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Research Note—

One Decade of Active Avian Influenza Wild Bird Surveillance in Belgium Showed a Higher Viroprevalence in Hunter-Harvested Than in Live-Ringed Birds

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SUMMARY. Active monitoring of avian influenza (AI) viruses in wild birds was initiated in Belgium in 2005 in response to the first highly pathogenic avian influenza (HPAI) H5N1 outbreaks occurring in Europe. In Belgium, active wild bird surveillance that targeted live-ringed and hunter-harvested wild birds was developed and maintained from 2005 onward. After one decade, this program assimilated, analyzed, and reported on over 35,000 swabs. The 2009–2014 datasets were used for the current analysis because detailed information was available for this period. The overall prevalence of avian influenza (AI) in samples from live-ringed birds during this period was 0.48% whereas it was 6.12% in hunter-harvested samples. While the ringing sampling targeted a large number of bird species and was realized over the years, the hunting sampling was mainly concentrated on mallard (*Anas platyrhynchos*) during the hunting season, from mid-August to late January. Even when using just AI prevalence for live-ringed *A. platyrhynchos* during the hunting season, the value remained significantly lower (2.10%) compared to that detected for hunter-harvested mallards. One explanation for this significant difference in viroprevalence in hunter-harvested mallards was the game restocking practice, which released captive-bred birds in the wild before the hunting period. Indeed, the released game restocking birds, having an AI-naïve immune status, could act as local amplifiers of AI viruses already circulating in the wild, and this could affect AI epidemiology. Also, the release into the wild of noncontrolled restocking birds might lead to the introduction of new strains in the natural environment, leading to increased AI presence in the environment. Consequently, the release of naïve or infected restocking birds may affect AI dynamics.

RESUMEN. *Nota de investigación* - Una década de vigilancia activa para el virus de la influenza aviar en aves silvestres de Bélgica demostró una prevalencia viral mayor en las aves recolectadas por cacería en comparación con la observada en las aves en libertad identificadas con anillos.

La vigilancia activa del virus de la influenza aviar en aves silvestres en Bélgica se inició en el año 2005 como respuesta a los primeros brotes de influenza aviar altamente patógena H5N1 que se presentaron en Europa. En Bélgica, a partir del año 2005 se desarrolló la vigilancia activa de aves silvestres que se enfocó en aves en libertad identificadas con anillos y en las aves recolectadas por cacería y desde entonces esta vigilancia se ha mantenido. Después de una década, este programa ha recabado, analizado y reportado los resultados de más de 35,000 hisopos. El conjunto de datos de los años 2009 al 2014 se utilizaron para el análisis actual, porque se contó con información más detallada para este período. La prevalencia global de la influenza aviar en muestras de aves identificadas con anillos durante este período fue de 0.48%, mientras que fue del 6.12% en las muestras recolectadas por cacería. Mientras que el muestreo de aves con anillos estuvo dirigido a un gran número de especies aviares y se realizó por varios años, la recolección de muestras por cacería se concentró principalmente en patos de collar (*Anas platyrhynchos*) durante la temporada de caza, desde mediados de agosto hasta finales de enero. Aun cuando se utilizó solo prevalencia de influenza aviar para aves de la especie *A. platyrhynchos* anilladas durante la temporada de caza, el valor se mantuvo significativamente más bajo (2.10%) en comparación con la detectada por los patos recolectados por cazadores. Una explicación de esta diferencia significativa en la prevalencia viral en patos silvestres en las aves recolectadas por cacería fue la práctica de repoblación de aves de caza, que liberó a la vida silvestre, aves criadas en cautividad antes de la temporada de caza. De hecho, las aves de caza para repoblación que poseían un sistema inmune no expuesto al virus de influenza aviar, pudieron actuar como amplificadores locales de virus de influenza aviar que ya circulaban en la naturaleza, y esto podría afectar a la epidemiología de esta enfermedad. Además, la liberación en el medio natural de las aves de reposición sin control, podría conducir a la introducción de nuevas cepas en el medio natural, lo que podría ocasionar una mayor presencia de influenza aviar en el medio ambiente. En consecuencia, la liberación de las aves susceptibles o infectadas para reposición podría modificar la dinámica de la influenza aviar.

Key words: avian influenza, active surveillance, ringing, hunting, game restocking

Abbreviations: AI = avian influenza; EC = European Commission; HPAI = highly pathogenic avian influenza; LPAI = low pathogenic avian influenza; RBINS = Royal Belgian Institute of Natural Sciences; RRT-PCR = real-time reverse-transcription polymerase chain reaction

Wild bird surveillance allows the prevalence of the low pathogenic avian influenza (LPAI) to be detected in bird populations. The biologic and genetic characterization of these circulating strains such as assessing the virulence of strains in poultry, determining the level of adaptation, and ease of transmission to and among poultry can contribute to a better understanding of the LPAI epidemiology (7,8,9). In addition, the surveillance of wild bird populations may serve as an early warning system of the introduction of highly pathogenic avian influenza (HPAI) viruses such as the Asian H5N1 and, recently, the H5N8 viruses.

The active monitoring of avian influenza (AI) viruses among wild birds in Belgium was initiated in 2005 after HPAI H5N1 was first

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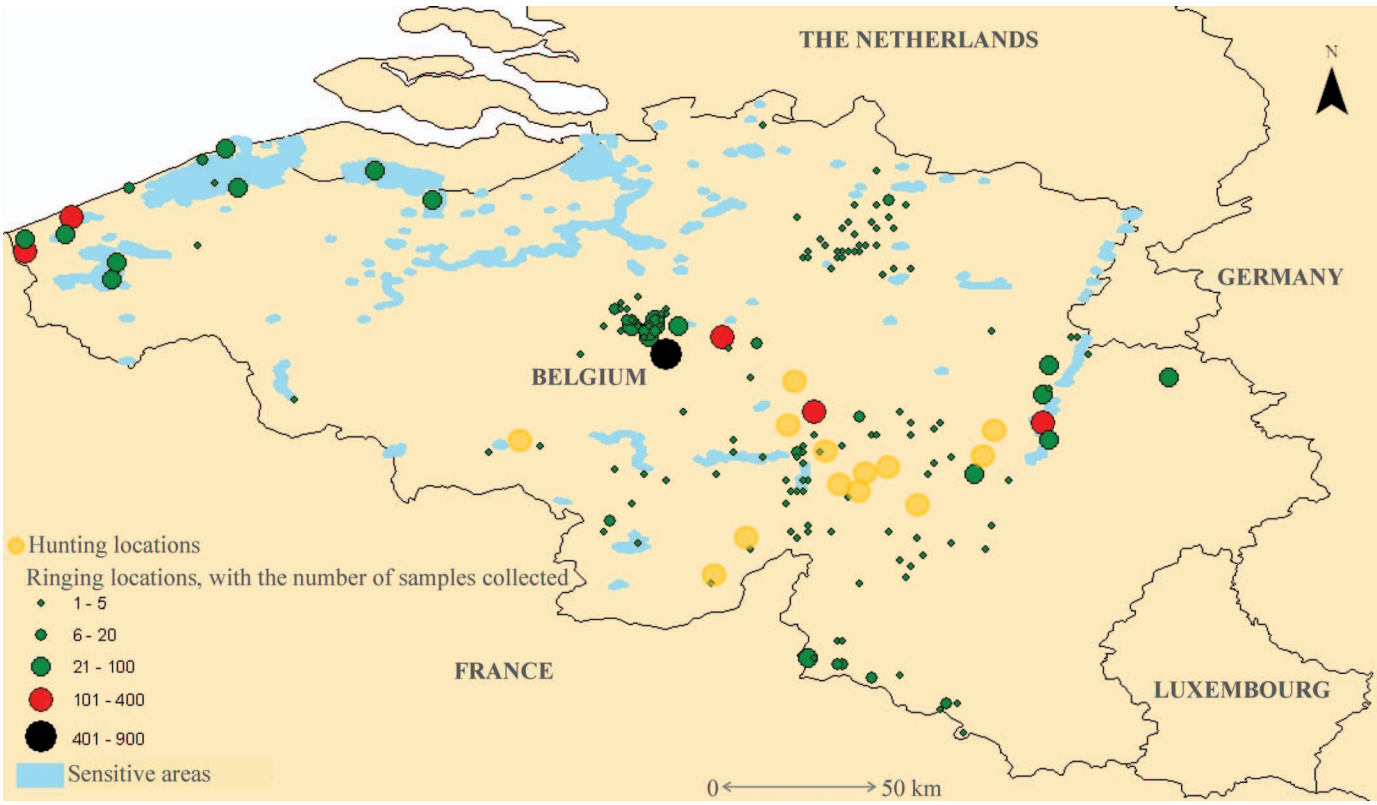

Fig. 1. The sampling sites of the Belgian wild bird AI active surveillance program: yellow dots represent the 13 hunting sites while green-red-blue dots represent the bird-ringing sites.

detected in Europe. Two types of active monitoring systems were implemented following the European Commission (EC) guidelines 2005/94/EC, 2007/268/EC, and 2009/437/EC (2,4,5). Firstly, live birds of different species were sampled by certified collaborators of the Royal Belgian Institute of Natural Sciences (RBINS) within the framework of the bird-ringing network. This sampling is performed year-round in all 10 provinces of Belgium, resulting in over 30,000 samples being collected from 2005 to 2014, of which most were cloacal (73%). Secondly, hunted *Anatidae* (mainly mallards, *Anas platyrhynchos*) were sampled by the staff of the Surveillance Network of Wildlife Diseases, University of Liège, Belgium, during the hunting season from mid-August to late January. This sampling of dead birds is performed in the five southern provinces of Belgium where game restocking is permitted. The hunter-harvested system obtained over 5000 samples between 2005–2014, again mostly of cloacal origin (82%).

Here, we present an overview of the results obtained following one decade of the active wild bird surveillance program in Belgium which compared AI viroprevalence in live-ringed and hunter-harvested populations.

MATERIALS AND METHODS

Bird sampling systems. *Live ringed birds.* Birds were sampled year-round during bird-ringing activities at over 130 sites throughout Belgium (Fig. 1). Greater sampling effort was focused in sensitive wetland zones (Fig. 1). To capture wild birds without inflicting damage, different species and season-adapted trapping techniques were used such as funnel cages, cannon nets, mist nets, and clap traps. Captured birds were ringed (if not already ringed) and provided with unique identification tags. The birds were assessed for their health status with the species, sex, and age being identified, and when possible weight and wing length was recorded. In addition, the date and location of capture was recorded. *Anatidae* (n = 8654; mainly *A. platyrhynchos* [n = 2828] and *Branta canadensis* [Canada goose; n = 2880]), *Laridae* (n = 1577; mainly *Larus ridibundus* [black-headed gull; n = 454] and *Sterna hirundo* [common tern; n = 402]), and *Accipitridae* (n = 1231; mainly *Accipter gentilis* [northern goshawk; n = 580]) were the most-sampled species. Birds were either swabbed in the cloacal region (73%), as LPAI is mostly excreted by this route, or in both the opharyngeal and cloacal region (27%). The oropharyngeal swabbing was targeted at certain species with a demonstrated sensitivity to HPAI H5N1, considering the tracheal tropism of this virus.

Hunter-harvested birds. Samples were collected from hunted mallards at 13 sites located in the five southern provinces of Belgium where the restocking of game mallards is permitted (Fig. 1). Game restocking birds are released into the wild after which wild and restocking populations cannot be differentiated. No official information about the characteristics of the farms that rear these ducks, nor the health status and identification of these birds, is currently available. In Belgium, the official limit for game restocking is set at 30 days before the opening of the hunting season, which extends from mid-August to late January. All the samples were collected from the cloaca (82%), with the remainder being collected from both the cloaca and oropharynx, within 2 hr after shooting. The hunting site and date were recorded; however, no other bird identification or body characteristics were recorded.

AI detection by real-time reverse-transcription PCR (RRT-PCR). Each swab was immediately placed in viral transport medium and stored at <−70 C until further analysis. Viral RNA extraction was performed using the MagMax AI/ND viral nucleic acid extraction kit (Life Technologies, Carlsbad, CA), according to the manufacturer's recommendations, on a semi-automated Kingfisher platform (Thermo Fisher, Erembodegem, Belgium). Virus detection was competed by TaqMan® RRT-PCR using an oligoset targeting the matrix encoding gene, allowing all avian matrix genes to be detected, as previously described by Spackman *et al.* (18). The AgPath-ID™ One-Step RT-PCR kit (Life Technologies) was used for amplification on a LightCycler®480 real-time PCR system (Roche, Mannheim, Germany). Sample quality and extraction efficacy were analyzed by detecting the beta-actine housekeeping gene as previously described (19).

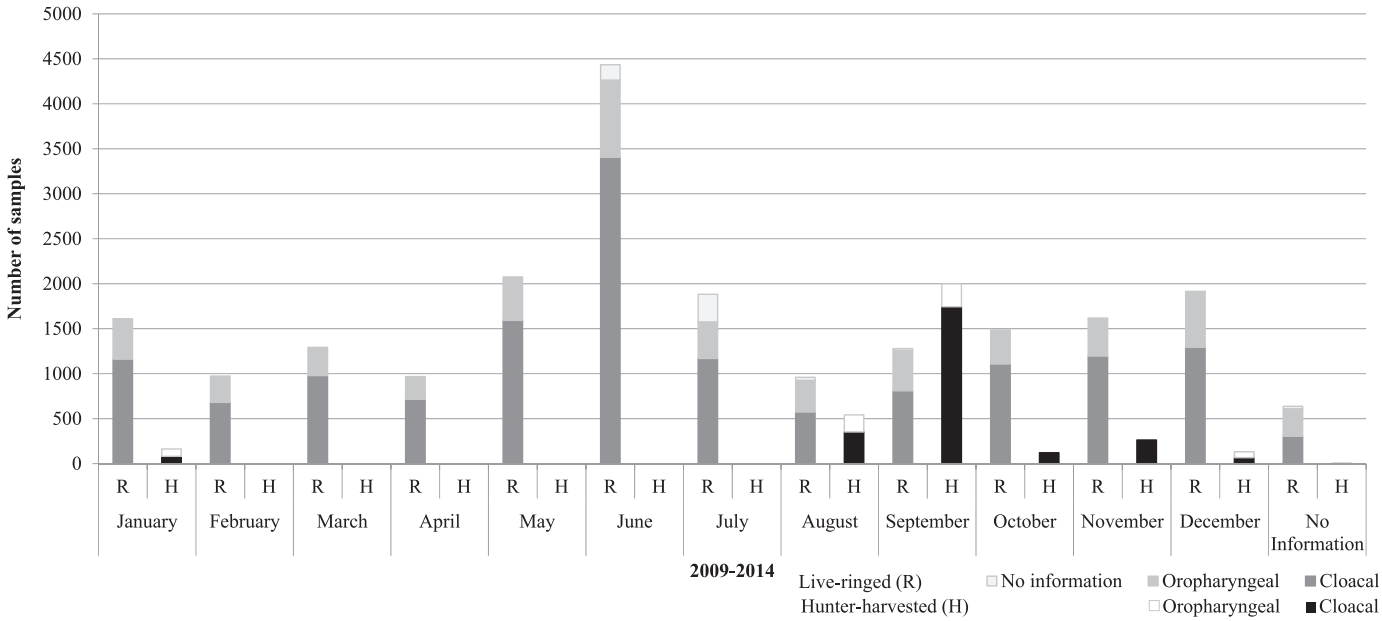

Fig. 2. The cumulative number of birds sampled from 2009 to 2014 during the Belgian active surveillance, for each month and by swab type, for live-ringed (R) and hunter-harvested (H) samples.

Samples that were positive for matrix-AI influenza A were tested for the presence of Eurasian H5 and H7 viruses by RRT-PCR (17,20). All AI-positive samples were further processed for virus isolation by inoculating specific-pathogen-free (SPF) embryonated chicken eggs at embryonation day 9 and by passaging after 5 days using standard procedures (14). Subtyping of hemagglutinating allantoic fluids was completed by hemagglutination inhibition (HI) test using reference sera (14) and partial sequencing of the HA and NA genes. H5 and H7 subtyping was performed by sequencing the cleavage site region (amplification primers and sequencing protocols are made available on request).

RESULTS

Active wild bird AI surveillance in Belgium. From 2005 to 2014, 35,544 samples were collected by active surveillance, with 30,477 samples being collected from the ringing program and 5067 samples being collected from the hunting surveillance system. The annual number of live-ringed samples increased significantly from 2005 to 2009 and stabilized at around 3500 samples from 2010 onward. The number of hunter-harvested samples remained fairly stable over the years at around 550 samples per year.

From 2009 onward, additional detailed data were assimilated for each live-captured bird, facilitating robust analysis. Therefore, only the 2009–2014 data were used to evaluate the surveillance data, which consisted of 20,822 live-ringed and 3220 hunter-harvested samples. The monthly overview of collected samples showed that live bird ring sampling peaked in June (Fig. 2). This peak coincided with the sampling of Canada geese (*B. canadensis*), which aggregate during the annual flightless moulting period. Most live-ringed swabs (73%) were cloacal. Overall, 56.21% of the live-ringed samples were collected from adult birds (>first moult), while just 19.93% were collected from juvenile birds (2 mo–first moult) and nestlings, with the

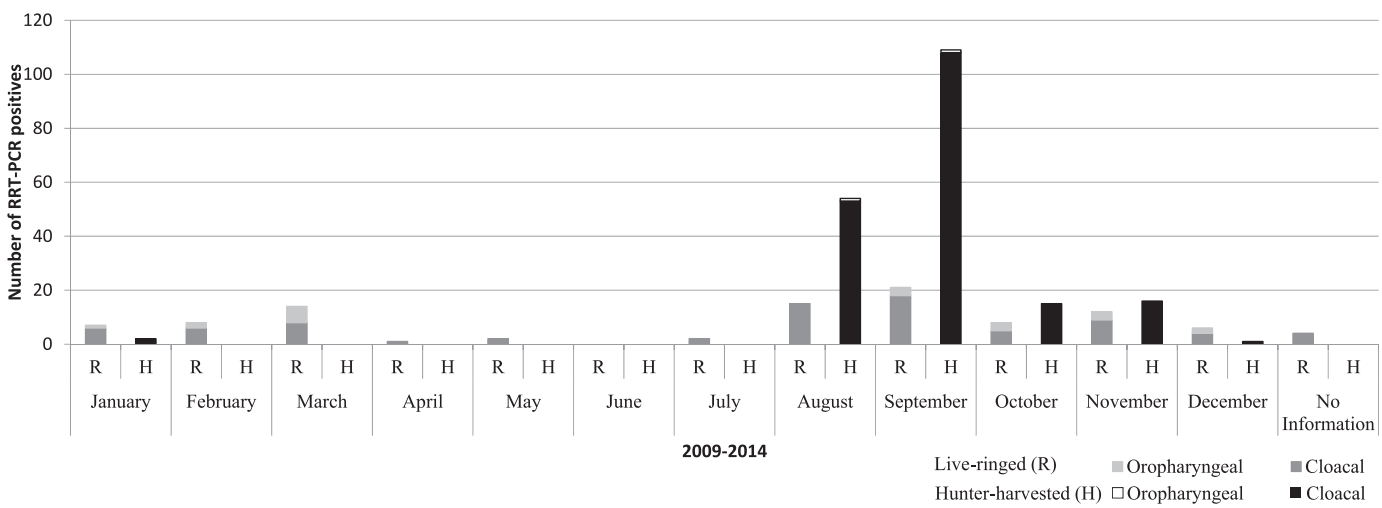

Fig. 3. Cumulative number of RRT–PCR-positive samples, per month and by swab type between 2009–2014, for live-ringed (R) and hunter-harvested (H) birds.

Table 1. Overview of the number and percentages of samples taken from live birds per family from 2009–2014. In addition, the number and percentages for the RRT–PCR-positive species are indicated as well.

Order[A]	Family and species[A]	Number of samples	% Total samples	RRT-PCR+	% RRT-PCR+ species samples
Accipitriformes	Accipitridae	1231	8.30	0	
Anseriformes	Anatidae	8654	58.37	84	0.97
	Anas platyrhynchos [Mallard]	2828		38	1.34
	Aythya ferina [common pochard]	144		1	0.69
	Aythya fuligula [tufted duck]	1111		9	0.81
	Branta canadensis [Canada goose]	2880		2	0.07
	Callonetta leucophrys [ringed teal]	2		2	100.00
	Cygnus bewickii [tundra swan]	21		1	4.76
	Cygnus olor [mute swan]	875		17	1.94
	Tadorna tadorna [common shelduck]	386		13	3.37
Charadriiformes	Charadriidae	1083	7.31	2	0.18
	Pluvialis apricaria [Eurasian golden plover]	1044		2	0.19
	Laridae	1577	10.64	4	0.25
	Larus argentatus [European herring gull]	132		2	1.52
	Larus canus [mew gull]	151		1	0.66
	Larus melanocephalus [Mediterranean gull]	285		1	0.35
	Scolopacidae	462	3.12	7	1.52
	Actitis hypoleucos [common sandpiper]	30		1	3.33
	Arenaria interpres [ruddy turnstone]	187		6	3.21
	Haematopodidae	10	0.07	0	
Ciconiiformes	Ciconiidae	20	0.13	0	
Falconiformes	Falconidae	374	2.52	0	
Galliformes	Phasianidae	46	0.31	0	
Gruiformes	Rallidae	655	4.42	3	0.46
	Fulica atra [common coot]	611		3	0.49
Pelicaniformes	Ardeidae	196	1.32	0	
	Threskiornithidae	16	0.11	0	
Podicipediformes	Podicipedidae	38	0.26	0	
Strigiformes	Strigidae	290	1.96	0	
Suliformes	Phalacrocoracidae	10	0.07	0	
Unknown	Unknown	163	1.10	0	

[A]Following the BirdLife taxonomic checklist version 8, October 2015 (http://www.birdlife.org/datazone/info/taxonomy).

age not being determined for 23.86% sampled birds. Birds from 80 species and 15 bird families were represented in the live-ringed sampling. All hunter-harvested birds were *A. platyrhynchos*, with 82% cloacal samples and no recording of age categories.

AI viroprevalence in live-ringed and hunter-harvested birds. From 2009 to 2014, 297 swabs tested RRT–PCR-positive for AI, of which 100 were live-ringed birds and 197 were hunted birds, corresponding to a viroprevalence of 0.48% and 6.12%, respectively (Fig. 3).

Out of the live-ringed bird samples, 80% of the positives were obtained from cloacal swabs, while just 20% of the positives were obtained from oropharyngeal swabs, corresponding to a ratio of 0.53% and 0.36%, respectively. For hunter-harvested birds, 195

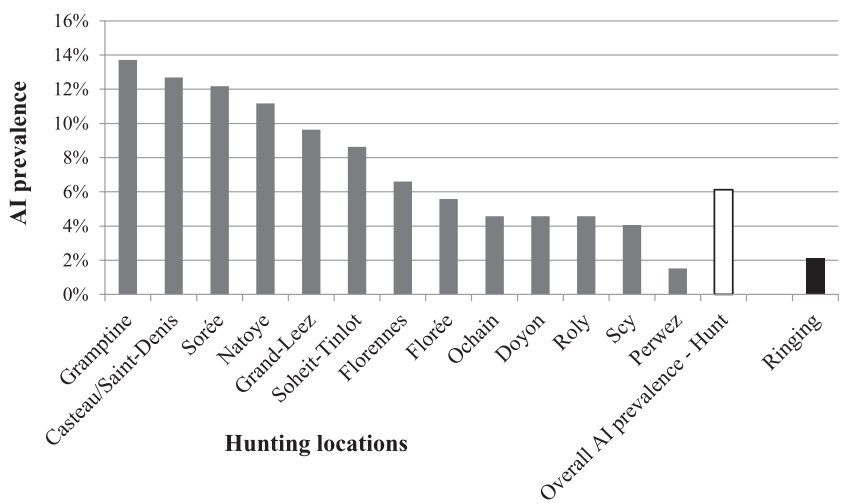

Fig. 4. Viroprevalence at the 13 Belgian hunting sites compared to the live-ringed *Anas platyrhynchos* AI viroprevalence during the parallel hunting season period. Viroprevalence was calculated as the number of RRT-PCR positives over the total number of samples.

Table 2. A list of AI-subtypes isolated from hunting and ringing, organized by species (the number of isolates and the year of isolation). All isolates were of low pathogenic pathotype (LPAI), with notifiable LPAI subtypes in bold.

Family and species	Method	Subtypes isolated (year)	Year
Anatidae			
Anas platyrhynchos	Hunting	H4N6	2005, 2× 2009, 2010, 3× 2011, 4× 2013, 3× 2014
		H3N8	2006, 2× 2010, 2× 2011, 2014
		H12N2	2007
		H10N4	2008
		H1N1	3× 2010, 2× 2012, 2× 2014
		H6N8	2011, 2× 2012
		H10N7	2011
		H3N6	2× 2012
		H7N7	2× 2012
		H5N1	2× 2012
		H9N2	2012
		H2N8	2012
		H5N8	2012
		H2N2	2× 2013
		H12N5	2013
		H3N2	2013
		H5N2	2013
		H2	2014
		H2N2	2014
	Ringing	H3N8	3× 2007, 2009, 2013, 3× 2014
		H1N1	2007, 2008, 2010, 2012
		H6N5	2008, 2009
		H4N6	2008, 2009, 2010
		H5N1	**2008**
		H5N3	**2008**
		H10N7	2009
		H11N9	2009
		H2N6	2009
		H12N5	2009
		H2N3	2010, 3× 2011
		H12N7	2010
		H4N1	2011
		H4N8	2012
		H1N2	2014
Anas acuta [Northern pintail]	Ringing	H11	2007
Aythya fuligula	Ringing	H1N1	2008
		H10N8	2011
		H6N8	2012
Branta canadensis	Ringing	**H7N7**	**2010**
Callonetta leucophrys	Ringing	H1N1	2× 2014
Cygnus olor	Ringing	H4N8	2009
		H9N2	2009, 2011
		H6N2	2011
Tadorna tadorna	Ringing	H4N6	2007
		H3N2	2008
		H10N3	2009
		H7N1	**2009**
		H2N1	2009
		H4N5	2011
Laridae			
Larus argentatus	Ringing	H16	2008
		H13	2008
		N6	2008
		H6N1	2010
		H5N2	**2010**
Larus ridibundus [black-headed gull]	Ringing	H16N8	2008
Larus fuscus [lesser black-headed gull]	Ringing	H13N8	2008
Larus canus	Ringing	H11	2013
Larus michahellis [yellow-legged gull]	Ringing	N6	2008
Scolopacidae			
Arenaria interpres	Ringing	H3N8	2008
		H11N9	2009
		H12N5	2010
		H3N5	2013
Rallidae			
Fulica atra	Ringing	H10N8	2009, 2011
		H6N8	2014

out of 197 positives were from cloacal swabs, with a ratio of 7.43% for cloacal and 0.34% for oropharyngeal swabs.

The AI viroprevalence of live-ringed birds peaked in September and August at 1.64% and 1.56%, respectively. For hunter-harvested birds, AI viroprevalence was highest in October (12.4%) followed by August (9.96%), November (6.11%), and September (5.46%).

The species of live-ringed birds that most often tested RRT-PCR positive for AI were *Callonetta leucophrys* (ringed teal; 2/2), *Cygnus bewickii* (Bewicks swan; 1/21), *Tadorna tadorna* (common shelduck; 13/386), *Actitis hypoleucos* (common sandpiper; 1/30), and *Arenaria interpres* (ruddy turnstone; 6/187; Table 1). The overall viroprevalence for live-ringed mallard was 1.34% (38/2828; Table 1). For mallards, which are the most-targeted species for AI surveillance in Belgium, the viroprevalence during the hunting season in the live-ringed individuals of this species (2.10%) is exceeded at all except one hunting sites (range: 1.52%– 13.71%; Fig. 4).

Virus isolation from live-ringed and hunter-harvested birds. A large diversity of LPAI subtypes was isolated from both live-ringed and hunter-harvested birds (Table 2). H5 and H7 subtypes were isolated from both live-ringed (H5N1, H5N2, H7N1, and H7N7) and hunter-harvested (H7N7, H5N1, H5N8, and H5N2) samples. The H5 and H7 isolates were identified as the low pathogenic pathotype by sequencing the cleavage site.

DISCUSSION

In this study, cloacal sampling was more common for both systems of active surveillance, justified by the higher LPAI detection in cloacal samples (16). For live-ringed birds, oropharyngeal sampling was maintained for specific species to allow HPAI early detection (11), while oropharyngeal sampling of hunted birds was stopped in 2010 as it was no longer considered effective.

Previous studies have shown that AI viroprevalence ranges from 0% to over 40% depending on the targeted bird species, the season of sampling, migratory status of the birds, and the region of sampling (21,22). Our study showed that the percentage of RRT-PCR AI viroprevalence was significantly higher in hunter-harvested birds (6.12%) compared to live-ringed birds (0.48%). The hunter-harvested data were comparable to the 5.4% viroprevalence detected by a study in the Camargue, which is a wetland in the south of France through which several migratory routes converge and where the restocking of game birds for hunting is implemented (13). Admittedly, hunting data could be biased compared to general active AI surveillance data, as samples are collected in autumn (considered the normal AI infection season) and from just one species (*A. platyrhynchos*) that is a known reservoir for LPAI viruses (12,15,24). However, our data did not support this suggestion because when the live-ringed surveillance data is limited to the hunting season and to *A. platyrhynchos* sampling, a significantly lower viroprevalence remained compared to the hunter-harvested data. Therefore, timing and target species did not explain the observed differences of AI viroprevalence data. For the live-ringed sampling data, highest detection rates were recorded in the known AI reservoirs, the orders of Anseriformes and Charadriiformes (Table 1) (1).

The age data from the current study were limited or absent and therefore insufficient to confirm a higher viroprevalence in younger birds (10,21).

Possibly, the higher viroprevalence detected for hunter-harvested birds by the current study could be linked to the game restocking practices. Indeed, naïve birds, without pre-existing immunity against AI, may function as amplifiers of local circulating strains. This phenomenon causes AI presence to increase in the environment, leading to further dispersion (22,23). Captive-bred ducks in Belgium are serologically analyzed following the EC request (Directives 2005/94/EC, 2005/734/EC, 2007/268/EC, and 2010/367/EU) that the population is checked at least once a year when bird numbers exceed 200 individuals at a given site (2,3,4,6). However, depending on their origin (Belgian farms with $<$200 birds or foreign farms without AI control), this does not mean that all game restocking birds are checked for AI viral presence before release in the wild. Releasing noncontrolled birds into the wild may lead to the introduction of new strains in the natural environment. This issue can lead to increased AI in the environment and allow new reassortments to occur with wild AI strains. Consequently, the release of naïve or infected restocking birds may affect AI dynamics, with potentially negative consequences on the local AI epidemiology. Ultimately, more-stringent controls or increased restraints on the release of domestic fowl in hunting regions might need to be implemented.

The active wild bird surveillance system in Belgium, a combination of live-ringed and hunter-harvested birds, allowed the isolation of a wide range of LPAI subtypes including H5 and H7 viruses. Detailed analysis of these strains is recommended to be able to inform governments and decision makers about the risk of transmission to commercial holdings. For instance, detailed analysis of the 2009–2014 samples from this study, consisting of 20,822 live-ringed and 3220 hunter-harvested samples, indicated a higher viroprevalence in hunter-harvested than in live-ringed birds, which could be related to exacerbation of AI prevalence by game restocking practices.

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