-1/2014 (H5N8)	Erasmus MC	Erasmus MC	Fouchier *et al.*
EPI552768	HA	Netherlands	2014-Nov-24	A/eurasian wigeon/Netherlands/emc-2/2014 (H5N8)	Erasmus MC	Erasmus MC	Fouchier *et al.*
EPI552770	NA	Netherlands	2014-Nov-24	A/eurasian wigeon/Netherlands/emc-2/2014 (H5N8)	Erasmus MC	Erasmus MC	Fouchier *et al.*
EPI552776	HA	Netherlands	2014-Nov-21	A/chicken/Netherlands/emc-3/2014 (H5N8)	Erasmus MC	Erasmus MC	Fouchier *et al.*
EPI552778	NA	Netherlands	2014-Nov-21	A/chicken/Netherlands/emc-3/2014 (H5N8)	Erasmus MC	Erasmus MC	Fouchier *et al.*
EPI547678	HA	Netherlands	2014-Nov-14	A/Chicken/Netherlands/14015526/2014 (H5N8)	Central Veterinary Institute	Central Veterinary Institute	Heutink *et al.*

Segment ID	Seg.	Country	Collection date	Isolate name	Originating laboratory	Submitting laboratory	Authors
EPI547683	NA	Netherlands	2014-Nov-14	A/Chicken/Netherlands/14015526/2014 (H5N8)	Central Veterinary Institute	Central Veterinary Institute	Heutink *et al.*
EPI548623	HA	Netherlands	2014-Nov-15	A/chicken/Netherlands/14015531/2014 (H5N8)	Central Veterinary Institute	Central Veterinary Institute	Heutink *et al.*
EPI548626	NA	Netherlands	2014-Nov-15	A/chicken/Netherlands/14015531/2014 (H5N8)	Central Veterinary Institute	Central Veterinary Institute	Heutink *et al.*
EPI544756	HA	Germany	2014-Nov-04	A/turkey/Germany-MV/R2472/2014 (H5N8)	Friedrich-Loeffler-Institut	Friedrich-Loeffler-Institut	NA
EPI544759	NA	Germany	2014-Nov-04	A/turkey/Germany-MV/R2472/2014 (H5N8)	Friedrich-Loeffler-Institut	Friedrich-Loeffler-Institut	NA
EPI552746	HA	Germany	2014-Nov-04	A/turkey/Germany/R2474-L00899/2014 (H5N8)	Friedrich-Loeffler-Institut	Friedrich-Loeffler-Institut	NA
EPI552748	NA	Germany	2014-Nov-04	A/turkey/Germany/R2474-L00899/2014 (H5N8)	Friedrich-Loeffler-Institut	Friedrich-Loeffler-Institut	NA
EPI547673	HA	United Kingdom	2014-Nov-14	A/duck/England/36254/14 (H5N8)	Animal and Plant Health Agency (APHA)	Animal and Plant Health Agency (APHA)	Hanna *et al.*
EPI547675	NA	United Kingdom	2014-Nov-14	A/duck/England/36254/14 (H5N8)	Animal and Plant Health Agency (APHA)	Animal and Plant Health Agency (APHA)	Hanna *et al.*
EPI553144	HA	Italy	2014-Dec-15	A/turkey/Italy/14VIR7898-10/2014 (H5N8)	Istituto Zooprofilattico Sperimentale Delle Venezie	Istituto Zooprofilattico Sperimentale Delle Venezie	Luca *et al.*
EPI555068	NA	Italy	2014-Dec-15	A/turkey/Italy/14VIR7898-10/2014 (H5N8)	Istituto Zooprofilattico Sperimentale Delle Venezie	Istituto Zooprofilattico Sperimentale Delle Venezie	Luca *et al.*
EPI553349	HA	Russia	2014-Sep-25	A/wigeon/Sakha/1/2014 (H5N8)	State Research Center of Virology and Biotechnology Vector	State Research Center of Virology and Biotechnology Vector	Susloparov *et al.*
EPI553350	NA	Russia	2014-Sep-25	A/wigeon/Sakha/1/2014 (H5N8)	State Research Center of Virology and Biotechnology Vector	State Research Center of Virology and Biotechnology Vector	Susloparov *et al.*
EPI548485	HA	Japan	2014-Nov-18	A/duck/Chiba/26-372-48/2014 (H5N8)	National Institute of Animal Health	National Institute of Animal Health	NA
EPI548487	NA	Japan	2014-Nov-18	A/duck/Chiba/26-372-48/2014 (H5N8)	National Institute of Animal Health	National Institute of Animal Health	NA
EPI548493	HA	Japan	2014-Nov-18	A/duck/Chiba/26-372-61/2014 (H5N8)	National Institute of Animal Health	National Institute of Animal Health	NA
EPI548495	NA	Japan	2014-Nov-18	A/duck/Chiba/26-372-61/2014 (H5N8)	National Institute of Animal Health	National Institute of Animal Health	NA
EPI553208	HA	Japan	2014-Nov-23	A/crane/Kagoshima/KU1/2014 (H5N8)	Kagoshima University	Kagoshima University	NA
EPI553210	NA	Japan	2014-Nov-23	A/crane/Kagoshima/KU1/2014 (H5N8)	Kagoshima University	Kagoshima University	NA
EPI553343	HA	Japan	2014-Dec-16	A/chicken/Miyazaki/7/2014 (H5N8)	National Institute of Animal Health	National Institute of Animal Health	NA
EPI553345	NA	Japan	2014-Dec-16	A/chicken/Miyazaki/7/2014 (H5N8)	National Institute of Animal Health	National Institute of Animal Health	NA
EPI553362	HA	Japan	2014-Dec-01	A/environment/Kagoshima/KU-ngr-H/2014 (H5N8)	Kagoshima University	Kagoshima University	NA
EPI553364	NA	Japan	2014-Dec-01	A/environment/Kagoshima/KU-ngr-H/2014 (H5N8)	Kagoshima University	Kagoshima University	NA
KJ476669	HA	China	2013-Nov-14	A/duck/Zhejiang/W24/2013 (H5N8)	NA	Other database import	Wu *et al.*
KJ476673	NA	China	2013-Nov-14	A/duck/Zhejiang/W24/2013 (H5N8)	NA	Other database import	Wu *et al.*
EPI507673	HA	China	2013-Nov-18	A/mallard_duck/Shanghai/SH-9/2013 (H5N8)	Institute of Military Veterinary, Academy of Military Medical Sciences	Institute of Laboratory Animal Sciences, Chinese Academy	Fan *et al.*

Segment ID	Seg.	Country	Collection date	Isolate name	Originating laboratory	Submitting laboratory	Authors
EPI507675	NA	China	2013-Nov-18	A/mallard_duck/Shanghai/SH-9/2013 (H5N8)	Institute of Military Veterinary, Academy of Military Medical Sciences	Institute of Laboratory Animal Sciences, Chinese Academy	Fan *et al.*
JQ973694	HA	China	2010-Dec-05	A/duck/Jiangsu/k1203/2010 (H5N8)	NA	Other database import	Zhao *et al.*
JQ973696	NA	China	2010-Dec-05	A/duck/Jiangsu/k1203/2010 (H5N8)	NA	Other database import	Zhao *et al.*
KJ413842	HA	South Korea	2014-Jan-17	A/broiler_duck/Korea/Buan2/2014 (H5N8)	NA	Other database import	Lee *et al.*
KJ413844	NA	South Korea	2014-Jan-17	A/broiler_duck/Korea/Buan2/2014 (H5N8)	NA	Other database import	Lee *et al.*
KJ413850	HA	South Korea	2014-Jan-17	A/baikal_teal/Korea/Donglim3/2014 (H5N8)	NA	Other database import	Lee *et al.*
KJ413852	NA	South Korea	2014-Jan-17	A/baikal_teal/Korea/Donglim3/2014 (H5N8)	NA	Other database import	Lee *et al.*
KJ746111	HA	South Korea	2014-Feb-05	A/mallard/Korea/W452/2014 (H5N8)	NA	Other database import	Choi *et al.*
KJ746113	NA	South Korea	2014-Feb-05	A/mallard/Korea/W452/2014 (H5N8)	NA	Other database import	Choi *et al.*
AJM70554	HA	US	2014-Dec-29	A/American green-winged teal/ Washington/195750/2014 (H5N1)	NA	Diagnostic Virology Laboratory, NVSL, USDA	Killian *et al.*
AJE30344	HA	US	2014-Dec-08	A/Northern pintail/Washington/40964/2014 (H5N2)	NA	Diagnostic Virology Laboratory, NVSL, USDA	Ip *et al.*; Killian *et al.*
AJM70576	HA	US	2014-Dec-16	A/chicken/Oregon/41613-2/2014 (H5N8)	NA	Diagnostic Virology Laboratory, NVSL, USDA	Killian, M.L.
AJM70578	NA	US	2014-Dec-16	A/chicken/Oregon/41613-2/2014 (H5N8)	NA	Diagnostic Virology Laboratory, NVSL, USDA	Killian, M.L.
AJM70609	HA	US	2014-Dec-30	A/chicken/Washington/61-9/2014 (H5N2)	NA	Diagnostic Virology Laboratory, NVSL, USDA	Killian, M.L.
AJM70598	HA	US	2014-Dec-30	A/domestic duck/Washington/61-16/2014 (H5N2)	NA	Diagnostic Virology Laboratory, NVSL, USDA	Killian, M.L.
AJM70565	HA	US	2014-Dec-16	A/guinea fowl/Oregon/41613-1/2014 (H5N8)	NA	Diagnostic Virology Laboratory, NVSL, USDA	Killian, M.L.
AJM70567	NA	US	2014-Dec-16	A/guinea fowl/Oregon/41613-1/2014 (H5N8)	NA	Diagnostic Virology Laboratory, NVSL, USDA	Killian, M.L.
AJE30333	HA	US	2014-Dec-08	A/gyrfalcon/Washington/41088-6/2014 (H5N8)	NA	Diagnostic Virology Laboratory, NVSL, USDA	Ip *et al.*; Killian *et al.*
AJE30335	NA	US	2014-Dec-08	A/gyrfalcon/Washington/41088-6/2014 (H5N8)	NA	Diagnostic Virology Laboratory, NVSL, USDA	Ip *et al.*; Killian *et al.*
AJE30360	HA	Canada	2014-Dec-02	A/turkey/BC/FAV10/2014 (H5N2)	NA	Canadian Food Inspection Agency, National Centre for Foreign Animal Disease	Pasick *et al.*
AJM70587	HA	US	2014-Dec-30	A/turkey/Washington/61-22/2014 (H5N2)	NA	Diagnostic Virology Laboratory, NVSL, USDA	Killian, M.L.

were collected from flocks of single species. The number of faeces droppings sampled per flock was on average less than 40% of the total number of birds in the flock with at least one metre in between each dropping (to limit sampling the same individual twice).

Virus detection, isolation and characterisation

Samples for virus detection were analysed for presence of H5N8 virus using a matrix-specific and H5-specific polymerase chain reaction (PCR) followed by H5 sequencing. Samples that tested positive in matrix-specific PCR were used for virus isolation in embryonated chicken eggs as described previously (151).

Virus sequencing and phylogeny

Of the HPAI H5N8 viruses isolated within this study, the sequences of the complete genome were obtained and deposited in a public database (158, 160). Sequencing was performed using specific primers as described previously (159). Nucleotide (nt) sequences were supplemented with sequences of HPAI H5 viruses of clade 2.3.4.4 detected globally in 2014 and with sequences of HPAI H5N8 viruses detected in China before 2014. These additional sequences were obtained from public databases as of 3 March 2015, which included the Global Initiative on Sharing Avian Influenza Data database (160) (Table 2) and GenBank (158). Sequences retrieved from GenBank had the following accession numbers: AJE30335; AJE30344; AJE30360; AJM70554; AJE30333; AJM70565; AJM70567; AJM70576; AJM70578; AJM70587; AJM70598; AJM70609. Maximum Likelihood (ML) phylogenetic trees were constructed based on the HA gene of 1,545 nt in length (position: 108–1,652) and the NA gene of 1,377 nt in length (position: 1–1,377). ML trees were generated using the PhyML package version 3.1 using the general time-reversible model with the proportion of invariant sites (GTR + I model) of nt substitution, performing a full heuristic search and subtree pruning and regrafting (SPR) searches. The best-fit model of nt substitution was determined with jModelTest (162). The reliability of the phylogenetic grouping was assessed with 1,000 boot- strap replicates. Trees were visualised using Figtree version 1.4.0 (163).

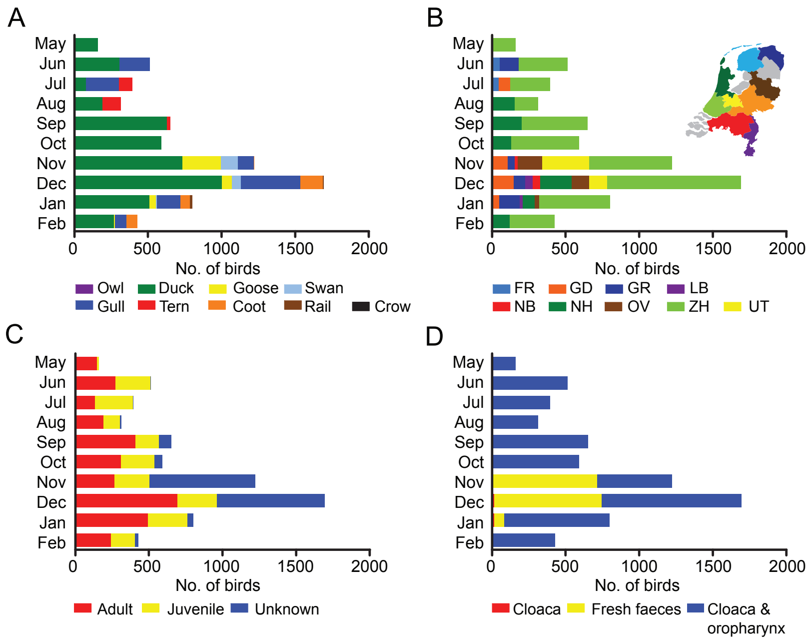


Figure 1. Monthly sampling of wild birds for H5N8 virus detection, by species, location, age, and sample type, the Netherlands, 14 May 2014–20 February 2015 (n = 6,763). FR, Friesland; GD, Gelderland; GR, Groningen; LB, Limburg; NB, Noord-Brabant; NH, Noord-Holland; OV, Overijssel; UT, Utrecht; ZH, Zuid-Holland. Locations were categorised according to Dutch provinces.

RESULTS

Wild bird surveillance activities to detect H5N8 virus in the Netherlands: newly acquired and historical data

Surveillance of avian influenza virus in wild birds in the Netherlands has been in place in the country since 1998. After the first HPAI H5N8 detection in poultry on 14 November 2014, activities to detect the virus were increased and a total of 4,018 wild birds of 25 different species belonging to five orders were sampled (Table 3). Of those, 623 birds (16%) were sampled within 10 km of farms previously affected by HPAI H5N8-virus. In the six months before the first detection of HPAI H5N8 in poultry, a total of 2,745 wild birds of nine different species belonging to three orders had also been sampled for HPAI H5 virus detection (Table 3). Results of the surveillance before and after mid-November 2014 are presented, covering a period from 14 May 2014 to 20 February 2015.

Taking into consideration the whole sampling period (May 2014 to February 2015), most avian influenza viruses were detected in ducks (719 of 4,495; 16%), swans (23 of 183; 13%) and gulls (254 of 1,185; 21%). Avian influenza viruses of the H5 subtype

were detected in common teal, Eurasian wigeon and mallard, whereby most H5 viruses were LPAI viruses (27 of 29; 93%). On 24 November 2014, HPAI H5N8 virus was isolated from two of 52 faecal samples collected from 150 Eurasian wigeons foraging on grassland between Kamerik and Kockengen (52 °08’35.5”N, 4°55’22.7”E). The birds were located ca 15 to 28 km away from three of five H5N8-virus-infected poultry farms; the remaining two H5N8-virus-infected farms were located ca 80 km away. In the Netherlands, the affected poultry farms were located in wild-bird-rich areas where water is abundant and with low to medium poultry densities. The distribution in time of sampled birds is shown per age, location, sample type and species in Figure 1.

Genetic analyses of H5N8 viruses

Genetic analyses of the HA and NA gene showed that H5N8 viruses from Europe and Russia were genetically most closely related to H5N8 viruses detected in Japan in November and December of 2014 followed by viruses detected in South Korea in 2014 (Figure 2). Also, genetic analyses of the HA gene showed that H5N8 viruses from North America were genetically most closely related to HPAI H5N2 and H5N1 viruses detected in North America followed by H5N8 virus detected in South Korea and Japan. The NA of North American H5N8 viruses was genetically most closely related to H5N8 viruses from South Korea and Japan (i.e. A/crane/Kagoshima/KU1/2014, Figure 2).

Genetic analyses of all gene segments showed that the gene constellation of H5N8 viruses from domestic and wild birds in Europe and from birds in North America was very similar to H5N8 viruses from domestic and wild birds in South Korea and Japan (data not shown). Of these viruses, four of eight gene segments (i.e. basic polymerase 2 (PB2), HA, nucleoprotein (NP) and NA) were derived from viruses similar to A/Duck/Jiangsu/k1203/2010 (H5N8). Of those, PB2 and HA genes were derived from viruses of the HPAI H5 GsGd lineage. The remaining four gene segments (i.e. basic polymerase 1 (PB1), acidic polymerase (PA), matrix protein (MP) and non-structural protein (NS)) were derived from common LPAI viruses (274, 280). Nucleotide sequence identity per segment between European, North American and the genetically closest Asian relatives was high (i.e. 99 to 100% identical). Two genetic lineages (A and B) of H5N8 virus were identified in both domestic and wild birds from South Korea in January 2014, of which lineage A was more frequently detected in both domestic and wild birds (279, 280, 288). H5N8 viruses detected in Europe (Germany, Italy, the Netherlands, and the UK), Russia and in North America belonged to lineage A based on analyses of the HA gene (279). The close genetic relationship between European, Asian and North American isolates suggested that these H5N8 viruses have a common origin.

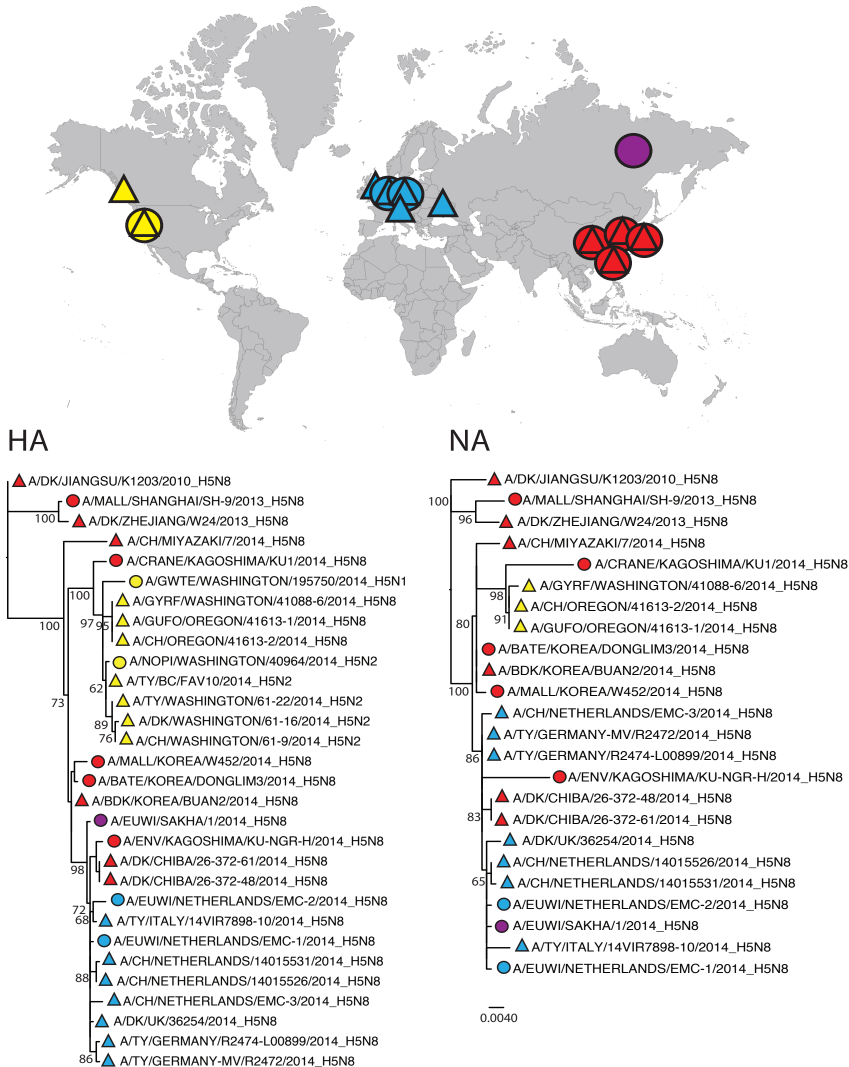


Figure 2. Phylogenetic analysis of haemagglutinin (HA) and neuraminidase (NA) genes from highly pathogenic avian influenza (HPAI) H5N8 viruses recovered in China in 2010–2013 together with respective HA and NA genes from HPAI H5N8 and other HPAI viruses belonging to the H5 clade 2.3.4.4, detected in poultry and wild birds in Asia, Europe, Russia and North America in 2014. BATE: Baikal teal; BDK: broiler duck; CH: chicken; DK: duck; ENV: environment; EUWI: Eurasian wigeon; GUFO: guinea fowl; GWTE: green-winged teal; GYRF: gyrfalcon; HPAI: highly pathogenic avian influenza; MALL: mallard; NOPI: northern pintail; TY: turkey. Maximum likelihood trees were based on the haemaggluitinin gene (HA; 1,545 nucleotides) and neuraminidase gene (NA; 1,377 nucleotides). Bootstrap values are shown if >60%.

Distribution and migratory flyways of H5N8-virus-positive bird species

Migrating birds from which H5N8 viruses have been isolated (Table 1) and that have circumpolar breeding grounds (e.g. northern pintail, *Anas acuta*) or that cover multiple major migratory flyways (e.g. Eurasian wigeon) are of specific interest with respect to global H5N8 virus epidemiology (Figure 3). Most of those species can be divided into distinct populations based on their geographically separate wintering areas. However, less is known about the degree of mixing among these populations in their breeding areas in Russia, and to which degree birds are loyal to their wintering areas.

Ring recoveries suggest that some waterfowl species (including ducks and geese) with populations wintering in East Asia and populations wintering in western Europe may have overlapping breeding grounds. For instance, ring recoveries of Eurasian wigeon and northern pintail ringed in Japan indicate that they migrate mostly north to north-east to the Russian Far East during spring migration, but a minority strays more north-west, some as far as the Western Siberian Lowlands (289) (Figure 3A and 3B). Here, ring recoveries indicate that some conspecifics originating from western Europe also may be found (290) (Figure 3A and 3B). Hence, although the probability of an actual meeting between east and west seems low, ring recoveries suggest it is not impossible. Furthermore, ring recoveries of Eurasian wigeon and northern pintail indicated a direct migratory connection between north Russia and north India (Figure 3A and 3B). Baikal teal and spot- billed duck, from which H5N8 viruses have also been isolated, have more restricted ranges, but could be involved in transport of virus from wintering grounds to breeding grounds in north-eastern Russia (Figure 3C and 3D). Mallards and teals have extensive ranges, and potentially can also be involved in transport of virus, but ring-recovery data from Russia were not available (Figure 3E and 3F).

Ring recoveries and satellite tracking have shown various waterfowl species from East Asia to be in indirect and sometimes even direct migratory connection with North America. Satellite tracking and colour banding of various waterfowl species, including emperor goose (*Chen canagica*) (293), black brant (*Branta bernicla nigricans*) (294), lesser snow goose (*Chen caerulescens caerulescens*) (295) and northern pintail have shown them to cross the Bering Strait (296). Ring recoveries of northern pintail in particular show that the connection between East Asia and North America is quite strong, albeit most likely still indirect with contact zones in the Russian Far East and Wrangel Island (289, 297). The same is true for some other species than waterfowl, which have not been identified as H5N8 virus hosts, but may play a role in the epidemiology of influenza, such as waders (298, 299).

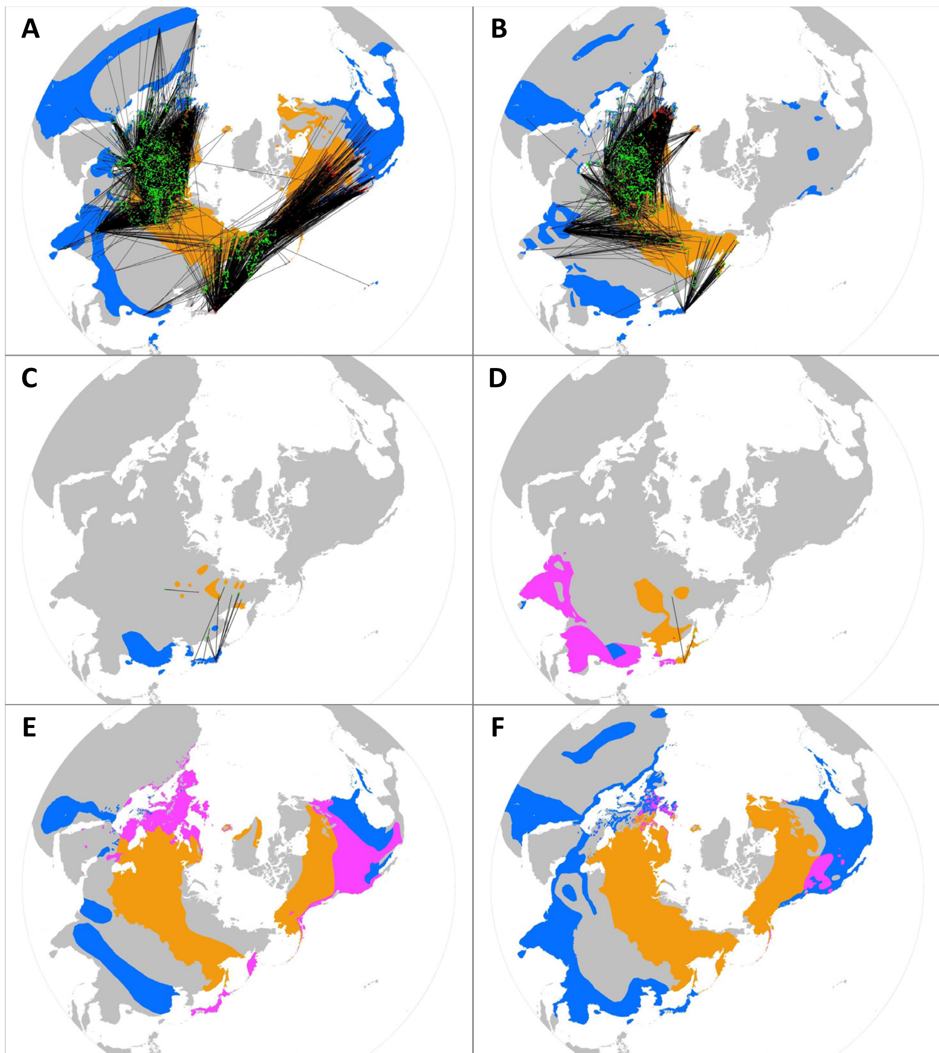


Figure 3. Breeding and wintering range and ring recoveries from 1940–2010a of wild duck species from which highly pathogenic avian influenza (HPAI) H5N8 viruses have been isolated. Top: wide range, long-distance migratory species northern pintail (*Anas acuta*) (A) and Eurasian wigeon (*Anas penelope*) (B); Middle: restricted range, short-distance migratory or resident species Baikal teal (*Anas formosa*) (C) and spot-billed duck (*Anas poecilorhyncha*) (D); Bottom: wide-range, long-distance migratory or resident species mallard (*Anas platyrhynchos*) (E), and teal (*Anas crecca / carolinensis*) (F). Orange: summer (breeding) range, blue: wintering range, purple: all-year (resident) range. Lines in maps A, B, C and D connect ringing locations (red dots) and recovery locations (green dots). The majority of ring recoveries were conducted during 1960–1990. Data source: Lines in maps A, B, C and D are based on ring-recovery data from the database of the Russian ringing scheme and are reprinted with permission from the Waterfowl Migration Atlas from the Bird Ringing Centre of Russia database and OMPO. Breeding and wintering ranges are reproduced from (291). Breeding ranges of Baikal teal and spot-billed duck have been updated from (292).

Table 3. Wild bird species sampled for highly pathogenic avian influenza (HPAI) H5N8 virus before and after the first detection of HPAI H5N8 virus in poultry on 14 November 2014, the Netherlands, May 2014–February 2015 (n = 6,763)

		Sample period	May 14th to November 13th 2014				November 14th 2014 to February 20th 2015			
Order	Family	Species	No. birds sampled	No. birds AIV+	No. birds H5+	Type	No. birds sampled	No. birds AIV+	No. birds H5+	Type
Anseriformes	Ducks		2071	455	19	LPAI	2424	261	10	2 HPAI; 8 LPAI
		Common teal (*Anas crecca*)	19	3	1	LPAI	85	19	1	LPAI
		Egyptian goose (*Alopochen aegyptiaca*)					40	0	0	
		Eurasian wigeon (*Anas penelope*)	140	26	8	LPAI	1185	33	2	HPAI
		Gadwall (*Anas strepera*)	18	2	0		127	1	0	
		Mallard (*Anas platyrhynchos*)	1876	422	10	LPAI	979	205	7	LPAI
		Northern pintail (*Anas acuta*)	2	0	0					
		Northern shoveler (*Anas clypeata*)	16	2	0		4	2	0	
		Red-breasted merganser (*Mergus serrator*)					1	1	0	
		Tufted duck (*Aythya fuligula*)					3	0	0	
	Geese						340	3	0	
		Barnacle goose (*Branta leucopsis*)					38	2	0	
		Brent goose (*Branta bernicla*)					39	1	0	
		Greylag goose (*Anser anser*)					17	0	0	
		Greater white-fronted goose (*Anser albifrons*)					246	0	0	
	Swans						183	23	0	
		Bewick's swan (*Cygnus columbianus bewickii*)					72	4	0	
		Mute swan (*Cygnus olor*)					109	18	0	
		Whooper swan (*Cygnus cygnus*)					2	1	0	

AIV, avian influenza virus; HPAI, highly pathogenic avian influenza; LPAI, low pathogenic avian influenza

Table 3 continued

		Sample period	May 14th to November 13th 2014				November 14th 2014 to February 20th 2015			
Order	Family	Species	No. birds sampled	No. birds AIV+	No. birds H5+	Type	No. birds sampled	No. birds AIV+	No. birds H5+	Type
Charadriiformes	Gulls		434	219	0		751	35	0	
		Black-headed gull (*Chroicocephalus ridibundus*)	434	219	0		611	22	0	
		Caspian gull (*Larus cachinnans*)					3	0	0	
		Common gull (*Larus canus*)					35	2	0	
		Great black-backed gull (*Larus marinus*)					10	0	0	
		Herring gull (*Larus argentatus*)					85	10	0	
		Lesser black-backed gull (*Larus fuscus*)					7	1	0	
	Terns		240	1	0					
		Black tern (*Chlidonias niger*)	176	1	0					
		Common tern (*Sterna hirundo*)	64	0	0					
Gruiformes	Coots						298	9	0	
		Common coot (*Fulica atra*)					298	9	0	
	Rails						20	0	0	
		Common moorhen (*Gallinula chloropus*)					20	0	0	
Passeriformes	Crows						1	0	0	
		Carrion crow (*Corvus corone*)					1	0	0	
Strigiformes	Owls						1	0	0	
		Barn owl (*Tyto alba*)					1	0	0	
Total			2745	675	19		4018	331	10	

DISCUSSION

The detection of the newly emerging HPAI H5N8 virus in at least 17 migratory bird species in Asia, Europe and North America, emphasises the need to study the role of migratory birds in the epidemiology of these H5N8 viruses. After the first detection of H5N8 virus in poultry in the Netherlands, wild bird sampling activities were intensified and HPAI H5N8 virus was detected in samples from two of 4,018 birds sampled within a three months period. The virus was isolated from Eurasian wigeons exclusively, whereas other bird species like mallards, greater white-fronted geese (*Anser albifrons*), black-headed gulls (*Chroicocephalus ridibundus*) and common coots (*Fulica atra*) also had been sampled intensively. The Eurasian wigeon is a long-distance migrant in which species H5N8-virus-specific antibodies had been detected in South Korea in 2014 (279). As HPAI H5N8 virus, like other avian influenza viruses, causes an infection of short duration in birds (118), the chance of detection is low and large sample sizes are needed to determine its presence in the population. The chance of detection of H5N8-virus-specific antibodies in wild bird sera is much higher, and serology can be used as a tool to target surveillance and determine past exposure to H5N8 virus, as H5 viruses of the HPAI GsGd lineage differ antigenically from common LPAI H5 viruses (285).

The H5N8 viruses isolated from wild birds in the Netherlands were genetically closely related to and had the same gene constellation as H5N8 viruses detected elsewhere in Europe, in Asia and in North America, suggesting a common origin. In wild and domestic birds in North America, HPAI reassortant viruses of the subtypes H5N2 and H5N1 have been detected. These viruses contain genes originating from both HPAI H5N8 and LPAI viruses. Reassortant viruses of the subtypes H5N2 and H5N3 have been detected in domestic birds in Taiwan. In Europe, no reassortant viruses with HPAI H5N8 genes have been detected so far. Monitoring wild birds to detect H5N8 virus and derived reassortants is warranted given their potential to cause severe disease and mortality in poultry and some species of wild birds (e.g. eagles and hawks).

Ring recoveries of migratory duck species from which H5N8 viruses have been isolated provide evidence for indirect migratory connections between East Asia and western Europe and between East Asia and North America. In addition, ring recoveries of northern pintails and Eurasian wigeons demonstrated a direct migratory connection between north India and north Russia and between north India and Europe. If these species are involved in the global spread of H5N8 virus, we hypothesise that H5N8 viruses may also spread to north India as occurred previously with HPAI H5N1 virus of clade 2.2 (300). During large-scale surveillance activities in north India from 2009 to 2011, no avian influenza viruses had been detected in 3,522 wild bird samples (301). To

which extent migrating bird populations of different flyways come in direct or indirect contact (e.g. using the same water source during stop over) with each other needs further study. To understand the role of wild birds in the epidemiology of H5N8 virus, sampling activities need to aim at detection of both the virus and specific antibodies with an emphasis on migrating birds in north-east Europe, Russia, and north China.

ACKNOWLEDGEMENTS

The authors thank Theo Bestebroer, Stefan van Vliet, Stefan van Nieuwkoop, Pascal Lexmond, Gerard Müskens, Teun de Vaal, Bert Pellegrom, Jan Berkouwer, Arie Keijzer, Henk ten Klooster, Jan Slijkerman, Lilian Slijkerman, Manon Kaandorp, Cynthia Lange, Joanne Malotaux, Jan Beekman, Alwin Hut, Peter Volten, Evert-Jan Epping, Harma Scholten, Ton Eggenhuizen, Henk Koffijberg, Gerben Tijsma, Erik Kleyheeg, Jan van der Winden, Sjoerd Dirksen and Ger van der Water for providing wild bird samples and technical and logistical assistance. We gratefully acknowledge the anonymous reviewers, and authors, originating and submitting laboratories of the sequences from GISAID's EpiFluTM Database on which this research is based. All submitters of data may be contacted directly via the GISAID website (160). This work was supported by The Dutch Ministry of Economic Affairs, European Research Council project FLUPLAN (250136), NIAID/NIH contract HHSN272201400008C, and Horizon 2020 project COMPARE.

CHAPTER 4

Josanne H. Verhagen

Summarizing discussion

The presence of highly pathogenic avian influenza (HPAI) H5 viruses in migrating birds and the dispersed spatial pattern of virus detections globally are worrisome. The impact of HPAI H5 virus outbreaks on poultry health and industry can be immense, and can pose a threat to human health. Several long-distance migratory birds—such as ducks of the *Anas* species—proved to be susceptible to HPAI H5 virus infection and to excrete virus in their feces without developing detectable disease. However, the dispersal of these HPAI viruses by migratory birds is difficult to investigate as the determination of the day of infection and inferring the health status of single-captured wild birds is rather challenging. The active role of migratory birds in local and long-distance HPAI H5 virus dispersal is expected to vary between locations, species, populations and hosts. The unprecedented intercontinental spread of HPAI H5 viruses in 2014 demonstrated the prospect of the emerging capacities of the constant evolving HPAI H5N1 viruses, and highlighted the need for identification of fundamental mechanisms of the dispersal and evolution of HPAI, and genetically closely related and widespread low pathogenic avian influenza (LPAI) viruses in wild birds.

Ecology and evolution of influenza A viruses in waterfowl

Since the mid-1970s, influenza A virus infection in wild birds has been studied more intensively by virologists and veterinarians, revealing an enormous diversity of influenza A viruses, and spatial and temporal patterns of influenza virus infections in wild birds. More recently, techniques have significantly improved, and increased number of studies combines virological and ecological techniques. The current available virological techniques that facilitate high-throughput molecular screening (151, 302) and the ecological techniques available for characterization of foraging habitat and/or migration origin of the host, such as stable isotope analyses and GPS tracking devices (145, 303, 304), allow for an integrative and comparative approach that is crucial to better understand the ecology and epidemiology of avian influenza viruses.

Avian influenza wild bird surveillance programs are used to detect emerging influenza A viruses, and to ultimately better understand the ecology and evolution of influenza A viruses in wild birds. Since 1998, the Department of Viroscience of Erasmus MC runs a wild bird surveillance program in close cooperation with many ornithologists. Within this program, approximately 15,000 samples from birds in the Netherlands and approximately 4,000 samples from birds outside the Netherlands are analyzed for virus detection each year. Bird species that have been sampled most frequently belong to the order of Anseriformes (mainly ducks, geese and swans) or Charadriiformes (mainly gulls, terns and waders). Live birds are sampled for virus detection in cloaca and oropharynx routinely, and additional blood samples are collected for influenza A virus-specific antibody detection. The sampling of live birds for virus and antibody detection allows the identification of the active role that birds play in the dispersal of influenza A viruses. Increased efforts to obtain and publish full-genome sequences of influenza A viruses and parallel sampling for virology and serology, will allow surveillance studies to go beyond who is infected when and where, and will shed new light on the underlying mechanisms that explain the ecology and evolution of influenza A viruses in wild birds.

Migratory birds can act as local LPAI virus amplifiers and as vectors for LPAI virus dispersal. Previously, the difference between migratory and non-migratory birds has been investigated in birds belonging to different species (141, 142). More recently, and as part of this thesis, the roles of migratory and resident birds have been investigated within a single species, the mallard (*Anas platyrhynchos*), which showed that the role of migrants is not mutually exclusive, i.e. they can both act as local amplifiers (chapter 2.1 (43)) as well as vectors for virus dispersal (145, 305). However, the studies on the roles of migratory and resident mallards exemplify the difficulty of elucidating the roles of migratory and resident hosts in infectious disease dynamics in wildlife, but

provide encouraging indications that the used multifaceted approach may provide new opportunities to study these processes.

Movement of LPAI virus genes within and between continents has been described, yet little is known about the conditions supporting long-distance gene flow. Birds' migration phenology—i.e. timing, direction, distance, use of stopover sites—combined with birds' physiology and immune status presumably shape the dispersal of influenza A viruses. Globally, influenza A viruses are largely divided into two main genetic lineages: Eurasian and American. Genetic analyses of influenza A viruses in wild birds have been proven to be useful for recognizing newly introduced pathogens when compared with long-term historic surveillance data. Influenza A viruses carrying a mix of Eurasian and American genes have been isolated from gulls, shorebirds and long-distance migratory ducks, predominantly in Alaska (306-308). To date, no study has detected a complete LPAI virus genome belonging to either clade into the other continent, which is not surprising given the transient genome constellation of influenza A viruses in wild birds (92, 309). Within North America, gene flow has been described along migratory flyways, as well as between the different major migratory flyways (145, 181). Within Eurasia, gene flow of most gene segments of LPAI viruses occurred in the direction from Western to Eastern Eurasia (chapter 2.2). Future more high-resolution studies will show if this is a consistent pattern, and may help to shed light on underlying mechanisms (e.g. species, timing of bird migration). Long-distance gene flow followed by local establishment is likely to be influenced by migratory connectivity and timing in relation to LPAI virus-specific herd-immunity in wild bird populations upon arrival, and/or direct competition with local circulating virus strains upon arrival. To assess the implication of migration patterns on the genetic diversity of influenza A viruses in Eurasia, future whole-genome sequencing should be directed towards increased numbers of samples within a short time frame in locations along the different flyways (chapter 2.2). Incorporating metadata such as host species, location and date of sampling, age, sex, and immune and migratory status will illuminate future host-focused studies by including the impact of ecological factors like individual species diversity and life cycle on influenza A virus genetic diversity (e.g. genetic sweeps in relation to virus incursions and/or immune evasion of viruses (309)).

The host immune system shapes AIV dynamics and presumably evolution in wild birds (92, 172, 190). A temporal structure of HA subtypes, based on HA-phylogenetic relatedness has been described in free-living mallards (172). In addition, mallards infected with a particular subtype were unlikely to become infected with the same subtype within the same season (190). To obtain better insights in the mechanisms that are responsible for the long-term spatial and temporal patterns of avian influenza virus subtypes and lineages, improved knowledge is needed on avian immunology, in

particular the specificity, strength and duration of influenza A virus-specific immunity in wild birds. Studies that investigate the effect of serial LPAI virus infections in wild birds are rare, use diverse methods to investigate virus excretion and AIV-specific immune responses, and are therefore difficult to compare. Black-headed gulls (*Chroicocephalus ridibundus*) are natural hosts for avian influenza A viruses of the subtypes H13 and H16, in which they cause annual outbreaks at breeding colony sites at the end of summer, when juveniles leave the nest (chapter 2.3). Serial infections of juvenile gulls with LPAI H13 and H16 viruses over a period of more than one year showed that re-infection with the same virus results in progressively less virus excretion (chapter 2.4). These observational and experimental studies demonstrated that the epidemiological cycle of LPAI virus in black-headed gulls is mainly determined by the presence of first-year birds. For how many years partial protection lasts remains unknown, but given the likelihood of annual boost of the birds' immune system with LPAI H13 and/or H16 viruses it may result in life-long partial to complete protection. To investigate how the immune system acts as a selection pressure modifying the virus pool in wild birds, the translation of genotype to phenotype, and the link with local herd-immunity may further elucidate the long-distance movement of influenza A virus genes.

To better understand the interplay between virus and host immunity at the population level, immune parameters with a predictable value for the susceptibility to infection will be needed, e.g. to use in high-resolution time series on the interaction between virus and host immunity. The sampling of black-headed gulls in an experimental setting for both LPAI virus-specific antibodies and LPAI viruses revealed that presence or titer of serum antibodies as detected at day of inoculation were not associated with decreased virus excretion (chapter 2.4). As LPAI virus infection in wild aquatic birds is predominantly a digestive tract infection (140, 231), AIV-specific antibodies as detected in serum may have limited protective value. Alternative measures of protection, like mucosal (active secretion or passive transfer from parent to young) and cellular immunity, may be worth investing in future studies to improve our understanding of influenza A virus immunity and protection (310).

The use of influenza A virus-specific antibody detection in blood may be limited for diagnostic or research purposes in wild birds. New influenza A virus host species have been identified based on serum antibody detection (70, 311), but serum antibodies may be insufficient to i) investigate past exposure of known AIV-host species in relation to current infection, ii) investigate past exposure as predictor for host susceptibility to subsequent infections with LPAI viruses of the same or different subtypes, and, iii) confirm AIV negative status prior to experimental infection of birds with unknown life-history. AIV-specific serum antibodies were no longer detected after 5 months in the

majority of black-headed gulls once or twice exposed to LPAI viruses (chapter 2.4), demonstrating limited value of seronegative status of host based on absence of serum AIV-specific antibodies, a frequently used method in field sampling activities. Compared to the classic influenza antibody test (i.e. the hemagglutination inhibition assay), sensitivity may be higher when using alternative HA-specific tests, e.g. protein micro-array (312), although such tests have other limitations. Thus, despite the need for more knowledge on the prior exposure of the bird, the use of serum antibodies, and classic influenza antibody tests, within surveillance programs may result in an underestimation of AIV-infected birds due to the relatively rapid decrease of detectable serum antibodies. Given the lack of correlation between AIV-specific serum antibodies and protection against LPAI virus infection, further research is required to elucidate the mechanism and duration of protection against LPAI virus infection and which parameters (e.g., mucosal antibody levels, cell-mediated immunity) can be used as correlates of protection for LPAI virus infections in wild birds.

Ecology and evolution of influenza A viruses in gulls: a potential model for influenza A viruses in ducks?

Birds of the Laridae family, such as gulls, are widespread and numerous, but until recently little was known about LPAI viruses in birds of this family. Genetic analyses revealed their role to allow reassortment of LPAI viruses (308). Annual outbreaks of H13 and H16 LPAI viruses have been described in black-headed gulls (chapter 2.3). For some birds of the Laridae family that have been sampled longitudinally during a similar time period and/or development stage (e.g. close to fledgling) the same timing of LPAI virus circulation (39) has been detected. Interestingly, in the Republic of Georgia, LPAI viruses have been detected in black-headed gulls predominantly in spring (183). For other bird species belonging to the Laridae family (n = 53 (313)), approximately in 26 (49%) of these species LPAI virus or antibodies have been detected, mainly H13 (16/26) and H16 (9/26) (314). Less is known about influenza A virus circulation in terns, although recently high H16 virus prevalence was detected in terns (315). Additional research is needed to further elucidate if the same AIV dynamics apply to other gull and tern species and other geographic areas.

Despite the high subtype diversity of endemic influenza A viruses in ducks, in contrast to the low diversity of endemic influenza A viruses in gulls ((26, 39, 40), chapter 2.3), some of the underlying mechanisms that shape the interaction between AIV and their natural hosts may be similar for birds belonging to the family of Anatidae (e.g. ducks) and Laridae (e.g. black-headed gulls). Field surveillance activities demonstrated

annual LPAI virus outbreaks in mallard (26, 146, 172, 316) and in gull populations ((39, 40), chapter 2.3). The epidemiological cycle of avian influenza depends mainly on infection of first-year black-headed gulls (chapter 2.3; 2.4) and juvenile mallards (146, 190, 228). Based on natural infections, there is evidence that LPAI viruses mainly infect cells lining the digestive tract in mallards (231) and black-headed gulls (140). Given these similarities, the lower subtype diversity of endemic viruses in gulls, and the higher feasibility to study gull populations, birds of the Laridae family may be a useful model to identify specific ecological, immunological and/or virological mechanisms fundamental to AIV emergence and establishment. Such presumably less complex host-pathogen interaction studies may facilitate studies on the fundamental processes of LPAI virus ecology and evolution in Laridae, such as the determination of conditions of virus persistence (e.g. metapopulation size and numbers, single host or multi-host systems, timing of annual stages), and/or the effect of prior exposure on susceptibility and virus excretion of subsequent exposure. The information generated as part of the experimental study in black-headed gulls (e.g., quantity of virus excreted and duration of excretion of infectious virus after the first, second, and third infections with homologous or heterologous viruses) can be used for top down modeling purposes to develop integrative and predictive multiscale models to e.g. identify the conditions for persistence of influenza A virus lineages in wild birds.

Wild – domestic interface studies on influenza A viruses: the identification of host species and routes

Viral epidemics in wild migrating birds may directly impact bird populations in urbanized areas. Worldwide surveillance activities in wild birds take place mainly in rural areas, whereas since 2007 more people live in cities than in rural areas worldwide (244). Within cities, avian influenza viruses were detected in similar wild bird species as outside cities, however with lower virus prevalence (chapter 3.1). Urban bird populations infected with LPAI viruses were not separated completely from populations of long-distance migrants, indicating that wild birds play a role in introduction of LPAI viruses into cities (chapter 3.1). Thus, urban bird populations should not be excluded as a source of influenza A virus infection for humans and animals, although highest prevalence and diversity may be expected in rural areas where large numbers of birds aggregate.

Given seasonal mass migration of birds and the high frequency of apparently mild LPAI virus infections in free-living birds and fecal virus excretion, it is expected that domestic birds become exposed to wild bird LPAI viruses more frequently than we have been able to detect so far. Detections of these LPAI virus introductions from wild birds to

poultry may be limited due to surveillance programs in poultry being focused on H5 and H7, and/or insufficient surveillance sample frequency and size (129, 133). Knowledge based on comparative studies that investigate which LPAI virus subtypes or genotypes are able to infect, and subsequently transmit between domestic birds, and how, is very limited. Investigations on the genetic diversity of LPAI viruses of wild versus domestic birds and susceptibility of poultry to various LPAI viruses isolated from wild and domestic birds will clarify this.

High LPAI virus prevalence in a wild bird species does not make it a high-risk species for poultry (chapter 3.2). Despite high LPAI virus prevalence in summer, multi-year and genetic studies on LPAI viruses in black-headed gulls showed they have no role as maintenance host for LPAI viruses ancestral to poultry outbreaks, although a role as bridge species or species in which reassortant occurs preferentially cannot be excluded (chapter 2.3, (99)). Based on Dutch surveillance data, the LPAI virus subtype distribution differs between wild birds and poultry (chapter 3.2), suggesting a difference in exposure and/or susceptibility to LPAI viruses between poultry and wild birds. Furthermore, the LPAI virus subtype distribution of geese and ducks other than mallards, showed highest similarity with the LPAI virus subtype distribution as detected in poultry. Year-round wild bird distribution and behavior studies, on a local scale in different habitats near poultry farms may clarify if e.g. geese and ducks other than mallards indeed are more prevalent near farms than other species like mallards, and therefore may form a direct risk for LPAI virus introduction into farms. In addition, specific wild bird species at the source of LPAI virus introductions into poultry farms may not have been identified yet. The genetic characterization of all LPAI viruses isolated from wild and domestic birds, not only H5 and H7, is extremely valuable to increase our understanding of the transmission of LPAI viruses between wild birds and poultry.

Although strict biosecurity is the most effective preventive measure for LPAI viruses to enter poultry farms, knowledge on the distribution and behavior of specific wild birds that are infected with LPAI viruses and may act as LPAI virus bridge species (317) is crucial to target such measures. Targeted measures such as putting specific plants, trees or fences around poultry farms that attract or keep off specific wild bird species may lower exposure to LPAI viruses. Wild birds that forage around outdoor facilities at night can be kept away through night-time measures when poultry are indoors. In addition, non-avian virus vectors, like rodents, have shown to be infected with a wide range of pathogens in urban and agricultural areas (318, 319), and measures to keep rodents out may lower infection risks. Prospective evaluation of measures like these will result in better knowledge on the risks of introducing LPAI virus and other poultry pathogens into poultry farms. To further optimize preventive measures of virus introductions into

poultry farms, a close collaboration between poultry farmers, industry, veterinarians, virologists and ornithologists is crucial, and will require investments of all parties.

Re-emerging HPAI viruses "the other way around"?

Since the emergence of HPAI H5N1 viruses of the A/Goose/Guandong/1/96 (GsGd) lineage in Southeast Asia, the virus has managed to continuously circulate in domestic birds, with frequent transmission to wild birds. During the circulation and spread of the H5N1 viruses, the HA genes diversified into multiple genetic lineages ("clades"), without evidence of gene exchange between the influenza viruses (271). However, this changed from 2009 onward, when HPAI viruses of subtypes H5N2, H5N5, H5N6, and H5N8 were found to contain the H5 gene of the GsGd lineage, together with NA and various other genes of LPAI virus origin (272-275). After numerous poultry outbreaks in eastern Asia and occasional detection in wild birds, these viruses spread into Europe and, for the first time, North America by December 2014 ((276, 279, 320), chapters 3.4; 3.4). HPAI H5N8 viruses of the same genome constellation were detected in free-living bird populations at geographically distant locations (chapter 3.4). In addition, intercontinental spread was followed by reassortment of HPAI H5N8 viruses with North American LPAI viruses, resulting in widespread distribution of HPAI H5N1 and HPAI H5N2 viruses in North America. Field observations as well as experimental work with ducks of the *Anas* species infected with HPAI H5N8 virus suggested absent or mild pathogenicity of HPAI H5N8 in some ducks (276, 280), which is similar to what has been demonstrated for HPAI H5N1 virus infections in mallards previously (118). Decreased pathogenicity for ducks of the *Anas* species potentially facilitated increased geographical spread through migratory ducks, meanwhile preserving some pathogenicity for chicken and turkeys. HPAI H5N8-specific antibodies were detected in blood samples from live ducks with varying migratory strategies (279), suggesting an active potential role for ducks in the—local or long-distance—dispersal of this HPAI H5N8 lineage. Prior to HPAI H5N8 detection, HPAI H5-biased sera (i.e. stronger reactivity with HPAI H5 virus than with LPAI H5 virus) have been demonstrated for bar-headed goose (*Anser indicus*), bean goose (*Anser fabalis*), swan goose (*Anser signoides*), whooper swan (*Cygnus Cygnus*), ruddy shelduck (*Tadorna ferruginea*) and tufted duck (*Aythya fuligula*) (285). Whether migratory birds encounter HPAI viruses locally, or whether they move them, directly or indirectly, along migratory flyways like with LPAI virus genes (145, 306) remains a topic of debate, partly due to rarity of longitudinal studies on virus circulation along migratory flyways, and to the lack of available data on pathogen dispersal by poultry manure trade. Therefore, the extent to which migrating bird populations of different flyways come in direct or indirect contact

(e.g. using the same water source during stop over) with each other needs further study. Despite the currently low public health risk of HPAI H5N8 viruses (321), outbreaks like these should be monitored closely, given that several animal species are susceptible (280) and that influenza viruses are generally unpredictable. Longitudinal sampling, and characterization of both viruses and immune response of birds along migratory flyways at geographically distant aggregation sites with high bird densities and high population turnover rates may be most efficient, and are still needed, to further unravel the long-distance movement of these HPAI viruses and other avian influenza viruses by migratory birds (133, 320).

CONCLUSION

Few viruses are as heterogenic as influenza A viruses. This heterogeneity enables them to infect a wide range of hosts with strongly varying virulence, ranging from a mild or subclinical infection in migrating ducks to severe disease and death in commercially kept chickens. Increased knowledge on structure and function of reservoir populations is needed to protect target populations (e.g. poultry) from becoming infected. Collaborative observational and experimental studies carried out by ecologists and virologists have identified important traits related to influenza A virus ecology and evolution. These traits revealed different susceptibility between host populations and species, as well as seasonal and geographic patterns in influenza A virus dynamics. These findings facilitated more targeted sampling of water birds for the purpose of virus harvesting, but at the same time gave rise to many more questions. Little is known about the underlying mechanisms of within- and between-host influenza A virus dynamics. How does prior exposure affect influenza A virus dynamics and evolution? What are determining factors of the influenza A virus genotype with respect to host range and transmission in wild birds? To 'unravel' the complex system of multiple virus lineages circulating in multiple hosts, long-term hypothesis-driven studies that bring together virology, pathology, ecology, immunology and evolutionary biology are needed, designed and collected by an interdisciplinary team of researchers including (molecular) virologists, pathologists, (animal) ecologists, ornithologists, immunologists and mathematical modelers.

Dings.

CHAPTER 5

References

1. Munster, V.J. and R.A. Fouchier, *Avian influenza virus: of virus and bird ecology.* Vaccine, 2009. **27**(45): p. 6340-4.
2. Stallknecht, D.E., et al., *Persistence of avian influenza viruses in water.* Avian Dis, 1990. **34**(2): p. 406-11.
3. Siembieda, J.L., et al., *Influenza A viruses in wild birds of the Pacific flyway, 2005-2008.* Vector Borne Zoonotic Diseases, 2010. **10**(8): p. 793-800.
4. Munster, V.J., et al., *Towards improved influenza A virus surveillance in migrating birds.* Vaccine, 2006. **24**(44-46): p. 6729-33.
5. Fereidouni, S.R., et al., *Avian influenza virus monitoring in wintering waterbirds in Iran, 2003-2007.* Virology Journal, 2010. **7**: p. 43.
6. Gaidet, N., et al., *Avian influenza viruses in water birds, Africa.* Emerg Infect Dis, 2007. **13**(4): p. 626-9.
7. Sharshov, K., et al., *Genetic and biological characterization of avian influenza H5N1 viruses isolated from wild birds and poultry in Western Siberia.* Arch Virol, 2010. **155**(7): p. 1145-50.
8. Chen, H., et al., *Establishment of multiple sublineages of H5N1 influenza virus in Asia: implications for pandemic control.* Proc Natl Acad Sci U S A, 2006. **103**(8): p. 2845-50.
9. Haynes, L., et al., *Australian surveillance for avian influenza viruses in wild birds between July 2005 and June 2007.* Australian Veterinary Journal, 2009. **87**(7): p. 266-72.
10. Kou, Z., et al., *The survey of H5N1 flu virus in wild birds in 14 Provinces of China from 2004 to 2007.* PLoS One, 2009. **4**(9): p. e6926.
11. Dusek, R.J., et al., *Surveillance for high pathogenicity avian influenza virus in wild birds in the Pacific Flyway of the United States, 2006-2007.* Avian Dis, 2009. **53**(2): p. 222-30.
12. Webster, R.G., et al., *Evolution and ecology of influenza A viruses.* Microbiol Rev, 1992. **56**(1): p. 152-79.
13. Tong, S., et al., *A distinct lineage of influenza A virus from bats.* Proceedings of the National Academy of Sciences of the United States of America, 2012. **109**(11): p. 4269-74.
14. Tong, S., et al., *New world bats harbor diverse influenza A viruses.* PLoS Pathogens, 2013. **9**(10): p. e1003657.
15. Short, K.R., et al., *One health, multiple challenges: The inter-species transmission of influenza A virus.* One Health, 2015. **1**: p. 1-13.
16. Fouchier, R.A., et al., *Characterization of a novel influenza A virus hemagglutinin subtype (H16) obtained from black-headed gulls.* J Virol, 2005. **79**(5): p. 2814-22.
17. Wright, K. and R.G. Webster, *Orthomyxoviruses*, in *Fields Virology*, D.M. Knipe and P.M. Howley, Editors. 2001, Lippincott Williams & Williams: Philadelphia, PA. p. 1533-1579.
18. Lamb, R.A. and R.M. Krug, *Orthomyxoviridae: the viruses and their replication*, in *Fields Virology*, D.M. Knipe and P.M. Howley, Editors. 2001, Lippincott Williams & Williams: Philadelphia, PA. p. 1487-1531.
19. Alexander, D.J., *A review of avian influenza in different bird species.* Vet Microbiol, 2000. **74**(1-2): p. 3-13.
20. Webster, R.G. and R. Rott, *Influenza virus A pathogenicity: the pivotal role of hemagglutinin.* Cell, 1987. **50**(5): p. 665-6.
21. Olsen, B., et al., *Global patterns of influenza a virus in wild birds.* Science, 2006. **312**(5772): p. 384-8.
22. Stallknecht, D.E. and S.M. Shane, *Host range of avian influenza virus in free-living birds.* Vet Res Commun, 1988. **12**(2-3): p. 125-41.
23. Van Dijk, J.G.B., R.A.M. Fouchier, and J.H. Verhagen, *Wetenschappelijk onderzoek in eendenkooien in Nederland: eenden en vogelgriepvirussen*, in *Blauwgoed, helen en halven: 100 jaar ringwerk in eendenkooien*, D. Karelse and F. Mandigers, Editors. 2013,

Werkgroep Ringwerk Eendenkooien Nederland (WREN). p. 109-120.

24. Wallensten, A., et al., *High prevalence of influenza A virus in ducks caught during spring migration through Sweden.* Vaccine, 2006. **24**(44-46): p. 6734-5.
25. Wallensten, A., et al., *Surveillance of influenza A virus in migratory waterfowl in northern Europe.* Emerg Infect Dis, 2007. **13**(3): p. 404-11.
26. Munster, V.J., et al., *Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds.* PLoS Pathogens, 2007. **3**(5): p. e61.
27. Krauss, S., et al., *Influenza A viruses of migrating wild aquatic birds in North America.* Vector Borne Zoonotic Diseases, 2004. **4**(3): p. 177-89.
28. Hanson, B.A., et al., *Avian influenza viruses in Minnesota ducks during 1998-2000.* Avian Dis, 2003. **47**(3 Suppl): p. 867-71.
29. Ellstrom, P., et al., *Sampling for low-pathogenic avian influenza A virus in wild Mallard ducks: oropharyngeal versus cloacal swabbing.* Vaccine, 2008. **26**(35): p. 4414-6.
30. Hinshaw, V.S., et al., *The ecology of influenza viruses in ducks and analysis of influenza viruses with monoclonal antibodies.* Comp Immunol Microbiol Infect Dis, 1980. **3**(1-2): p. 155-64.
31. Hinshaw, V.S., et al., *Genetic reassortment of influenza A viruses in the intestinal tract of ducks.* Virology, 1980. **102**(2): p. 412-9.
32. Parmley, E.J., et al., *Wild bird influenza survey, Canada, 2005.* Emerging Infectious Diseases, 2008. **14**(1): p. 84-7.
33. Nolting, J., et al., *Recovery of H14 influenza A virus isolates from sea ducks in the Western Hemisphere.* PLoS Currents, 2012. **4**: p. RRN1290.
34. Halvorson, D.A., C.J. Kelleher, and D.A. Senne, *Epizootiology of avian influenza: effect of season on incidence in sentinel ducks and domestic turkeys in Minnesota.* Applied and Environmental Microbiology, 1985. **49**(4): p. 914-9.
35. del Hoyo, J., et al., *Handbook of the Birds of the World*. 1996, Barcelona: Lynx Editions.
36. Hinshaw, V.S., et al., *Antigenic and genetic characterization of a novel hemagglutinin subtype of influenza A viruses from gulls.* J Virol, 1982. **42**(3): p. 865-72.
37. Kawaoka, Y., et al., *Is the gene pool of influenza viruses in shorebirds and gulls different from that in wild ducks?* Virology, 1988. **163**(1): p. 247-50.
38. Gaidet, N., et al., *Investigating avian influenza infection hotspots in old-world shorebirds.* PLoS One, 2012. **7**(9): p. e46049.
39. Velarde, R., et al., *Avian influenza virus H13 circulating in ring-billed gulls (Larus delawarensis) in southern Ontario, Canada.* Avian Dis, 2010. **54**(1 Suppl): p. 411-9.
40. Graves, I.L., *Influenza viruses in birds of the Atlantic flyway.* Avian Diseases, 1992. **36**(1): p. 1-10.
41. Iamnikova, S.S., et al., *(Circulation of the influenza A virus of H13 serosubtype among seagulls in the Northern Caspian (1979-1985)).* Voprosy Virusologii, 1989. **34**(4): p. 426-30.
42. Kleijn, D., et al., *Dynamics and ecological consequences of avian influenza virus infection in greater white-fronted geese in their winter staging areas.* Proceedings of the Royal Society B: Biological Sciences, 2010. **277**(1690): p. 2041-8.
43. Hoye, B.J., et al., *Reconstructing an annual cycle of interaction: natural infection and antibody dynamics to avian influenza along a migratory flyway.* Oikos, 2011. **120**(5): p. 748-755.
44. Krauss, S., et al., *Coincident ruddy turnstone migration and horseshoe crab spawning creates an ecological 'hot spot' for influenza viruses.* Proceedings of the Royal Society B: Biological Sciences, 2010. **277**(1699): p. 3373-9.
45. Winker, K., E. Spackman, and D.E. Swayne, *Rarity of influenza A virus in spring shorebirds, southern Alaska.* Emerg Infect Dis, 2008. **14**(8): p. 1314-6.
46. Hurt, A.C., et al., *Isolation of avian influenza viruses from two different*

transhemispheric migratory shorebird species in Australia. Arch Virol, 2006. **151**(11): p. 2301-9.

47. Gronesova, P., et al., *Using nested RT-PCR analyses to determine the prevalence of avian influenza viruses in passerines in western Slovakia, during summer 2007.* Scand J Infect Dis, 2008. **40**(11-12): p. 954-7.
48. Homme, P.J. and B.C. Easterday, *Avian influenza virus infections. IV. Response of pheasants, ducks, and geese to influenza A-turkey-Wisconsin-1966 virus.* Avian Dis, 1970. **14**(2): p. 285-90.
49. Brown, J., et al., *Susceptibility of avian species to North American H13 low pathogenic avian influenza viruses.* Avian Diseases, 2012. **56**(4 Suppl): p. 969-75.
50. Slemons, R.D. and B.C. Easterday, *Virus replication in the digestive tract of ducks exposed by aerosol to type-A influenza.* Avian Dis, 1978. **22**(3): p. 367-77.
51. Webster, R.G., et al., *Intestinal influenza: replication and characterization of influenza viruses in ducks.* Virology, 1978. **84**(2): p. 268-78.
52. Kida, H., R. Yanagawa, and Y. Matsuoka, *Duck influenza lacking evidence of disease signs and immune response.* Infection and Immunity, 1980. **30**(2): p. 547-53.
53. Brown, J.D., et al., *Intestinal excretion of a wild bird-origin H3N8 low pathogenic avian influenza virus in mallards (Anas Platyrhynchos).* J Wildl Dis, 2012. **48**(4): p. 991-8.
54. Daoust, P.Y., et al., *Replication of 2 subtypes of low-pathogenicity avian influenza virus of duck and gull origins in experimentally infected Mallard ducks.* Vet Pathol, 2013. **50**(3): p. 548-59.
55. Bahl, A.K. and B.S. Pomeroy, *Experimental exposure of Franklins' gulls (Larus pipixan) and Mallards (Anas platyrhynchos) to a turkey influenza A virus A/Turkey/Minn/BF/72 (Hav6Neq2).* J Wildl Dis, 1977. **13**(4): p. 420-6.
56. Costa, T.P., et al., *Variation in viral shedding patterns between different wild bird species infected experimentally with low-pathogenicity avian influenza viruses that originated from wild birds.* Avian Pathol, 2011. **40**(2): p. 119-24.
57. Costa, T.P., et al., *The effect of age on avian influenza viral shedding in mallards (Anas platyrhynchos).* Avian Diseases, 2010. **54**(1 Suppl): p. 581-5.
58. VanDalen, K.K., et al., *Shedding light on avian influenza H4N6 infection in mallards: modes of transmission and implications for surveillance.* PLoS One, 2010. **5**(9): p. e12851.
59. Costa, T.P., et al., *Effect of a prior exposure to a low pathogenic avian influenza virus in the outcome of a heterosubtypic low pathogenic avian influenza infection in mallards (Anas platyrhynchos).* Avian Diseases, 2010. **54**(4): p. 1286-91.
60. Fereidouni, S.R., et al., *Highly pathogenic avian influenza virus infection of mallards with homo- and heterosubtypic immunity induced by low pathogenic avian influenza viruses.* PLoS One, 2009. **4**(8): p. e6706.
61. Jourdain, E., et al., *Influenza virus in a natural host, the mallard: experimental infection data.* PLoS One, 2010. **5**(1): p. e8935.
62. Latorre-Margalef, N., et al., *Effects of influenza A virus infection on migrating mallard ducks.* Proceedings of the Royal Society B: Biological Sciences, 2009. **276**(1659): p. 1029-36.
63. Hubalek, Z., *An annotated checklist of pathogenic microorganisms associated with migratory birds.* J Wildl Dis, 2004. **40**(4): p. 639-59.
64. Lebarbenchon, C., et al., *Isolation of influenza A viruses from wild ducks and feathers in Minnesota (2010-2011).* Avian Dis, 2013. **57**(3): p. 677-80.
65. Sharma, J.M. and I. Tizard, *Avian cellular immune effector mechanisms—a review.* Avian Pathology, 1984. **13**(3): p. 357-76.
66. Barber, M.R., et al., *Association of RIG-I with innate immunity of ducks to influenza.* Proc Natl Acad Sci U S A, 2010. **107**(13): p. 5913-8.

67. Cornelissen, J.B., et al., *Differential innate responses of chickens and ducks to low-pathogenic avian influenza.* Avian Pathology, 2012. **41**(6): p. 519-29.
68. Huang, Z., et al., *Differential cellular immune responses between chickens and ducks to H9N2 avian influenza virus infection.* Vet Immunol Immunopathol, 2012. **150**(3-4): p. 169-80.
69. Homme, P.J. and B.C. Easterday, *Avian influenza virus infections. III. Antibody response by turkeys to influenza A-turkey-Wisconsin-1966 virus.* Avian Diseases, 1970. **14**(2): p. 277-84.
70. Brown, J.D., et al., *Prevalence of antibodies to type a influenza virus in wild avian species using two serologic assays.* Journal of Wildlife Diseases, 2010. **46**(3): p. 896-911.
71. Kapczynski, D.R. and D.E. Swayne, *Influenza vaccines for avian species.* Curr Top Microbiol Immunol, 2009. **333**: p. 133-52.
72. Magor, K.E., et al., *Secretory immune system of the duck (Anas platyrhynchos). Identification and expression of the genes encoding IgA and IgM heavy chains.* Eur J Immunol, 1998. **28**(3): p. 1063-8.
73. Magor, K.E., et al., *Opposite orientation of the alpha- and upsilon-chain constant region genes in the immunoglobulin heavy chain locus of the duck.* Immunogenetics, 1999. **49**(7-8): p. 692-5.
74. Magor, K.E., *Immunoglobulin genetics and antibody responses to influenza in ducks.* Dev Comp Immunol, 2011. **35**(9): p. 1008-16.
75. Warr, G.W., K.E. Magor, and D.A. Higgins, *IgY: clues to the origins of modern antibodies.* Immunol Today, 1995. **16**(8): p. 392-8.
76. Maxted, A.M., et al., *Annual survival of ruddy turnstones is not affected by natural infection with low pathogenicity avian influenza viruses.* Avian Dis, 2012. **56**(3): p. 567-73.
77. Kuiken, T., *Is low pathogenic avian influenza virus virulent for wild waterbirds?* Proceedings of the Royal Society B: Biological Sciences, 2013. **280**(1763): p. 20130990.
78. Munster, V.J., et al., *Mallards and highly pathogenic avian influenza ancestral viruses, northern Europe.* Emerg Infect Dis, 2005. **11**(10): p. 1545-51.
79. Krauss, S., et al., *Influenza in migratory birds and evidence of limited intercontinental virus exchange.* PLoS Pathogens, 2007. **3**(11): p. e167.
80. Rohani, P., D.J. Earn, and B.T. Grenfell, *Opposite patterns of synchrony in sympatric disease metapopulations.* Science, 1999. **286**(5441): p. 968-71.
81. Scott, D.A. and P.M. Rose, *Atlas of Anatidae Populations in Africa and Western Eurasia.* 1996, Wageningen, Nederlands: Wetlands International.
82. Bentz, P.G., *Studies on some urban Mallard Anas platyrhynchos populations in Scandinavia. Part I: cause of death, mortality and longevity among Malmo Mallards as shown by ringing recoveries.* Fauna Norvegica Series, 1985. **8**(1): p. 44-56.
83. Tsiodras, S., et al., *Human infections associated with wild birds.* J Infect, 2008. **56**(2): p. 83-98.
84. Stallknecht, D.E., et al., *Effects of pH, temperature, and salinity on persistence of avian influenza viruses in water.* Avian Dis, 1990. **34**(2): p. 412-8.
85. Lebarbenchon, C., et al., *Viral replication, persistence in water and genetic characterization of two influenza A viruses isolated from surface lake water.* PLoS One, 2011. **6**(10): p. e26566.
86. Nazir, J., et al., *Long-term study on tenacity of avian influenza viruses in water (distilled water, normal saline, and surface water) at different temperatures.* Avian Dis, 2010. **54**(1 Suppl): p. 720-4.
87. Brown, J.D., et al., *Avian influenza virus in water: infectivity is dependent on pH, salinity and temperature.* Vet Microbiol, 2009. **136**(1-2): p. 20-6.

88. Zarkov, I.S., *Survival of avian influenza viruses in filtered and natural surface waters of different physical and chemical parameters.* Revue de medecine Veterinaire, 2006. **157**: p. 471-476.
89. Brown, J.D., et al., *Persistence of H5 and H7 avian influenza viruses in water.* Avian Dis, 2007. **51**(1 Suppl): p. 285-9.
90. Macken, C.A., R.J. Webby, and W.J. Bruno, *Genotype turnover by reassortment of replication complex genes from avian influenza A virus.* J Gen Virol, 2006. **87**(Pt 10): p. 2803-15.
91. Hatchette, T.F., et al., *Influenza A viruses in feral Canadian ducks: extensive reassortment in nature.* J Gen Virol, 2004. **85**(Pt 8): p. 2327-37.
92. Dugan, V.G., et al., *The evolutionary genetics and emergence of avian influenza viruses in wild birds.* PLoS Pathogens, 2008. **4**(5): p. e1000076.
93. Widjaja, L., et al., *Matrix gene of influenza a viruses isolated from wild aquatic birds: ecology and emergence of influenza a viruses.* J Virol, 2004. **78**(16): p. 8771-9.
94. Wallensten, A., et al., *Multiple gene segment reassortment between Eurasian and American lineages of influenza A virus (H6N2) in Guillemot (Uria aalge).* Arch Virol, 2005. **150**(8): p. 1685-92.
95. Wahlgren, J., et al., *Gene segment reassortment between American and Asian lineages of avian influenza virus from waterfowl in the Beringia area.* Vector Borne Zoonotic Diseases, 2008. **8**(6): p. 783-90.
96. Makarova, N.V., et al., *Transmission of Eurasian avian H2 influenza virus to shorebirds in North America.* J Gen Virol, 1999. **80 (Pt 12)**: p. 3167-71.
97. Koehler, A.V., et al., *Genetic evidence of intercontinental movement of avian influenza in a migratory bird: the northern pintail (Anas acuta).* Molecular Ecology, 2008. **17**(21): p. 4754-62.
98. Pearce, J.M., et al., *Avian influenza at both ends of a migratory flyway: characterizing viral genomic diversity to optimize surveillance plans for North America.* Evolutionary Applications, 2009. **2**(4): p. 457-68.
99. Wille, M., et al., *Extensive geographic mosaicism in avian influenza viruses from gulls in the northern hemisphere.* PLoS One, 2011. **6**(6): p. e20664.
100. Van Borm, S., et al., *Phylogeographic analysis of avian influenza viruses isolated from Charadriiformes in Belgium confirms intercontinental reassortment in gulls.* Arch Virol, 2012. **157**(8): p. 1509-22.
101. zu Dohna, H., et al., *Invasions by Eurasian avian influenza virus H6 genes and replacement of the virus' North American clade.* Emerg Infect Dis, 2009. **15**(7): p. 1040-5.
102. Bahl, J., et al., *Gene flow and competitive exclusion of avian influenza A virus in natural reservoir hosts.* Virology, 2009. **390**(2): p. 289-97.
103. Fereidouni, S.R., et al., *Failure of productive infection of Mallards (Anas platyrhynchos) with H16 subtype of avian influenza viruses.* Influenza and Other Respiratory Viruses, 2014. **8**(6): p. 613-6.
104. Alexander, D.J., *An overview of the epidemiology of avian influenza.* Vaccine, 2007. **25**(30): p. 5637-44.
105. Voslamber, B., *Wilde vogels op en rond pluimveebedrijven*, in *Sovon-informatierapport 2005/13*. 2005, Sovon Vogelonderzoek Nederland: Nijmegen.
106. Voslamber, B., *Wilde vogels op en rond pluimveebedrijven, juli/augustus 2006*, in *Sovon-informatierapport 2006/8*. 2006, Sovon Vogelonderzoek Nederland: Nijmegen.
107. Cappelle, J., et al., *Characterizing the interface between wild ducks and poultry to evaluate the potential of transmission of avian pathogens.* International Journal of Health Geographics, 2011. **10**: p. 60.
108. Alexander, D.J. and I.H. Brown, *History of highly pathogenic avian influenza.* Rev Sci

Tech, 2009. **28**(1): p. 19-38.
109. OIE. *Update on highly pathogenic avian influenza in animals (type H5 and H7)*. 31 Dec 2012); Available from: http://www.oie.int/animal-health-in-the-world/update-on-avian-influenza/2012/.
110. Chen, H., et al., *The evolution of H5N1 influenza viruses in ducks in southern China.* Proc Natl Acad Sci U S A, 2004. **101**(28): p. 10452-7.
111. de Jong, J.C., et al., *A pandemic warning?* Nature, 1997. **389**(6651): p. 554.
112. Ellis, T.M., et al., *Investigation of outbreaks of highly pathogenic H5N1 avian influenza in waterfowl and wild birds in Hong Kong in late 2002.* Avian Pathology, 2004. **33**(5): p. 492-505.
113. Sims, L.D., et al., *Avian influenza in Hong Kong 1997-2002.* Avian Dis, 2003. **47**(3 Suppl): p. 832-8.
114. Kilpatrick, A.M., et al., *Predicting the global spread of H5N1 avian influenza.* Proc Natl Acad Sci U S A, 2006. **103**(51): p. 19368-73.
115. Starick, E., et al., *Phylogenetic analyses of highly pathogenic avian influenza virus isolates from Germany in 2006 and 2007 suggest at least three separate introductions of H5N1 virus.* Vet Microbiol, 2008. **128**(3-4): p. 243-52.
116. Si, Y., et al., *Spatio-temporal dynamics of global H5N1 outbreaks match bird migration patterns.* Geospatial Health, 2009. **4**(1): p. 65-78.
117. Feare, C.J. and M. Yasue, *Asymptomatic infection with highly pathogenic avian influenza H5N1 in wild birds: how sound is the evidence?* Virol J, 2006. **3**: p. 96.
118. Keawcharoen, J., et al., *Wild ducks as long-distance vectors of highly pathogenic avian influenza virus (H5N1).* Emerg Infect Dis, 2008. **14**(4): p. 600-7.
119. Kalthoff, D., et al., *Highly pathogenic avian influenza virus (H5N1) in experimentally infected adult mute swans.* Emerg Infect Dis, 2008. **14**(8): p. 1267-70.
120. Brown, J.D., et al., *Susceptibility of North American ducks and gulls to H5N1 highly pathogenic avian influenza viruses.* Emerg Infect Dis, 2006. **12**(11): p. 1663-70.
121. Brown, J.D., D.E. Stallknecht, and D.E. Swayne, *Experimental infection of swans and geese with highly pathogenic avian influenza virus (H5N1) of Asian lineage.* Emerg Infect Dis, 2008. **14**(1): p. 136-42.
122. Hesterberg, U., et al., *Avian influenza surveillance in wild birds in the European Union in 2006.* Influenza and Other Respiratory Viruses, 2009. **3**(1): p. 1-14.
123. Globig, A., et al., *Epidemiological and ornithological aspects of outbreaks of highly pathogenic avian influenza virus H5N1 of Asian lineage in wild birds in Germany, 2006 and 2007.* Transboundary and Emerging Diseases, 2009. **56**(3): p. 57-72.
124. Becker, W.B., *The isolation and classification of Tern virus: influenza A-Tern South Africa-1961.* Journal of Hygiene, 1966. **64**(3): p. 309-20.
125. Sturm-Ramirez, K.M., et al., *Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks.* J Virol, 2004. **78**(9): p. 4892-901.
126. Chen, H., et al., *Avian flu: H5N1 virus outbreak in migratory waterfowl.* Nature, 2005. **436**(7048): p. 191-2.
127. Liu, J., et al., *Highly pathogenic H5N1 influenza virus infection in migratory birds.* Science, 2005. **309**(5738): p. 1206.
128. Reperant, L.A., A.D.M.E. Osterhaus, and T. Kuiken, *Influenza Virus Infections*, in *Infectious Diseases of Wild Mammals and Birds in Europe*, D. Gavier-Widen, et al., Editors. 2012, Blackwell Publishing Ltd. p. 37-58.
129. Artois, M., et al., *Outbreaks of highly pathogenic avian influenza in Europe: the risks associated with wild birds.* Rev Sci Tech, 2009. **28**(1): p. 69-92.
130. Chen, H., et al., *Properties and dissemination of H5N1 viruses isolated during an influenza outbreak in migratory waterfowl in western China.* J Virol, 2006. **80**(12): p. 5976-83.

131. Chen, H.X., et al., *Seroprevalance and identification of influenza A virus infection from migratory wild waterfowl in China (2004-2005).* Journal of Veterinary Medicine. B, Infectious Diseases and Veterinary Public Health, 2006. **53**(4): p. 166-70.
132. Gaidet, N., et al., *Evidence of infection by H5N2 highly pathogenic avian influenza viruses in healthy wild waterfowl.* PLoS Pathogens, 2008. **4**(8): p. e1000127.
133. Hoye, B.J., et al., *Surveillance of wild birds for avian influenza virus.* Emerg Infect Dis, 2010. **16**(12): p. 1827-34.
134. Altizer, S., R. Bartel, and B.A. Han, *Animal migration and infectious disease risk.* Science, 2011. **331**(6015): p. 296-302.
135. Rappole, J.H. and Z. Hubalek, *Migratory birds and West Nile virus.* J Appl Microbiol, 2003. **94 Suppl**: p. 47S-58S.
136. Leroy, E.M., et al., *Human Ebola outbreak resulting from direct exposure to fruit bats in Luebo, Democratic Republic of Congo, 2007.* Vector Borne Zoonotic Diseases, 2009. **9**(6): p. 723-8.
137. Leighton, F.A., *Health risk assessment of the translocation of wild animals.* Rev Sci Tech, 2002. **21**(1): p. 187-95.
138. Waldenstrom, J., et al., *Cross-species infection of blood parasites between resident and migratory songbirds in Africa.* Mol Ecol, 2002. **11**(8): p. 1545-54.
139. Haydon, D.T., et al., *Identifying reservoirs of infection: a conceptual and practical challenge.* Emerging Infectious Diseases, 2002. **8**(12): p. 1468-73.
140. Höfle, U., et al., *Tissue tropism and pathology of natural influenza virus infection in black-headed gulls (Chroicocephalus ridibundus).* Avian Pathology, 2012. **41**(6): p. 547-53.
141. Stallknecht, D.E., et al., *Avian influenza viruses from migratory and resident ducks of coastal Louisiana.* Avian Dis, 1990. **34**(2): p. 398-405.
142. Ferro, P.J., et al., *Multiyear surveillance for avian influenza virus in waterfowl from wintering grounds, Texas coast, USA.* Emerg Infect Dis, 2010. **16**(8): p. 1224-30.
143. Lack, D., *The problem of partial migration.* British Birds, 1943. **37**: p. 122-130.
144. Chapman, B.B., et al., *The ecology and evolution of partial migration.* Oikos, 2011. **120**(12): p. 1764-1775.
145. Hill, N.J., et al., *Migration strategy affects avian influenza dynamics in mallards (Anas platyrhynchos).* Molecular Ecology, 2012. **21**(24): p. 5986-99.
146. van Dijk, J.G., et al., *Juveniles and migrants as drivers for seasonal epizootics of avian influenza virus.* Journal of Animal Ecology, 2014. **83**(1): p. 266-75.
147. Henaux, V. and M.D. Samuel, *Avian influenza shedding patterns in waterfowl: implications for surveillance, environmental transmission, and disease spread.* J Wildl Dis, 2011. **47**(3): p. 566-78.
148. Payne-Gallwey, R., *The book of duck decoys, their construction, management, and history*. 1886, London: J. van Voorst. 154.
149. Boyd, H., J. Harrison, and A. Allison, *Duck wings: a study of duck production*. 1975, Chester, Great Britain: A WAGBI Publication. Marley Ltd., and the Harrison Zoological Museum.
150. Fouchier, R.A. and V.J. Munster, *Epidemiology of low pathogenic avian influenza viruses in wild birds.* Rev Sci Tech, 2009. **28**(1): p. 49-58.
151. Munster, V.J., et al., *Practical considerations for high-throughput influenza A virus surveillance studies of wild birds by use of molecular diagnostic tests.* J Clin Microbiol, 2009. **47**(3): p. 666-73.
152. Hoye, B.J., *Variation in postsampling treatment of avian blood affects ecophysiological interpretations.* Methods in Ecology and Evolution, 2012. **3**: p. 162-167.
153. Peterson, B.J. and B. Fry, *Stable isotopes in ecosystem studies.* Annual Review of Ecology, Evolution, and Systematics, 1987. **18**: p. 293-320.

154. Hobson, K.A., *Tracing origins and migration of wildlife using stable isotopes: a review.* Oecologia, 1999. **120**: p. 314–326.
155. Hobson, K.A. and L.I. Wassenaar, *Linking breeding and wintering grounds of neotropical migrant songbirds using stable hydrogen isotopic analysis of feathers.* Oecologia, 1997. **109**: p. 142-148.
156. van Dijk, J.G.B., W. Meissner, and M. Klaassen, *Improving provenance studies in migratory birds when using feather hydrogen stable isotopes.* Journal of Avian Biology, 2014. **45**: p. 103–108.
157. Fouchier, R.A., et al., *Avian influenza A virus (H7N7) associated with human conjunctivitis and a fatal case of acute respiratory distress syndrome.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(5): p. 1356-61.
158. *GenBank*. Available from: http://www.ncbi.nlm.nih.gov.
159. Hoffmann, E., et al., *Universal primer set for the full-length amplification of all influenza A viruses.* Arch Virol, 2001. **146**(12): p. 2275-89.
160. *GISAID EpiFlu*. Available from: http://platform.gisaid.org/.
161. *MAFFT version 7*. Available from: mafft.cbrc.jp/alignment/software/.
162. Posada, D., *jModelTest: phylogenetic model averaging.* Molecular Biology and Evolution, 2008. **25**(7): p. 1253-6.
163. *Figtree version 1.4.0*. Available from: http://tree.bio.ed.ac.uk/software/figtree.
164. *BEAST version 1.8.0*. Available from: http://beast.bio.ed.ac.uk.
165. *Tracer version 1.6*. Available from: http://tree.bio.ed.ac.uk/software/tracer/.
166. Westgeest, K.B., et al., *Genomewide analysis of reassortment and evolution of human influenza A(H3N2) viruses circulating between 1968 and 2011.* J Virol, 2014. **88**(5): p. 2844-57.
167. Hirst, G.K., *Studies of Antigenic Differences among Strains of Influenza a by Means of Red Cell Agglutination.* Journal of Experimental Medicine, 1943. **78**(5): p. 407-23.
168. Team, R.D.C. *R: A Language and Environment for Statistical Computing*. 2012; Available from: https://www.r-project.org/.
169. Bates, D., M. Maechler, and B. Bolker. *Lme4:linear mixed-effects models using S4 classes. R package version 0.999999-0*. 2012; Available from: http://CRAN.R-project.org/package=lme4.
170. Hothorn, T., F. Bretz, and P. Westfall, *Simultaneous inference in general parametric models.* Biometrical Journal, 2008. **50**: p. 346–363.
171. Hardin, G., *The competitive exclusion principle.* Science, 1960. **131**(3409): p. 1292-7.
172. Latorre-Margalef, N., et al., *Long-term variation in influenza A virus prevalence and subtype diversity in migratory mallards in northern Europe.* Proc Biol Sci, 2014. **281**(1781): p. 20140098.
173. Curran, J.M., et al., *Variation in the responses of wild species of duck, gull, and wader to inoculation with a wild-bird-origin H6N2 low pathogenicity avian influenza virus.* Avian Dis, 2013. **57**(3): p. 581-6.
174. Newman, S.H., et al., *Eco-virological approach for assessing the role of wild birds in the spread of avian influenza H5N1 along the Central Asian Flyway.* PLoS One, 2012. **7**(2): p. e30636.
175. van de Kam, J., P. de Goeij, and S.J. Moore, *Shorebirds: An Illustrated Behavioural Ecology*. 2004, Zeist: KNNV.
176. Olson, S.H., et al., *Sampling strategies and biodiversity of influenza A subtypes in wild birds.* PLoS One, 2014. **9**(3): p. e90826.
177. Takekawa, J.Y., et al., *Migration of waterfowl in the East Asian flyway and spatial relationship to HPAI H5N1 outbreaks.* Avian Diseases, 2010. **54**(1 Suppl): p. 466-76.
178. Takekawa, J.Y., et al., *Movements of wild ruddy shelducks in the Central Asian Flyway*

and their spatial relationship to outbreaks of highly pathogenic avian influenza H5N1. Viruses, 2013. **5**(9): p. 2129-52.
179. Vijaykrishna, D., et al., *The recent establishment of North American H10 lineage influenza viruses in Australian wild waterfowl and the evolution of Australian avian influenza viruses.* J Virol, 2013. **87**(18): p. 10182-9.
180. Chen, R. and E.C. Holmes, *Frequent inter-species transmission and geographic subdivision in avian influenza viruses from wild birds.* Virology, 2009. **383**(1): p. 156-61.
181. Bahl, J., et al., *Influenza a virus migration and persistence in North American wild birds.* PLoS Pathogens, 2013. **9**(8): p. e1003570.
182. Obenauer, J.C., et al., *Large-scale sequence analysis of avian influenza isolates.* Science, 2006. **311**(5767): p. 1576-80.
183. Lewis, N.S., et al., *Avian influenza virus surveillance in wild birds in Georgia: 2009-2011.* PLoS One, 2013. **8**(3): p. e58534.
184. Worobey, M., G.Z. Han, and A. Rambaut, *A synchronized global sweep of the internal genes of modern avian influenza virus.* Nature, 2014. **508**(7495): p. 254-7.
185. Munir, M., et al., *Alleles A and B of non-structural protein 1 of avian influenza A viruses differentially inhibit beta interferon production in human and mink lung cells.* Journal of General Virology, 2011. **92**(Pt 9): p. 2111-21.
186. Chen, R. and E.C. Holmes, *Hitchhiking and the population genetic structure of avian influenza virus.* J Mol Evol, 2010. **70**(1): p. 98-105.
187. Lemey, P., et al., *Bayesian phylogeography finds its roots.* PLoS Computational Biology, 2009. **5**(9): p. e1000520.
188. Lu, L., S.J. Lycett, and A.J. Leigh Brown, *Reassortment patterns of avian influenza virus internal segments among different subtypes.* BMC Evolutionary Biology, 2014. **14**: p. 16.
189. Roche, B., et al., *Adaptive evolution and environmental durability jointly structure phylodynamic patterns in avian influenza viruses.* PLoS Biology, 2014. **12**(8): p. e1001931.
190. Latorre-Margalef, N., et al., *Heterosubtypic immunity to influenza A virus infections in mallards may explain existence of multiple virus subtypes.* PLoS Pathogens, 2013. **9**(6): p. e1003443.
191. Jehl, J.R., *Aspects of the molt migration.*, in *Bird Migration*. 1990, Springer: Berlin. p. 102–113.
192. Zhou, B. and D.E. Wentworth, *Influenza A virus molecular virology techniques.* Methods Mol Biol, 2012. **865**: p. 175-92.
193. Zhou, B., et al., *Single-reaction genomic amplification accelerates sequencing and vaccine production for classical and Swine origin human influenza a viruses.* J Virol, 2009. **83**(19): p. 10309-13.
194. Djikeng, A., et al., *Viral genome sequencing by random priming methods.* BMC Genomics, 2008. **9**: p. 5.
195. Djikeng, A., et al., *Metagenomic analysis of RNA viruses in a fresh water lake.* PLoS One, 2009. **4**(9): p. e7264.
196. Guindon, S. and O. Gascuel, *A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood.* Systematic Biology, 2003. **52**(5): p. 696-704.
197. Zwickl, D.J., *Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion.* 2006, The University of Texas at Austin: Austin, TX, USA.
198. *Path-O-Gen version 1.3*. Available from: http://tree.bio.ed.ac.uk/software/tempest/.
199. Drummond, A., O.G. Pybus, and A. Rambaut, *Inference of viral evolutionary rates from molecular sequences.* Adv Parasitol, 2003. **54**: p. 331-58.
200. Drummond, A.J., et al., *Bayesian coalescent inference of past population dynamics*

from molecular sequences. Mol Biol Evol, 2005. **22**(5): p. 1185-92.
201. Drummond, A.J., et al., *Relaxed phylogenetics and dating with confidence.* PLoS Biology, 2006. **4**(5): p. e88.
202. Drummond, A.J. and A. Rambaut, *BEAST: Bayesian evolutionary analysis by sampling trees.* BMC Evolutionary Biology, 2007. **7**: p. 214.
203. Bahl, J., et al., *Temporally structured metapopulation dynamics and persistence of influenza A H3N2 virus in humans.* Proc Natl Acad Sci U S A, 2011. **108**(48): p. 19359-64.
204. Bielejec, F., et al., *SPREAD: spatial phylogenetic reconstruction of evolutionary dynamics.* Bioinformatics, 2011. **27**(20): p. 2910-2.
205. Parker, J., A. Rambaut, and O.G. Pybus, *Correlating viral phenotypes with phylogeny: accounting for phylogenetic uncertainty.* Infection, Genetics and Evolution, 2008. **8**(3): p. 239-46.
206. *TreeMap version 1.0*. Available from: http://taxonomy.zoology.gla.ac.uk/rod/treemap.html.
207. Hinshaw, V.S., et al., *Circulation of influenza viruses and paramyxoviruses in waterfowl originating from two different areas of North America.* Bull World Health Organ, 1985. **63**(4): p. 711-9.
208. Cramp, S., *Handbook of the Birds of Europe, the Middle East and North Africa: The Birds of the Western Palearctic.* Vol. 3. 1983, Oxford: Oxford University Press.
209. Verhagen, J.H., et al., *Avian influenza a virus in wild birds in highly urbanized areas.* PLoS One, 2012. **7**(6): p. e38256.
210. *RStudio version 0.95.265*. Available from: https://www.rstudio.com/.
211. Van Dijk, J.G.B., et al., *Reproductie van de Kokmeeuw in kust- en binnenlandkolonies.* Limosa, 2009(82): p. 13-22.
212. Van Dijk, K. and R. Oosterhuis, *Herkomst, aantallen en broedsucces van Kokmeeuwen op Griend.* Limosa, 2010(83): p. 21-35.
213. Lutterop, D. and G. Kasemir, *Griend Vogels en Bewaking 2008*. 2009, Vereniging Natuurmonumenten: ‘s-Graveland.
214. Lutterop, D. and G. Kasemir, *Griend Vogels en Bewaking 2009*. 2010, Vereniging Natuurmonumenten: ‘s-Graveland.
215. Lutterop, D. and G. Kasemir, *Griend Vogels en Bewaking 2010*. 2011, Vereniging Natuurmonumenten: ‘s-Graveland.
216. Boele, A., et al., *Broedvogels in Nederland in 2010*, in *Sovon-rapport 2012/01*. 2012, Sovon Vogelonderzoek Nederland: Nijmegen.
217. Van Dijk, A.J., et al., *Broedvogels in Nederland in 2008*, in *Sovon-monitoringsrapport 2010/01*. 2010, Sovon Vogelonderzoek Nederland: Beek-Ubbergen.
218. Boele, A., et al., *Broedvogels in Nederland in 2009*, in *Sovon-monitoringsraport 2011/01*. 2011, Sovon Vogelonderzoek Nederland: Nijmegen.
219. Stienen, E.W.M., et al., *Broedresultaten van kokmeeuwen in Nederland in 1997.* Sula, 1998. **12**(1): p. 1-11.
220. Speek, B.J. and G. Speek, *Thieme’s vogelatlas*. 1984, Zutphen: Thieme.
221. Palomares, L.E., et al., *Sex- and age-related biometric distribution of Black-headed Gulls Larus ridibundus in Western European populations.* Bird Study, 1997. **44**: p. 310-317.
222. Grant, P.J., *Gulls: A Guide to Identification*. 1986, Arrington, USA: Buteo books.
223. Peig, J. and A.J. Green, *New perspectives for estimating body condition from mass/length data: the scaled mass index as an alternative method.* Oikos, 2009. **118**: p. 1883-1891.
224. Verhagen, J.H., et al., *Epidemiology of influenza A virus among black-headed gulls, the Netherlands, 2006-2010.* Emerging Infectious Diseases, 2014. **20**(1): p. 138-41.
225. Chaise, C., et al., *Protection patterns in duck and chicken after homo- or hetero-subtypic*

reinfections with h5 and h7 low pathogenicity avian influenza viruses: a comparative study. PLoS One, 2014. **9**(8): p. e105189.
226. Pepin, K.M., et al., *Quantification of heterosubtypic immunity between avian influenza subtypes H3N8 and H4N6 in multiple avian host species.* Journal of General Virology, 2012. **93**(Pt 12): p. 2575-83.
227. Fereidouni, S.R., et al., *Dynamics of specific antibody responses induced in mallards after infection by or immunization with low pathogenicity avian influenza viruses.* Avian Diseases, 2010. **54**(1): p. 79-85.
228. Tolf, C., et al., *Individual variation in influenza A virus infection histories and long-term immune responses in Mallards.* PLoS One, 2013. **8**(4): p. e61201.
229. Stallknecht, D.E. and S.M. Shane, *Host range of avian influenza virus in free-living birds.* Veterinary Research Communications, 1988. **12**(2-3): p. 125-41.
230. Kuiken, T. and T. Harder, *H5N1 highly pathogenic avian influenza: breaking the rules in disease emergence*, in *New directions in conservation medicine: applied cases of ecological health*, A.A. Aguirre, R.S. Ostfeld, and P. Daszak, Editors. 2012, Oxford University Press: Oxford, UK. p. 228–238.
231. Daoust, P.Y., et al., *Replication of low pathogenic avian influenza virus in naturally infected Mallard ducks (Anas platyrhynchos) causes no morphologic lesions.* J Wildl Dis, 2011. **47**(2): p. 401-9.
232. Higgins, D.A., K.F. Shortridge, and P.L. Ng, *Bile immunoglobulin of the duck (Anas platyrhynchos). II. Antibody response in influenza A virus infections.* Immunology, 1987. **62**(3): p. 499-504.
233. Gelb, J., Jr., W.A. Nix, and S.D. Gellman, *Infectious bronchitis virus antibodies in tears and their relationship to immunity.* Avian Diseases, 1998. **42**(2): p. 364-74.
234. Takada, A. and H. Kida, *Protective immune response of chickens against Newcastle disease, induced by the intranasal vaccination with inactivated virus.* Veterinary Microbiology, 1996. **50**(1-2): p. 17-25.
235. Coulson, B.S., et al., *Role of coproantibody in clinical protection of children during reinfection with rotavirus.* Journal of Clinical Microbiology, 1992. **30**(7): p. 1678-84.
236. Matson, D.O., et al., *Fecal antibody responses to symptomatic and asymptomatic rotavirus infections.* Journal of Infectious Diseases, 1993. **167**(3): p. 577-83.
237. Reperant, L.A., et al., *Highly pathogenic avian influenza virus H5N1 infection in a long-distance migrant shorebird under migratory and non-migratory states.* PLoS One, 2011. **6**(11): p. e27814.
238. Zohari, S., et al., *Phylogenetic analysis of the non-structural (NS) gene of influenza A viruses isolated from mallards in Northern Europe in 2005.* Virology Journal, 2008. **5**: p. 147.
239. Adams, S., et al., *The effect of avian influenza virus NS1 allele on virus replication and innate gene expression in avian cells.* Mol Immunol, 2013. **56**(4): p. 358-68.
240. Slemons, R.D., et al., *Type-A influenza viruses isolated from wild free-flying ducks in California.* Avian Dis, 1974. **18**(1): p. 119-24.
241. Webster, R.G., et al., *Ortho- and paramyxoviruses from migrating feral ducks: characterization of a new group of influenza A viruses.* J Gen Virol, 1976. **32**(2): p. 217-25.
242. Gronesova, P., A. Mizakova, and T. Betakova, *Determination of hemagglutinin and neuraminidase subtypes of avian influenza A viruses in urban pigeons by a new nested RT-PCR.* Acta Virol, 2009. **53**(3): p. 213-6.
243. Smietanka, K., et al., *Avian influenza H5N1 outbreak in a flock of mute swans in the city of Torun, Poland, in 2006.* Bulletin of the Veterinary Institute in Pulawy, 2008. **52**(4): p. 491-495.
244. UNFPA. *State of world population 2007: Unleashing the Potential of Urban Growth;*

United Nations Populations Fund. 2007; Available from: https://www.unfpa.org/sites/default/files/pub-pdf/695_filename_sowp2007_eng.pdf.

245. Hirst, M., et al., *Novel avian influenza H7N3 strain outbreak, British Columbia.* Emerging Infectious Diseases, 2004. **10**(12): p. 2192-5.
246. Arzey, G.G., et al., *Influenza virus A (H10N7) in chickens and poultry abattoir workers, Australia.* Emerging Infectious Diseases, 2012. **18**(5): p. 814-6.
247. Beigel, J.H., et al., *Avian influenza A (H5N1) infection in humans.* New England Journal of Medicine, 2005. **353**(13): p. 1374-85.
248. Ostrowsky, B., et al., *Low pathogenic avian influenza A (H7N2) virus infection in immunocompromised adult, New York, USA, 2003.* Emerging Infectious Diseases, 2012. **18**(7): p. 1128-31.
249. Chen, H., et al., *Clinical and epidemiological characteristics of a fatal case of avian influenza A H10N8 virus infection: a descriptive study.* Lancet, 2014. **383**(9918): p. 714-21.
250. Gao, R., et al., *Human infection with a novel avian-origin influenza A (H7N9) virus.* New England Journal of Medicine, 2013. **368**(20): p. 1888-97.
251. Peiris, M., et al., *Human infection with influenza H9N2.* Lancet, 1999. **354**(9182): p. 916-7.
252. Subbarao, K., et al., *Characterization of an avian influenza A (H5N1) virus isolated from a child with a fatal respiratory illness.* Science, 1998. **279**(5349): p. 393-6.
253. Berhane, Y., et al., *Highly pathogenic avian influenza virus A (H7N3) in domestic poultry, Saskatchewan, Canada, 2007.* Emerg Infect Dis, 2009. **15**(9): p. 1492-5.
254. de Wit, J.J., et al., *A cross-sectional serological survey of the Dutch commercial poultry population for the presence of low pathogenic avian influenza virus infections.* Avian Pathology, 2004. **33**(6): p. 565-70.
255. Lebarbenchon, C. and D.E. Stallknecht, *Host shifts and molecular evolution of H7 avian influenza virus hemagglutinin.* Virol J, 2011. **8**: p. 328.
256. Velkers, F.C., et al., *Outbreak of avian influenza H7N3 on a turkey farm in the Netherlands.* Veterinary Record, 2006. **159**(13): p. 403-5.
257. Si, Y., W.F. de Boer, and P. Gong, *Different environmental drivers of highly pathogenic avian influenza H5N1 outbreaks in poultry and wild birds.* PLoS One, 2013. **8**(1): p. e53362.
258. Gilbert, M. and D.U. Pfeiffer, *Risk factor modelling of the spatio-temporal patterns of highly pathogenic avian influenza (HPAIV) H5N1: a review.* Spatial and Spatio-temporal Epidemiology, 2012. **3**(3): p. 173-83.
259. Welby, S., et al., *Redesigning the serological surveillance program for notifiable avian influenza in Belgian professional poultry holdings.* Avian Diseases, 2010. **54**(1 Suppl): p. 597-605.
260. Gonzales, J.L., et al., *Rate of introduction of a low pathogenic avian influenza virus infection in different poultry production sectors in the Netherlands.* Influenza and Other Respiratory Viruses, 2013. **7**(1): p. 6-10.
261. Gonzales, J.L., et al., *Low-pathogenic notifiable avian influenza serosurveillance and the risk of infection in poultry–a critical review of the European Union active surveillance programme (2005-2007).* Influenza and Other Respiratory Viruses, 2010. **4**(2): p. 91-9.
262. Gonzales, J.L., *Surveillance of low pathogenic avian influenza in layer chickens: risk factors, transmission and early detection (dissertation)*, in *Central Veterinary Institute*. 2012, University of Utrecht: Utrecht (the Netherlands).
263. Sovon. 2-June-2015); Available from: https://www.sovon.nl/.
264. Hornman, M., et al., *Watervogels in Nederland in 2009/2010*, in *Sovon-rapport 2012/02*. 2012, Sovon Vogelonderzoek Nederland: Nijmegen.
265. Elbers, A.R., G. Koch, and A. Bouma, *Performance of clinical signs in poultry for*

the detection of outbreaks during the avian influenza A (H7N7) epidemic in The Netherlands in 2003. Avian Pathology, 2005. **34**(3): p. 181-7.
266. de Boer, G.F., W. Back, and A.D. Osterhaus, *An ELISA for detection of antibodies against influenza A nucleoprotein in humans and various animal species.* Arch Virol, 1990. **115**(1-2): p. 47-61.
267. European Council, *Council Directive 2005/94/EC of 20 December 2005 on Community measures for the control of avian influenza and repealing Directive 92/40/EEC. OJEU L10: 14.11.2006, p2016*. 2005.
268. Guindon, S., et al., *New algorithms and methods to estimate maximum-likelihood phylogenies: assessing the performance of PhyML 3.0.* Systematic Biology, 2010. **59**(3): p. 307-21.
269. *TOP10NL*. Available from: https://www.kadaster.nl/.
270. WHO. *H5N1 highly pathogenic avian influenza: Timeline of major events*. 2014.
271. Shepard, S.S., et al., *LABEL: fast and accurate lineage assignment with assessment of H5N1 and H9N2 influenza A hemagglutinins.* PLoS One, 2014. **9**(1): p. e86921.
272. Zhao, G., et al., *Novel reassortant highly pathogenic H5N2 avian influenza viruses in poultry in China.* PLoS One, 2012. **7**(9): p. e46183.
273. Liu, C.G., et al., *Emerging multiple reassortant H5N5 avian influenza viruses in ducks, China, 2008.* Veterinary Microbiology, 2013. **167**(3-4): p. 296-306.
274. Zhao, K., et al., *Characterization of three H5N5 and one H5N8 highly pathogenic avian influenza viruses in China.* Veterinary Microbiology, 2013. **163**(3-4): p. 351-7.
275. Wong, F.Y., et al., *Reassortant highly pathogenic influenza A(H5N6) virus in Laos.* Emerg Infect Dis, 2015. **21**(3): p. 511-6.
276. OIE. *Update on highly pathogenic avian influenza in animals (type H5 and H7)*. 06 Mar 2015); Available from: http://www.oie.int/animal-health-in-the-world/update-on-avian-influenza/2014/.
277. ProMED-mail. *Avian influenza*. 02 Feb 2015); Available from: http://www.promedmail.org/.
278. Adlhoch, C., et al., *Comparing introduction to Europe of highly pathogenic avian influenza viruses A(H5N8) in 2014 and A(H5N1) in 2005.* Eurosurveillance, 2014. **19**(50).
279. Jeong, J., et al., *Highly pathogenic avian influenza virus (H5N8) in domestic poultry and its relationship with migratory birds in South Korea during 2014.* Veterinary Microbiology, 2014. **173**(3-4): p. 249-57.
280. Kim, Y.I., et al., *Pathobiological features of a novel, highly pathogenic avian influenza A(H5N8) virus.* Emerging Microbes and Infections, 2014. **3**(10): p. e75.
281. Herfst, S., et al., *Airborne transmission of influenza A/H5N1 virus between ferrets.* Science, 2012. **336**(6088): p. 1534-41.
282. Munster, V.J., et al., *Pathogenesis and transmission of swine-origin 2009 A(H1N1) influenza virus in ferrets.* Science, 2009. **325**(5939): p. 481-3.
283. Li, Q., et al., *Novel H5 clade 2.3.4.6 viruses with both alpha-2,3 and alpha-2,6 receptor binding properties may pose a pandemic threat.* Vet Res, 2014. **45**: p. 127.
284. WHO. *Antigenic and genetic characteristics of zoonotic influenza viruses and development of candidate vaccine viruses for pandemic preparedness*. 2014; Available from: http://who.int/influenza/vaccines/virus/201409_zoonotic_vaccinevirusupdate.pdf?ua=1.
285. Gilbert, M., et al., *Serological Evidence for Non-Lethal Exposures of Mongolian Wild Birds to Highly Pathogenic Avian Influenza H5N1 Virus.* PLoS One, 2014. **9**(12): p. e113569.
286. Dusek, R.J., et al., *North Atlantic migratory bird flyways provide routes for intercontinental movement of avian influenza viruses.* PLoS One, 2014. **9**(3): p. e92075.
287. *USGS*. Available from: http://www.nwhc.usgs.gov.

288. Lee, Y.J., et al., *Novel reassortant influenza A(H5N8) viruses, South Korea, 2014.* Emerging Infectious Diseases, 2014. **20**(6): p. 1087-9.
289. Dobrynina, D.V. and S.P. Kharitonov, *The Russian waterbird migration atlas: temporal variation in migration routes*, in *Waterbirds around the world*, G.C.G. Boere, C.A.; Stroud, D.A., Editor. 2006, The Stationary Office: Edinbrugh. p. 582-589.
290. Atkinson, P.W., et al., *Migratory movements of waterfowl: a web-based mapping tool*, in *EURING report to the EU Commission*. 2007.
291. BirdLife International and Nature Serve, *Bird species distribution maps of the world*. 2014, Cambridge, UK/Arlington, USA: BirdLife International.
292. Syroechkovsky, E.E., *Goose, Swan and Duck Study Group of the Northern Eurasia*, in *Field Guide for Anseriformes of Russia*. 2011: Moscow, Russia
293. Hupp, J.W., et al., *Moult migration of emperor geese Chen canagica between Alaska and Russia.* Journal of Avian Biology, 2007. **38**: p. 462-470.
294. Derksen, D.V., et al., *Black Brant from Alaska staging and wintering in Japan.* Condor, 1996. **98**: p. 653-657.
295. Ely, C.R., J.Y. Takekawa, and M.L. Wege, *Distribution, abundance and age ratios of Wrangel Island Lesser Snow Geese Anser caerulescens during autumn migration on the Yukon-Kuskokwim Delta, Alaska.* Wildfowl, 1993. **44**: p. 24-32.
296. Miller, M.R., et al., *Spring migration of Northern Pintails from California's Central Valley wintering area tracked with satellite telemetry: routes, timing, and destinations.* Canadian Journal of Zoology, 2005. **83**: p. 1314-1332.
297. Flint, P.L., et al., *Breeding-season sympatry facilitates genetic exchange among allopatric wintering populations of northern pintails in Japan and California.* The Condor, 2009. **111**(4): p. 591-598.
298. Van Gils, J. and P. Wiersma, *Baird's Sandpiper (Calidris bairdii).* in *Handbook of the Birds of the World Alive*, J. del Hoyo, Elliott, A., Sargatal, J., Christie, D.A. & de Juana, E., Editor. 1996, Lynx Edicions: Barcelona.
299. Van Gils, J., P. Wiersma, and A. Bonan, *Buff-breasted Sandpiper (Calidris subruficollis)*, in *Handbook of the Birds of the World Alive*, J. del Hoyo, Elliott, A., Sargatal, J., Christie, D.A. & de Juana, E., Editor. 2013, Lynx Edicions: Barcelona.
300. Pandit, S.S., et al., *Modelling highly pathogenic avian influenza transmission in wild birds and poultry in West Bengal, India.* Scientific Reports, 2013. **3**(2175).
301. Pawar, S.D., et al., *Avian influenza surveillance reveals presence of low pathogenic avian influenza viruses in poultry during 2009-2011 in the West Bengal State, India.* Virology Journal, 2012. **9**(151).
302. Fereidouni, S.R., et al., *Saving resources: avian influenza surveillance using pooled swab samples and reduced reaction volumes in real-time RT-PCR.* J Virol Methods, 2012. **186**(1-2): p. 119-25.
303. Bridge, E.S., et al., *Bird Migration and Avian Influenza: A Comparison of Hydrogen Stable Isotopes and Satellite Tracking Methods.* Ecol Indic, 2014. **45**: p. 266-273.
304. Ogden, N.H., et al., *How far north are migrant birds transporting the tick Ixodes scapularis in Canada? Insights from stable hydrogen isotope analyses of feathers.* Ticks Tick Borne Dis, 2015. **6**(6): p. 715-20.
305. Ramey, A.M., et al., *Dispersal of H9N2 influenza A viruses between East Asia and North America by wild birds.* Virology, 2015. **482**: p. 79-83.
306. Huang, Y., et al., *Genetic structure of avian influenza viruses from ducks of the Atlantic flyway of North America.* PLoS One, 2014. **9**(1): p. e86999.
307. Huang, Y., et al., *Perpetuation and reassortment of gull influenza A viruses in Atlantic North America.* Virology, 2014. **456-457**: p. 353-63.
308. Wille, M., et al., *Reassortment of American and Eurasian genes in an influenza A virus isolated from a great black-backed gull (Larus marinus), a species demonstrated to*

move between these regions. Archives of Virology, 2011. **156**(1): p. 107-15.

309. Wille, M., et al., *Frequency and patterns of reassortment in natural influenza A virus infection in a reservoir host.* Virology, 2013. **443**(1): p. 150-60.
310. Kapczynski, D.R., *Detection of cell-mediated immune response to avian influenza viruses.* Methods Mol Biol, 2014. **1161**: p. 199-215.
311. Fouchier, R.A. and D.J. Smith, *Use of antigenic cartography in vaccine seed strain selection.* Avian Dis, 2010. **54**(1 Suppl): p. 220-3.
312. Freidl, G.S., et al., *Getting more out of less-a quantitative serological screening tool for simultaneous detection of multiple influenza A hemagglutinin-types in chickens.* PLoS One, 2014. **9**(9): p. e108043.
313. Pons, J.M., A. Hassanin, and P.A. Crochet, *Phylogenetic relationships within the Laridae (Charadriiformes: Aves) inferred from mitochondrial markers.* Molecular Phylogenetics and Evolution, 2005. **37**: p. 686-699.
314. Arnal, A., et al., *Laridae: A neglected reservoir that could play a major role in avian influenza virus epidemiological dynamics.* Crit Rev Microbiol, 2015. **41**(4): p. 508-19.
315. Lebarbenchon, C., et al., *Influenza a virus on oceanic islands: host and viral diversity in seabirds in the Western Indian ocean.* PLoS Pathogens, 2015. **11**(5): p. e1004925.
316. Runstadler, J.A., et al., *Using RRT-PCR analysis and virus isolation to determine the prevalence of avian influenza virus infections in ducks at Minto Flats State Game Refuge, Alaska, during August 2005.* Archives of Virology, 2007. **152**(10): p. 1901-10.
317. Caron, A., et al., *Bridge hosts, a missing link for disease ecology in multi-host systems.* Vet Res, 2015. **46**: p. 83.
318. Antoniou, M., et al., *Rats as indicators of the presence and dispersal of pathogens in Cyprus: ectoparasites, parasitic helminths, enteric bacteria, and encephalomyocarditis virus.* Vector Borne Zoonotic Diseases, 2010. **10**(9): p. 867-73.
319. Rothenburger, J.L., et al., *Respiratory Pathology and Pathogens in Wild Urban Rats (Rattus norvegicus and Rattus rattus).* Vet Pathol, 2015. **52**(6): p. 1210-9.
320. Machalaba, C.C., et al., *Global avian influenza surveillance in wild birds: a strategy to capture viral diversity.* Emerg Infect Dis, 2015. **21**(4): p. e1-7.
321. Richard, M., et al., *Low Virulence and Lack of Airborne Transmission of the Dutch Highly Pathogenic Avian Influenza Virus H5N8 in Ferrets.* PLoS One, 2015. **10**(6): p. e0129827.

Summary (English and Dutch)

Summary

The presence of influenza A viruses in long-distant migrating birds and the highly pathogenic properties of some of these viruses for poultry predominantly are worrisome. The impact of influenza A virus outbreaks on domestic poultry health, welfare and industry can be immense, and can pose a threat to human health. Influenza A viruses are mainly known as the cause of annual flu outbreaks and pandemics (worldwide outbreaks, such as H1N1 Spanish influenza in 1918) in humans and as the cause of bird flu outbreaks in poultry. Less known is that wild birds are naturally infected with a high variety of influenza viruses and that these viruses can be transmitted and cause disease in other animals, like poultry. Influenza A viruses are characterized based on their surface proteins: the hemagglutinin (HA) and the neuraminidase (NA). In wild birds 16 different HA subtypes (H1-H16) and 9 different NA subtypes (N1-N9) have been detected. The genome of influenza A viruses is segmented, facilitating exchange of these segments (so called genetic reassortment), resulting in high virus diversity. In addition, influenza A viruses in wild birds can be characterized based on their ability to cause disease in chickens; low pathogenic avian influenza (LPAI) viruses can cause no to mild disease, and highly pathogenic avian influenza (HPAI) viruses can cause severe disease and high mortality. Wild birds are mainly infected with LPAI viruses in which they cause no to mild disease in general. The research presented in this thesis focuses on the ecology and evolution of influenza viruses in wild aquatic birds—in particular gulls—and the virus transmission between wild and domestic birds. An introduction on influenza A viruses and the birds that are naturally infected with these viruses is described in chapter 1.

The ecology and evolution of influenza A viruses in wild aquatic birds

Few animals are as mobile as birds. This mobility makes them one of the most fascinating and important vectors for emerging infectious diseases, such as influenza A viruses. Wild birds with different migratory strategies are likely to differ in LPAI virus exposure and susceptibility, however this has rarely been investigated within single species. The role of long-distant migrants, local migrants and residential birds in the introduction and infection dynamics of LPAI virus during a LPAI virus outbreak in mallards was investigated (chapter 2.1). Within this study, we combined virological and ecological techniques which is an improvement over previous studies. The presence of migrating mallards was shown to amplify the virus outbreak while no evidence was obtained for the geographical dispersal by migrating mallards of the outbreak LPAI virus.

The epidemiology and evolution of influenza A viruses in Eurasian wild birds

were investigated based on genetic analyses of the complete virus genome of viruses collected at various geographic locations during a 15-year period and combined with temporal and spatial information (chapter 2.2). Frequent reassortment (or mixing of gene segments) and co-circulating lineages were observed for all eight gene segments. No apparent species-specific effect on influenza virus diversity was identified. There was a spatial and temporal relationship between the Eurasian sequences and significant viral migration of influenza viruses from West Eurasia towards Central Eurasia, however viral migration patterns differed between segments.

The ecology and evolution of avian influenza viruses in gulls: a potential model for influenza viruses in ducks?

Highest influenza A virus subtype diversity (H1-H12) has been detected in ducks of the *Anas* genus, such as the mallard. In contrast, birds belonging to the *Laridae* family, such as gulls, are endemically infected with a limited diversity of influenza A virus subtypes, largely confined to H13 and H16. In addition to migration behavior, breeding behavior affects the spread of influenza A viruses. Massive virus amplification was detected at dense breeding colony sites of black-headed gulls (*Chroicocephalus ridibundus*) at the end of the breeding season when juvenile birds left the nest (chapter 2.3). Furthermore, it was shown in an experimental setting that these immuno-naive juvenile birds were protected after single or multiple influenza A virus infections for a period of at least one year after infection (chapter 2.4), emphasizing an important role for juvenile birds in the epidemiology of influenza A viruses. These chapters provide valuable data to build analytical mathematical models describing the epidemiology and evolution of avian influenza in wild birds, which may form the basis for similar work to describe influenza epidemiology and evolution in Anseriformes species.

Wild – domestic interface studies on influenza A viruses: the identification of host species and routes

The mobility of birds enables them to move between a wide variety of environments and habitats—including agricultural areas and cities—potentially facilitating exposure of domestic animals and humans to wild bird viruses. Influenza A viruses were detected in birds sampled in cities, therefore demonstrating that feral and urban bird populations, and the pathogens they harbor, are not separated completely (chapter 3.1). Furthermore, a comparative study in wild birds and poultry of the Netherlands demonstrated that influenza A viruses as detected in poultry were genetically similar to influenza A viruses as detected in wild birds, but yielded different subtype distributions,

suggesting different susceptibility of wild birds and poultry to influenza A virus subtypes and strains (chapter 3.2). Upon the emergence of the HPAI H5N8 virus in Europe in 2014, the virus was detected in Eurasian wigeons (*Anas penelope*), a long-distant migratory duck species, suggesting a role of this species in the local and/or global spread of these HPAI viruses (chapter 3.3; 3.4). The underlying mechanisms that drive interspecies and geographical spread of HPAI H5 viruses, and their closely related low pathogenic avian influenza viruses, need further study in order to target preventive measures to prevent and limit virus spread.

This thesis shed new light on virus and host characteristics that shape the underlying mechanisms driving the geographic distribution of influenza A viruses by birds in time and space. The effect of birds' ecology on the epidemiology and genetic diversity of influenza A viruses needs further study based on more detailed datasets collected from wild birds along different migratory routes within a short period of time, including metadata such as the migratory status of the bird.

Nederlandse samenvatting

De aanwezigheid van griepvirussen in trekvogels en het sterk ziekteverwekkend vermogen van sommige van deze virussen voor met name pluimvee is zorgwekkend. Trekvogels leggen jaarlijks in gigantische aantallen enorme afstanden af, waarbij zij virussen kunnen verplaatsen over kleine en onvoorstelbaar grote afstanden, zelfs tussen continenten. Griepvirusuitbraken in pluimvee kunnen niet alleen drastische gevolgen hebben voor de gezondheid en het welzijn van de dieren en voor de pluimvee verwerkende industrie, maar kunnen ook een bedreiging vormen voor de volksgezondheid. Griepvirussen zijn vooral bekend als veroorzakers van pandemieën (wereldwijde uitbraken, zoals de H1N1 Spaanse griep in 1918) en van de jaarlijkse griepepidemieën bij de mens. Daarnaast zijn griepvirussen bekend als veroorzaker van uitbraken van vogelgriep bij pluimvee. Minder bekend is dat griepvirussen van nature veel in wilde vogels, zoals eenden, voorkomen en vanuit wilde vogels overgedragen kunnen worden en ziekte kunnen veroorzaken in andere dieren, waaronder pluimvee.

Griepvirussen worden ingedeeld op basis van twee eiwitten aan de buitenkant van het virus deeltje: het hemagglutinine (HA) en het neuraminidase (NA) eiwit. In wilde vogels zijn er zestien verschillende HA subtypen (H1-H16) en negen verschillende NA subtypen (N1-N9) aangetroffen. Daarnaast kunnen vogelgriepvirussen worden ingedeeld op basis van hun ziekteverwekkend vermogen in kippen; laag pathogene aviaire influenza (LPAI) virussen die geen of slechts milde ziekte veroorzaken, en hoog pathogene aviaire influenza (HPAI) virussen die ernstige ziekte en massale sterfte kunnen veroorzaken. Bij wilde vogels worden met name LPAI virussen aangetroffen die bij de wilde vogels zelf in het algemeen geen tot weinig ziekte veroorzaken. Kenmerkend voor griepvirussen is dat hun erfelijk materiaal gesegmenteerd is, dat wil zeggen dat het bestaat uit afzonderlijke stukjes, en die stukjes kunnen onderling uitgewisseld worden (zogenaamd ‘genetisch reassorteren’), waardoor griepvirussen van nature een divers en veranderlijk innerlijk en uiterlijk hebben. Dit veranderlijke uiterlijk maakt dat het afweersysteem het virus niet of minder gemakkelijk kan herkennen met als gevolg dat het virus zich gemakkelijker binnen een populatie kan verspreiden.

De verspreiding en de evolutie van griepvirussen hangt in grote mate af van de wisselwerking tussen het virus, de vogel en de omgeving, oftewel de ecologie. Het onderzoek beschreven in dit proefschrift richt zich op de ecologie en evolutie van griepvirussen in wilde watervogels—en in het bijzonder in meeuwen—en de virusoverdracht tussen wilde en gehouden vogels. Achtergrondinformatie over griepvirussen en de vogels waarin de virussen voorkomen is beschreven in hoofdstuk 1.

De ecologie en evolutie van griepvirussen in wilde watervogels

Weinig dieren verplaatsen zich over zulke grote afstanden en uiteenlopende richtingen als trekvogels. Dat maakt hen tot een van de meest fascinerende en belangrijke verspreiders van infectieziekten. Wilde vogels verschillen onderling sterk in de mate waarin zij zich verplaatsen. Binnen de soort wilde eend (*Anas platyrhyngus*) zijn er eenden die zeer plaatstrouw zijn (standvogels) en eenden die zich over kleine of grotere afstanden verplaatsen (korteafstand en langeafstand trekvogels). Over de vraag in hoeverre verschillende trekroutes en timing binnen een vogelsoort effect hebben op het voorkomen en de verspreiding van griepvirussen is weinig bekend. De mogelijk verschillende rol die langeafstand trekvogels, korteafstand trekvogels en standvogels spelen bij in het ontstaan en het verloop van een lokale griepuitbraak hebben wij in wilde eenden onderzocht (hoofdstuk 2.1). Daarbij bleek dat trekvogels een virusuitbraak lokaal kunnen versterken, maar werd er geen bewijs gevonden voor een rol van trekvogels in het verplaatsen van het betreffende griepvirus van de ene naar de andere locatie. Het resultaat van deze studie, in combinatie met bestaande literatuur, benadrukt dat trekvogels meerdere rollen kunnen hebben in de verspreiding van griepvirussen (te weten het verplaatsen van virussen tussen verschillende plekken en het lokaal versterken van een uitbraak) en belangrijk zijn om te bemonsteren binnen griepvirus onderzoek en surveillance programma's.

Griepvirussen worden wereldwijd in meer dan 105 vogelsoorten aangetroffen. De verspreiding en evolutie van griepvirussen afkomstig van Euraziatische vogels is in kaart gebracht op basis van analyses aan het complete erfelijk materiaal van virussen afkomstig van vogels van uiteenlopende locaties gedurende een periode van 15 jaar in combinatie met informatie over de locatie en datum van bemonsteren (hoofdstuk 2.2). Uit deze studie bleek dat delen van het erfelijk materiaal van de virussen onderling frequent werden uitgewisseld en dat virussen met deels hetzelfde erfelijk materiaal gelijktijdig in wilde vogels circuleerden. In deze studie werden geen aanwijzingen gevonden voor een effect van vogelsoort op de diversiteit van griepvirussen. Tot slot, verwant erfelijk materiaal van griepvirussen afkomstig van Euraziatische vogels groepeerden in tijd en ruimte, en er werd bewijs gevonden voor virus verplaatsing vanuit West Eurazië naar Centraal Eurazië, echter de virus verplaatsing patronen verschilden per onderdeel van het erfelijk materiaal.

De ecologie en evolutie van griepvirussen in meeuwen: een model voor griepvirussen in eenden?

De grootste verscheidenheid aan griepvirus subtypen (H1-H12) wordt gevonden in eenden van het *Anas* genus, zoals de wilde eend. Daarentegen zijn vogels behorende tot de Laridae familie, zoals meeuwen, endemisch ('van nature rijkelijk') geïnfecteerd met een beperkte diversiteit aan griepvirus subtypen, voornamelijk H13 en H16. In aanvulling op het migratiegedrag speelt het broedgedrag een rol in het voorkomen van griepvirussen. Om de verspreiding van griepvirussen door kokmeeuwen (*Chroicocephalus ridibundus*) beter in kaart te brengen zijn gedurende zes jaar kokmeeuwpopulaties in Nederland jaarrond bemonsterd op de aanwezigheid van griepvirussen en griepvirus-antilichamen (hoofdstuk 2.3). Binnen deze studie werd in dichtbevolkte kokmeeuw broedkolonies aan het eind van het broedseizoen, wanneer de kuikens uitvliegen, massale virusreplicatie waargenomen. Het resultaat van deze studie is waardevol voor het bepalen van het moment waarop in kolonie broedende vogels het beste op aanwezigheid van griepvirussen kunnen worden bemonsterd.

Het is aannemelijk dat de verspreiding en de grote verscheidenheid aan griepvirussen in wilde vogels deels gevormd wordt door blootstelling aan eerdere virusinfecties, maar over hoe dit precies werkt is weinig bekend. In een experimentele studie is het effect van een of meerdere H13 en H16 griepvirusinfecties in kokmeeuwen op de gevoeligheid voor infectie en virusuitscheiding in kaart gebracht (hoofdstuk 2.4). Deze studie liet zien dat de kokmeeuwkuikens na een of meerdere infecties beschermd bleken te zijn tegen een herinfectie met hetzelfde virus gedurende een periode van minstens een jaar. Door deze twee studies is de belangrijke rol van jonge vogels in de verspreiding van griepvirussen in kokmeeuwen voor het eerst overtuigend aangetoond.

Virus-overdracht tussen wild en gedomesticeerd: identificeren van gastheersoorten en routes

De mobiliteit van wilde vogels maakt dat zij zich bewegen tussen veel verschillende omgevingen—inclusief landbouw- en veeteelt gebieden en steden—als gevolg waarvan gehouden dieren en mensen het risico lopen te worden blootgesteld aan virussen afkomstig van wilde vogels. In een onderzoek naar het voorkomen van griepvirussen in steden, werden griepvirussen gevonden in vrijlevende stadsvogels, hetgeen suggereert dat stedelijke en landelijke vogelpopulaties, en de bijbehorende ziekteverwekkers, in contact staan met elkaar (hoofdstuk 3.1). Deze studie laat zien dat stadsvogels een mogelijke bron zijn voor griepvirus overdracht naar gehouden dieren en de mens.

Daarnaast liet een studie in wilde vogels en pluimvee in Nederland zien dat griepvirussen in pluimvee op basis van hun erfelijk materiaal vergelijkbaar zijn met griepvirussen uit wilde vogels, maar dat desondanks verschillende griepvirussen bij wilde vogels en pluimvee voorkomen. Dit laatste suggereert dat de gevoeligheid om geïnfecteerd te raken bij wilde vogels en pluimvee uiteenloopt voor verschillende griepvirus subtypen en lijnen (hoofdstuk 3.2). De gevoeligheid van wilde vogels en pluimvee voor infectie met verschillende subtypen en lijnen zal verder uitgezocht dienen te worden in experimentele infectie studies. Na het plots opduiken van hoog pathogeen H5N8 griepvirus in Europa in 2014, werd het virus gevonden in smienten (*Anas penelope*), een langeafstand trekvogel, hetgeen doet denken dat deze vogelsoort een rol vervult in de korte- en/of lange afstand verspreiding van hoog pathogene H5 virussen (hoofdstuk 3.3; 3.4). In hoeverre andere vogelsoorten dan de smient geïnfecteerd zijn geweest met het H5N8 griepvirus en in hoeverre het H5N8 griepvirus zal blijven circuleren in de wilde vogel populatie zal verder dienen te worden uitgezocht door het voortzetten van de wilde vogel bemonstering.

De mechanismen die de overdracht van hoog pathogene H5 griepvirussen, en hun genetisch nauw verwante laag pathogene griepvirussen, tussen diersoorten en locaties sturen, dienen verder bestudeerd te worden, zodat er meer gerichte maatregelen kunnen worden genomen om virus introductie te voorkomen en spreiding te beperken.

CHAPTER 7

Author's affiliations

Department of Viroscience, Erasmus MC, Rotterdam, the Netherlands
Ron A.M. Fouchier, Oanh Vuong, Theo M. Bestebroer, Pascal Lexmond, Thijs Kuiken, Geert van Amerongen, Marco W. van de Bildt, Job B.G. Stumpel, Guus F. Rimmelzwaan, Albert D.M.E. Osterhaus, Martin Schutten, Sander Herfst, Judith Guldemeester, Kim B. Westgeest, Miranda de Graaf

Department of Animal Ecology, Netherlands Institute of Ecology (NIOO-KNAW), Wageningen, the Netherlands
Jacintha G. B. van Dijk, Henk P. van der Jeugd, Bart A. Nolet, Peter P. de Vries

Vogeltrekstation - Dutch Centre for Avian Migration and Demography (NIOO-KNAW), Wageningen, the Netherlands
Henk P. van der Jeugd

Sovon Dutch Centre for Field Ornithology, Nijmegen, the Netherlands
Menno Hornman, Frank Majoor, Roy Slaterus

Natuurmonumenten Dutch Association for Nature Conservation and Management, 's-Graveland, the Netherlands
Giny Kasemir, Date Lutterop

Central Veterinary Institute (CVI) part of Wageningen UR, Lelystad, the Netherlands
Armin R.W. Elbers, Guus Koch

Centre for Ecology and Evolution in Microbial Model Systems, Linnaeus University, Kalmar, Sweden
Neus Latorre-Margalef, Jonas Waldenström

Department of Medical Sciences, Zoonosis Science Center, Uppsala University, Uppsala, Sweden
Björn Olsen

Department of Zoology, University of Cambridge, Cambridge, UK
Nicola Lewis

Department of Veterinary Medicine, University of Cambridge, Cambridge, UK
Colin A. Russell

SaBio Group, Instituto de Investigación en Recursos Cinegéticos (IREC)-CSIC-UCLM-JCCM, Cuidad Real, Spain
Ursula Höfle

Bird Ringing Centre of Russia, Moscow, Russia
Sergey P. Kharitonov

Institute of Ecology, Ilia State University, Tbilisi, Georgia
Zurab Javakhishvili

Laboratory of Virus Evolution, Program in Emerging Infectious Diseases, Duke-NUS Graduate Medical School, Singapore
Justin Bahl, Gavin Smith

Centre for Integrative Ecology, School of Life and Environmental Sciences, Deakin University, Geelong, Australia
Marcel Klaassen

Laboratory of Virology, Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, Montana, USA
Vincent J. Munster

Center for Infectious Diseases, The University of Texas School of Public Health, Houston, USA
Justin Bahl

Department of Population Health, College of Veterinary Medicine, Southeastern Cooperative Wildlife Disease Study, University of Georgia, Athens, USA
Neus Latorre-Margalef

J. Craig Venter Institute, Rockville, USA
Rebecca A. Halpin, Xudong Lin, Amy Ransier, Nadia B. Fedorova, Timothy B. Stockwell, David E. Wentworth

CHAPTER 8

About the author

Curriculum vitae

Josanne Hinke Verhagen was born on 29th of March 1981 in Gouderak, the Netherlands. After she finished pre-university secondary education at the St.- Antoniuscollege in Gouda, she started in 2000 to study veterinary medicine at Utrecht University in Utrecht. As a veterinary student she joined the research team of prof.dr. Thijs Kuiken (Department of Viroscience of the Erasmus MC, Rotterdam) during the legendary large-scale seal necropsies in Groningen in winter 2002 and spring 2003. From 2006 to 2007, during a 1-year internship with prof.dr. Guus Rimmelzwaan and dr. Joost Kreijtz (Department of Viroscience of the Erasmus MC, Rotterdam) she did experimental work on the development of an influenza vaccine based on the nucleoprotein of the influenza A virus. In the summer of 2007 and 2008 she had been given the opportunity by prof. dr. Ted Leighton and dr. Catherine Soos (University of Saskatchewan, Saskatoon, Canada) to join an amazing team of veterinarians, biologists and pathologists, and study avian foraging ecology and wildlife pathology, and perform outbreak and disease surveillance activities of avian cholera (*Pasteurella multocida*) and avian influenza A viruses in populations of double-crested cormorants (*Phalacrocorax auritus*), American white pelicans (*Pelecanus erythrorhynchos*), blue-winged teals (*Anas discors*) and eider ducks (*Somateria mollissima*), in the Canadian prairies, boreal forest and the Arctic tundra. The summers in Canada, and the Wildlife Disease Association conference in Colorado, USA, in 2007, had been a fantastic experience in the field of wildlife health and disease. While going after a dream of becoming a large animal vet, studying infectious disease dynamics in free-living animals proved to be much more interesting and fun. After completing veterinary medicine, she joined the Department of Viroscience of the Erasmus MC in Rotterdam in 2009 to coordinate the wild bird influenza virus surveillance. In 2011, she started as a PhD student within the same department under the supervision of prof.dr. Ron Fouchier, studying the role of wild birds in the dispersal and evolution of influenza A viruses, resulting in this thesis.

PhD portfolio

Name	Josanne Hinke Verhagen
Research department	Viroscience, Erasmus MC
Research school	Post-graduate Molecular Medicine
PhD period	2011-2016
Promotors	Prof.dr. Ron A.M. Fouchier and Prof.dr. Thijs Kuiken

Education

2011-2016 PhD program, Erasmus MC, Rotterdam, the Netherlands. PhD thesis: Influenza A Viruses in Migratory Birds: Ecology, evolution and the wild-domestic interface

2009-2011 Master of Science, Erasmus MC, Rotterdam, the Netherlands. Study: Infection and Immunology

2000-2008 Doctor of Veterinary Medicine (DVM). Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

In-depth courses

2012 Wellcome Trust Advanced Course Mathematical Models for Infectious Disease Dynamics, Hinxton, Cambridge, UK

2011 Photoshop and Illustrator CS5

2010 Basic course on 'R'

Poster presentations at international meetings

2014 Conference Jacques Monod "Infectious diseases as drivers of evolution: the challenges ahead", Centre National de la Recherche Scientifique (CNRS) Roscoff, France

2013 11th Conference of Ecology and Evolution of Infectious Diseases (EEID), State College, Pennsylvania, USA

2010 4th Annual NIAID CEIRS Network Meeting, Fairport, New York, USA

Oral presentations at international meetings

2015 3rd International One Health Congres, Amsterdam
2015 9th International Avian Influenza Symposium, Athene, USA

2014 11th Conference European Wildlife Disease Association (EWDA), Edinburgh, UK

2013 7th Annual NIAID CEIRS Network Meeting, Memphis, USA

2012 NIAID CEIRS Network Surveillance Meeting, Cape May, New Jersey, USA
2012 8th International Symposium Avian Influenza, London, UK

2011 NIAID CEIRS Network Meeting, Washington, USA
2011 5th Annual NIAID CEIRS Network Meeting, Stone Mountain, Atlanta, USA
2011 4th EWDA Student Workshop, Veyrier-du-Lac, France
2011 Annual EU AI and ND National Reference Lab Meeting, Brussel, Belgium
2011 The International Conference: "From DNA to phenotype", MolMed, Rotterdam

2010 9th Conference EWDA, Vlieland
2010 New Flu Bird Symposium, Montpellier, France

Attended

2011 15th Molecular Medicine Day 2011, Rotterdam

2010 Annual EU AI and ND National Reference Lab Meeting, Vienna, Austria
2010 KNAW Symposium Evolutionary Medicine, Amsterdam
2010 14th Molecular Medicine Day 2010, Rotterdam

Publications

2015 **J.H. Verhagen**, U. Höfle, G. van Amerongen, M.W. van de Bildt, F. Majoor, R.A.M. Fouchier, T. Kuiken. Long-term effect of serial infections with H13 and H16 low pathogenic avian influenza viruses in black-headed gulls. Journal of *Virology*. 2015. Nov 15;89(22):11507-22

J.H. Verhagen, H.P. van der Jeugd, B.A. Nolet, R. Slaterus, S.P. Kharitonov, P.P. de Vries, O. Vuong, F. Majoor, T. Kuiken and R.A.M. Fouchier. Wild bird surveillance around outbreaks of highly pathogenic avian influenza A(H5N8) virus in the Netherlands, 2014, within the context of global flyways. *Eurosurveillance*. 2015; 20(12): pii=21069

J.H. Verhagen, S. Herfst and R.A.M. Fouchier. Infectious disease. How a virus travels the world: Wild birds may spread the H5N8 virus. Perspective. *Science*. 2015; 347(6222): 623-625

N.S. Lewis, **J.H. Verhagen**, Z. Javakhishvili, C.A. Russell, P. Lexmond, K.B. Westgeest, T.M. Bestebroer, R.A. Halpin, X. Lin, A. Ransier, N.B. Fedorova, T.B. Stockwell, N. Latorre-Margalef, B. Olsen, G. Smith, J. Bahl, D.E. Wentworth, J. Waldenström, R.A.M. Fouchier and M. de Graaf. Influenza A virus evolution and spatio-temporal dynamics in Eurasian wild birds: A phylogenetic and phylogeographic study of whole-genome sequence data. *Journal of General Virology*. 2015; April 22

K.R. Short, M. Richard, **J.H. Verhagen**, D. van Riel, E.J.A. Schrauwen, J.M.A. van den Brand, B. Mänz, R. Bodewes & S. Herfst. One health, multiple challenges: The inter-species transmission of influenza A virus. *One Health*. 2015; 1: 1-13

R. Bodewes, T.M. Bestebroer, E. van der Vries, **J.H. Verhagen**, S. Herfst, M.P. Koopmans, R.A.M. Fouchier, V.M. Pfankuche, P. Wohlsein, U. Siebert, W. Baumgartner and A.D.M.E. Osterhaus. Avian influenza A(H10N7) virus–associated mass deaths among harbor seals (letter). *Emerging Infectious Diseases*. 2015; 21(4): 720-722

2014 **J.H. Verhagen**, F. Majoor, P. Lexmond, O. Vuong, G. Kasemir, D. Lutterop, R.A.M. Fouchier and T. Kuiken. Epidemiology of influenza A virus among black-headed gulls, the Netherlands, 2006–2010. *Emerging Infectious Diseases*. 2014; 20(1): 138-141

J.H. Verhagen, J.G.B. van Dijk, O. Vuong, T.M. Bestebroer, P. Lexmond, M. Klaassen, R.A.M. Fouchier. Migratory birds reinforce local circulation of avian influenza viruses. *PLoS One*. 2014; 9(11): e112366

J.G.B. Van Dijk, B.J. Hoye, **J.H. Verhagen**, B.A. Nolet, R.A.M. Fouchier, M. Klaassen. Juveniles and migrants as drivers for seasonal epizootics of avian influenza virus. *Journal of Animal Ecology*. 2014, 83, 266-275

2013 N.S. Lewis, Z. Javakhisvili, C.A. Russell, A. Machablishvili, P. Lexmond, **J.H. Verhagen**, O. Vuong, R.A.M. Fouchier. Avian influenza virus surveillance in wild birds in Georgia: 2009-2011. *PLoS One*. 2013; 8(3): e58534

J.G.B. van Dijk, R.A.M Fouchier and **J.H. Verhagen**. Wetenschappelijk onderzoek in eendenkooien in Nederland: eenden en vogelgriepvirussen. Bookchapter 5 in Blauwgoed, helen en halven: 100 jaar ringwerk in eendenkooien, edited by Desiré Karelse & Fons Mandigers. 2013 (Dutch)

2012 **J.H. Verhagen**, V.J. Munster, F. Majoor, P. Lexmond, O. Vuong, J.B.G. Stumpel, G.F. Rimmelzwaan, A.D.M.E. Osterhaus, M. Schutten, R. Slaterus and R.A.M. Fouchier. Avian influenza A virus in wild birds in highly urbanized areas. *PLoS One*. 2012; 7(6): e38256

U. Höfle, M.W. van de Bildt, L.M. Leijten, G. van Amerongen, **J.H. Verhagen**, R.A.M. Fouchier, A.D.M.E. Osterhaus, T. Kuiken. Tissue tropism and pathology of natural influenza virus infection in black-headed gulls (Chroicocephalus ridibundus). *Avian Pathology*. 2012 Dec; 41(6): 547-53

S. van Boheemen, T.M. Bestebroer, **J.H. Verhagen**, A.D.M.E. Osterhaus, S.D. Pas, S. Herfst, R.A.M. Fouchier. A family-wide RT-PCR assay for detection of paramyxoviruses and application to a large-scale surveillance study. *PLoS One*. 2012; 7(4): e34961

2011 **J.H. Verhagen**, R.A.M. Fouchier and V.J. Munster. Ecology of Avian Viruses. Bookchapter 14 in Viral Ecology, edited by C.J. Hirst. John Wiley & Sons, 2011

J.H. Verhagen, V.J. Munster and R.A.M. Fouchier. Ecology and Evolution of Avian Influenza Viruses. Bookchapter 28 in Genetics and Evolution of Infectious Diseases, edited by M. Tibayrenc. Elsevier, 2011