Epidemiology of Influenza A Virus among Black-headed Gulls, the Netherlands, 2006–2010

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We sampled 7,511 black-headed gulls 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.

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 (*1*). 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) (*2*) but is poorly known among gulls, despite their abundance and close association with humans (*3*). 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 (online Technical Appendix, wwwnc.cdc.gov/EID/article/20/1/13-0984-Techapp1.pdf). 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 (*4*,*5*). 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 (*6*). Statistics were performed by using software RStudio version 0.95.265 (www.r-project.org). Additional analyses on AIV prevalence among male versus female birds, dead versus live birds, recaptured birds, and capture bias were performed (online 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 (online Technical Appendix Table 1). 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, unpub. data).

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

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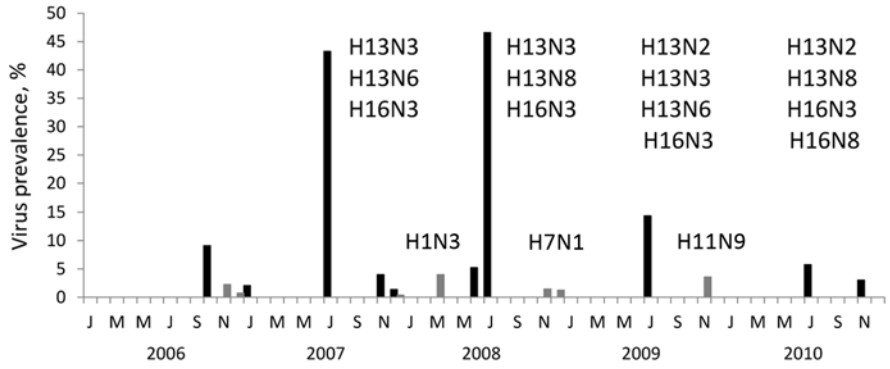

Figure 1. Avian influenza virus prevalence among 7,511 black-headed gulls, the Netherlands, 2006–2010. Cloacal and oropharyngeal samples were collected once from each gull for virus detection. Influenza virus subtypes detected are shown above virus positives. Bars indicate virus prevalence (No. PCR-positive 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.

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 (*7*). 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 (*8*) or histological lesions (*9*) 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 prevalence were found related to capture method, and no AIVs in dead BHGU were detected outside epidemics (online 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 (*10*). 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 (*11*). 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.

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

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*

	No. sampled											
Y, 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.

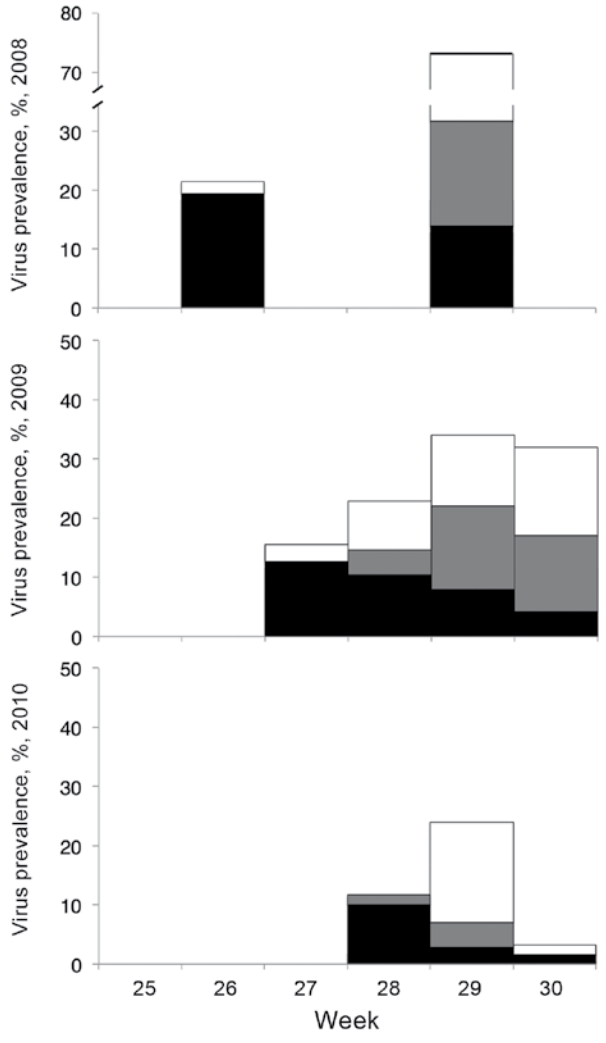

Figure 2. Avian influenza virus prevalence and hemagglutinin subtype (H) distribution of 871 first-year black-headed gulls sampled on the colony site of Griend during 2008–2010. Bars indicate virus prevalence (no. PCR-positive samples/no. sampled per week). Black bar sections, H13; gray bar sections,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

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 noninfected 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.

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

Month and week	No. samples		
	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.

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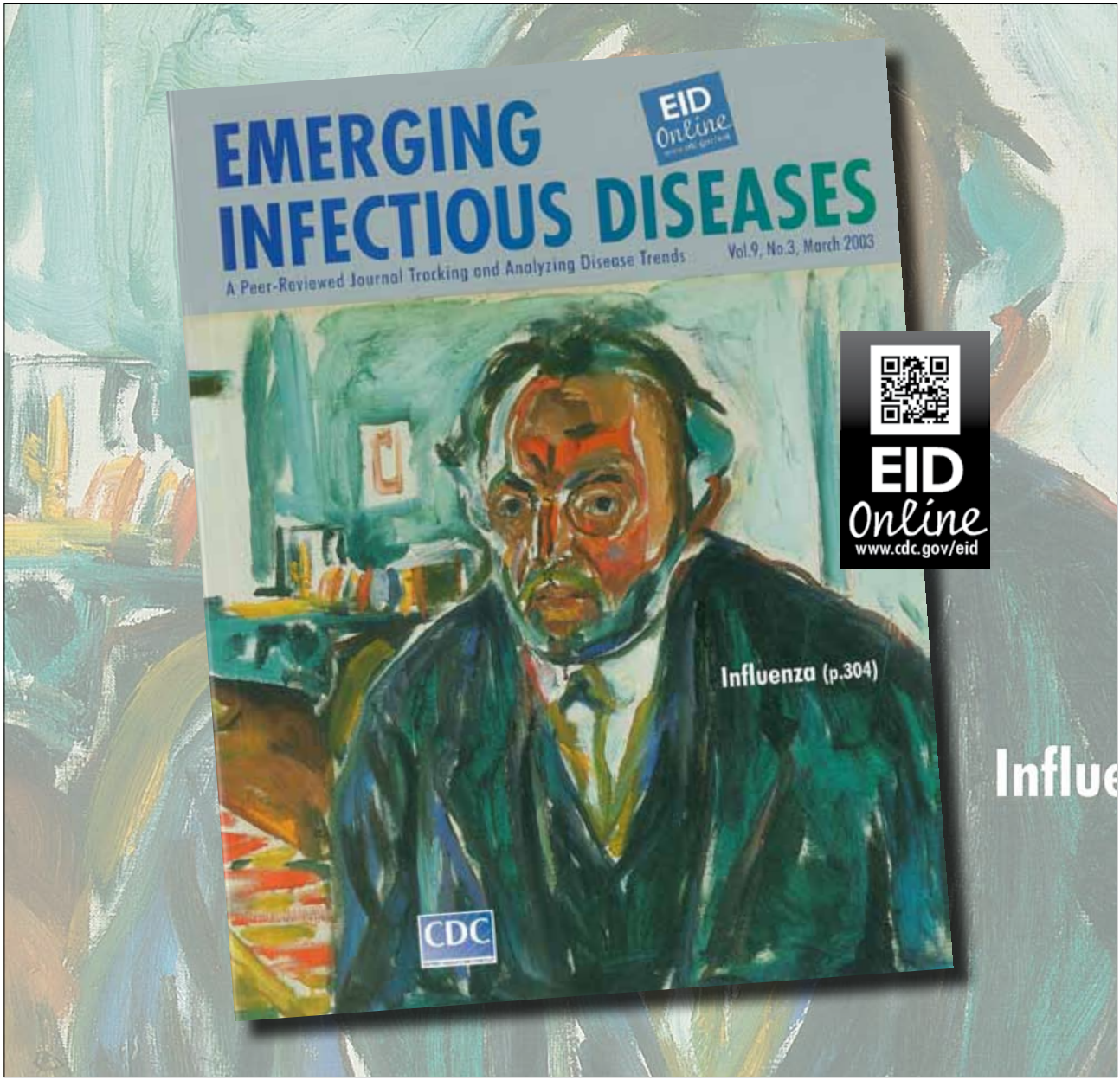

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Technical Appendix

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 (*1*). The colony size was estimated at 35,166 breeding pairs in 2008, 32,780 in 2009, and 31,408 in 2010 (*2-4*). 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 (*5*). 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 (*5*). 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 (*4*). 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 [*5*]), near Bargerveen (lat-y 52.681, long-x 7.032, 829 pairs in 2008 [*6*]), Blauwestad (lat-y 53.171, long-x 7.012, 1500 pairs in 2009 [*7*]), 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 (*8*) 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 (*1*). 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 (*9*). 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 (*10*). Birds were aged based on total head-bill length when captured before fledging (*11*) and based on plumage when captured after fledging (*12*). In addition, FY birds were categorized based on wing length as nestling (<200 mm) or fledgling (≥200 mm) (*11*). To calculate a scaled mass index of body condition (called body condition), body mass and head-bill length as length value were used (*13*).

Additional Analyses

Analysis of Gender Differences

Gender was determined for 4,356 of 7,511 sampled BHGU (58.0%). Of these 4356 gulls, 317 (7.3%) were FY birds sampled within the breeding season. Of 4,356 birds, 1,149 birds were female (26.3%) and 3207 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 Ct 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).

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Technical Appendix Table 1. Avian influenza virus prevalence in first-year Black-headed Gulls sampled at 3 breeding colony sites (Griend, De Kreupel, Veluwemeer) in the Netherlands during the 2006–2010 breeding seasons*†

	Griend					De Kreupel					Veluwemeer				
Year	Date	No. sampled	No. PCR positive	No. VI positives	No. each subtype	Date	No. sampled	No. PCR positive	No. VI positive	No. each subtype	Date	No. sampled	No. PCR positives	No. VI positives	No. each subtype
2006	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	6 May	6	0	0	NA
	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	4 Jun	199	0	0	NA
	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	12 Jun	94	0	0	NA
	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	23 Jun	72	0	0	NA
2007	ND	ND	ND	ND	ND	5 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
	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	10 Jun	144	0	0	NA
	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	30 Jun	23	0	0	NA
2008	28 Jun	98	20	19	H13N8 (19)	9 Jun	64	0	0	NA	7 May	1	0	0	NA
	14 Jul	100	72	32	H13N8 (14) H16N3 (18)	24 Jun	77	0	0	NA	6 Jun	126	0	0	NA
	ND	ND	ND	ND	ND	15 Jul	78	23	9	H13N8 (5) H16N3 (4)	25 Jun	18	0	0	NA
2009	17 Jun	46	0	0	NA	15 Jun	47	0	0	NA	ND	ND	ND	ND	ND
	24 Jun	70	0	0	NA	26 Jun	64	0	0	NA	1 Jul	29	0	0	NA
	2 Jul	71	11	9	H13N2 (9)	29 Jun	66	0	0	NA	ND	ND	ND	ND	ND
	9 Jul	48	11	7	H13N2 (4) H13N6 (1) H16N3 (2)	3 Jul	63	0	0	NA	ND	ND	ND	ND	ND
	15 Jul	50	17	11	H13N2 (3) H13N3 (1) H16N3 (7)	6 Jul	50	0	0	NA	ND	ND	ND	ND	ND
	22 Jul	47	15	8	H13N2 (1) H13N6 (1) H16N3 (6)	13 Jul	26	1	1	H13N2 (1)	ND	ND	ND	ND	ND
2010	23 Jun	44	0	0	NA	24 Jun	63	0	0	NA	ND	ND	ND	ND	ND
	30 Jun	33	0	0	NA	2 Jul	43	0	0	NA	5 Jun	30	0	0	NA
	7 Jul	73	0	0	NA	8 Jul	60	0	0	NA	4 Jul	41	0	0	NA
	14 Jul	60	7	7	H13N2 (1) H13N8 (5) H16N8 (1)	19 Jul	39	0	0	NA	ND	ND	ND	ND	ND
	21 Jul	71	17	5	H13N2 (1) H13N8 (1) H16N3 (3)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

	Griend					De Kreupel					Veluwemeer				
Year	Date	No. sampled	No. PCR positive	No. VI positives	No. each subtype	Date	No. sampled	No. PCR positive	No. VI positive	No. each subtype	Date	No. sampled	No. PCR positives	No. VI positives	No. each subtype
	26 Jul	60	2	1	H13N2 (1)	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
Total		871	172	99			776	40	20			783	0	0	

*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.

Technical Appendix Table 2**.** 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.

Technical Appendix Table 3. 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.

Technical Appendix Table 4. 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*

				Capture technique		Significance of difference between hand-captured and clap-net-captured birds	
Year	Date of sampling		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
	2-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
	9-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	Mann-Whitney Wilcoxon
		Mean SMI	188	182	199	p<0.05	Mann-Whitney Wilcoxon
		No. virus positive/No. sampled (%)	15/47 (32)	10/31 (32)	5/16 (31)	p=1	Fisher's exact
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
	7-Jul	Mean wing length	247	239	272	p<0.01	Mann-Whitney Wilcoxon
		Mean SMI	240	247	220	p<0.01	Mann-Whitney Wilcoxon
		No. virus positive/No. sampled (%)	0/73 (0)	0/56 (0)	0/17 (0)	p=1	Fisher's exact
	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	Mann-

	Mean SMI	186	183	190	p>0.05	Whitney Wilcoxon Mann-Whitney Wilcoxon
	No. virus positive/No. sampled (%)	17/71 (24)	17/39 (44)	0/32 (0)	p<0.01	Fisher's exact
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; NA, not applicable.