Avian Influenza Infection Dynamics in Minor Avian Species

Kateri Bertran i Dols

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Avian Influenza Infection Dynamics in Minor Avian Species

Tesi doctoral presentada per na **Kateri Bertran i Dols** per optar al grau de Doctora en Veterinària dins del programa de doctorat de Medicina i Sanitat Animals del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, sota la direcció de la Dra. **Natàlia Majó i Masferrer** i la Dra. **Roser Dolz i Pascual**.

Bellaterra, 2013

La Dra. NATÀLIA MAJÓ i MASFERRER, professora titular del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona i investigadora adscrita al Centre de Recerca en Sanitat Animal (CReSA), i la Dra. ROSER DOLZ i PASCUAL, investigadora del CReSA,

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Que la memòria titulada **"Avian Influenza Infection Dynamics in Minor Avian Species"** presentada per na Kateri Bertran i Dols per a l'obtenció del grau de Doctor, s'ha realitzat sota la seva direcció i supervisió i, considerant-la acabada, n'autoritzen la seva presentació per tal de ser avaluada per la comissió corresponent.

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Dra. Natàlia Majó i Masferrer	Dra. Roser Dolz i Pascual	Kateri Bertran i Dols
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Al Manelet,

el meu àngel de la guarda

Bl (www.flick.com)

“I, a vegades, ens en sortim”

Captatio benevolentiae

(Els millors professors europeus, Manel)

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... I la tekanagüen emprèn el vol...

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ABBREVIATIONS

AI	avian influenza
AIV(s)	avian influenza virus(es)
ABSL-3	animal biosafety level 3
AGID	agar gel immunodiffusion
C-ELISA	competitive enzyme-linked immunosorbent assay
CBC	cell blood count
CReSA	*Centre de Recerca en Sanitat Animal*
cRNA	complementary RNA
CS	cloacal swabs
Ct	cycle threshold
DIVA	differentiation of infected from vaccinated animals
DMEM	Dulbecco's Modified Eagle's Medium
dpi	days post-inoculation
EC	European Commission
EID_{50}	mean egg infective dose
ELD_{50}	mean egg lethal dose
ELISA	enzyme-linked immunosorbent assay
FP	feather pulp
HA	hemagglutinin
HE	hematoxylin/eosin
HI	hemagglutination inhibition
HPAI	highly pathogenic avian influenza
HPAIV(s)	highly pathogenic avian influenza virus(es)
hpi	hours post-inoculation
HPNAIV(s)	notifiable highly pathogenic avian influenza virus(es)
H5N1/HP	A/Great crested grebe/Basque Country/06.03249/2006
H7N1/HP	A/Chicken/Italy/5093/1999 (H7N1)
H7N2/LP	A/*Anas platyrhynchos*/Spain/1877/2009
H7N9/LP	A/*Anas crecca*/Spain/1460/2008
IF	immunofluorescence
IHC	immunohistochemistry
IREC	*Instituto de Investigación en Recursos Cinegéticos*
IVPI	intravenous pathogenicity index
IZSLER	*Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna*
GMT	geometric mean titers

ABBREVIATIONS

LPAI	low pathogenic avian influenza
LPAIV(s)	low pathogenic avian influenza virus(es)
LPM	live poultry market
LPNAIV(s)	notifiable low pathogenic avian influenza virus(es)
MAAII	*Maackia amurensis* agglutinin II
MDCK	Madin-Darby canine kidney
MDT(s)	mean death time(s)
mRNA	messenger RNA
M1	matrix protein
M2	membrane ion channel protein
NAIVs	notifiable avian influenza virus(es)
NEPs	nuclear export proteins
NA	neuraminidase
NI	neuraminidase inhibition
NP	nucleoprotein
NS1	nonstructural protein 1
NS2	nonstructural protein 2
OIE	World Organization for Animal Health
OS	oropharyngeal swabs
PA	polymerase acidic protein
PBS	phosphate buffer saline
PB1	polymerase basic protein 1
PB2	polymerase basic protein 2
qRRT-PCR	quantitative real time RT-PCR
rFPV	recombinant fowl poxvirus
rNDV	recombinant Newcastle disease virus
RNPs	ribonucleoproteins
RRT-PCR	real time RT-PCR
RT	room temperature
SNA	*Sambucus nigrans* agglutinin
SPF	specific pathogen free
SPSS	Statistical Package for the Social Sciences
$TCID_{50}$	mean tissue culture infectious dose
VI	virus isolation
vRNA	genomic viral RNA

Avian influenza (AI) has become one of the most important challenges that ever emerged from animal reservoirs. The constant outbreaks detected worldwide in domestic and wild bird species are of concern to the economics of the poultry industry, wildlife conservation, and animal and public health. Susceptibility to AI viruses (AIVs) varies deeply among avian species, as well as their possible role as sentinels, intermediate hosts or reservoirs. To date, several experimental studies and natural infections have assessed the susceptibility of numerous major and minor avian species to diverse highly (HPAIV) and low (LPAIV) pathogenic AIVs. Gallinaceous poultry are considered to be highly susceptible, whereas waterfowl have long been recognized as natural reservoirs. However, scarce information concerning the infection dynamics of AIV in the red-legged partridge (*Alectoris rufa*), European quail (*Coturnix c. coturnix*), and gyr-saker hybrid falcon (*Falco rusticolus* x *F. cherrug*) exist so far. Such non-conventional avian species may be of great interest in particular geographical regions, including the Iberian Peninsula. These species not only belong to the autochthonous wildlife, but they are also raised (normally within extensive farming systems) for various human benefits, which leads to close contact with both humans and local wildlife.

With the aim to understand the dynamics of AIV infection in these three non-conventional avian species, and their epidemiological role in an interspecies AI outbreak to better define surveillance strategies, three experimental infections were undertaken which have been presented in this dissertation. The pathogenesis of the infection with both LPAIV and HPAIV was determined in the red-legged partridge (**Study I**), European quail (**Study II**), and gyr-saker hybrid falcon (**Study III**), with special emphasis on describing the clinical disease, gross and microscopic lesions, together with the presence of viral antigen in tissues. In addition, viral shedding pattern for each avian species and each AIV was defined, and the likelihood of effective viral transmission among birds was assessed. **Study II** also represents a comparative study of infection dynamics of two different HPAIV subtypes (H7 and H5). In **Studies I** and **II**, special attention was paid on feathers as a potential origin of AIV dissemination. Besides, in **Study II** drinking water was investigated as for being a possible transmission route. Finally, in **Study III**, the natural AIV infection route in falcons (i.e., by ingestion of infected prey) was efficiently reproduced, and the influenza virus receptors' pattern in this species was elucidated for the first time.

SUMMARY/RESUM

In **Study I**, HPAIV-infected red-legged partridges displayed the first clinical signs at 3 days post-inoculation (dpi), and mortality started at 4 dpi, reaching 100% at 8 dpi. The presence of viral antigen in tissues and viral shedding were confirmed by immunohistochemistry (IHC) and quantitative real time RT-PCR (qRRT-PCR), respectively, in both HPAIV-inoculated and -contact partridges. Neither clinical signs nor histopathological findings were observed in LPAIV-infected partridges. In addition, only short-term viral shedding together with seroconversion was detected in some LPAIV-inoculated partridges. **Study I** demonstrates that the red-legged partridge is highly susceptible to the H7N1 HPAIV strain used, causing severe disease, mortality, and abundant viral shedding, and thus, contributing to the spread of a potential local outbreak of this virus. In contrast, our results concerning H7N9 LPAIV suggest that the red-legged partridge is not a reservoir species for this virus.

In **Study II**, severe neurological signs and mortality rates of 67% (H7N1/HP) and 92% (H5N1/HP) were observed in HPAIV-infected European quail. Although histopathological findings were present in both HPAIV-infected groups, H5N1/HP-quail displayed a broader viral antigen distribution and extent of microscopic lesions. Neither clinical nor pathological involvement was observed in LPAIV-infected quail. Consistent long-term viral shedding and effective transmission to naïve quail was demonstrated for the three studied AIVs. Drinking water arose as a possible transmission route and feathers as a potential origin of HPAIV dissemination. **Study II** demonstrates that European quail may play a major role in AI epidemiology, highlighting the need to further understand its putative role as an intermediate host for avian/mammalian reassortant viruses.

In **Study III**, falcons exhibited similar infection dynamics regardless the different routes of exposure (i.e., nasochoanal route or by ingestion of infected prey), demonstrating the effectiveness of *in vivo* feeding route. H5N1/HP-infected falcons died, or were euthanized, between 5–7 dpi after showing acute severe neurological signs. Presence of viral antigen in several tissues was confirmed by IHC and RRT-PCR, which was generally associated with significant microscopical lesions, mostly in the brain. Neither clinical signs, nor histopathological findings were observed in any of the H7N2/LP-infected falcons, although all of them had seroconverted by 11 dpi. Avian receptors were strongly present in the upper respiratory tract of the falcons, in accordance with the consistent oral viral shedding detected by RRT-PCR in both H5N1/HP- and H7N2/LP-infected falcons. **Study III**

demonstrates that gyr-saker hybrid falcons are highly susceptible to H5N1/HP virus infection, as previously observed, and that they may play a major role in the spreading of both HPAIV and LPAIV. For the first time in raptors, natural infection by feeding on infected prey was successfully reproduced. The use of avian prey species in falconry husbandry and wildlife rehabilitation facilities could put valuable birds of prey and humans at risk and, therefore, this practice should be closely monitored.

The present dissertation highlights the importance of studying the susceptibility, infection dynamics, and transmission likelihood of AIVs in host avian species at the domestic-wild interface. Such knowledge is crucial to effectively perform surveillance efforts, develop appropriate preventive measures, and successfully manage AI outbreaks when different avian species are involved.

La influença aviària (IA) s'ha convertit en un dels reptes més importants que mai hagi sorgit del reservori animal. Els constants brots que s'han detectat arreu del món en aus domèstiques i aus salvatges afecten en gran mesura a l'economia de la indústria avícola, a la conservació de les aus salvatges i a la sanitat animal i pública. El grau de susceptibilitat als virus d'IA (VIA) és extremadament diferent entre les espècies aviàries, igual que succeeix amb la seva possible funció com a sentinelles, hostes intermediaris o reservoris. Fins ara, diversos estudis experimentals i infeccions naturals han avaluat la susceptibilitat de nombroses espècies aviàries menors i majors a diferents VIA d'alta patogenicitat (VIAAP) o de baixa patogenicitat (VIABP). Es considera que els gal·liformes són altament susceptibles, mentre que les aus aquàtiques es consideren reservoris naturals. Tanmateix, existeix molt poca informació sobre la dinàmica d'infecció de la IA en la perdiu roja (*Alectoris rufa*), en la guatlla europea (*Coturnix c. coturnix*) i en el falcó híbrid sacre-grifó (*Falco rusticolus* x *F. cherrug*). Aquestes espècies aviàries no convencionals poden suscitar un gran interès a regions geogràfiques determinades, com ara la península Ibèrica. Les espècies esmentades no només pertanyen a la fauna autòctona, sinó que també es crien (normalment en sistemes agrícoles extensius) en benefici dels humans, la qual cosa comporta un estret contacte tant amb humans com amb la fauna local.

Amb la fi de comprendre la dinàmica de la infecció pel VIA en aquestes tres espècies aviàries no convencionals, així com la funció epidemiològica que exercirien en un brot interespècie d'IA per tal de definir millors estratègies de vigilància, es van realitzar tres infeccions experimentals, les quals s'han descrit en aquesta tesi. La patogènia de la infecció tant pel VIAAP com pel VIABP es va determinar per a la perdiu roja (**Estudi I**), la guatlla europea (**Estudi II**) i el falcó híbrid sacre-grifó (**Estudi III**), posant especial èmfasi en la descripció de la malaltia clínica, les lesions macroscòpiques i microscòpiques, i la presència de l'antigen víric als teixits. A més, en aquest treball s'ha descrit el patró d'excreció vírica de cada espècie aviària i cada VIA, i s'ha avaluat la probabilitat d'una eficaç transmissió vírica entre animals. L'**Estudi II** també representa un estudi comparatiu de la dinàmica d'infecció en dos subtipus diferents de VIABP (H7 i H5). En el cas dels **Estudis I** i **II**, es va prestar especial atenció en les plomes com a possible origen de propagació del VIA. A més, a l'**Estudi II** es va investigar l'aigua com a possible ruta de transmissió. Finalment, a l'**Estudi III** es va reproduir amb eficiència la infecció natural pel VIA en falcons (és a dir, mitjançant la ingestió de preses infectades) i es va dilucidar per primera vegada en aquesta espècie el patró dels receptors del virus de la grip.

SUMMARY/RESUM

A l'**Estudi I**, les perdius roges infectades pel VIAAP van presentar els primers signes clínics a 3 dies post-inoculació (dpi); a partir de 4 dpi van començar a morir i a 8 dpi ja no restava cap espècimen viu. Per mitjà d'immunohistoquímica (IHQ) i de PCR quantitativa a temps real (PCRq-TR), es va confirmar la presència de l'antigen víric en teixits i l'excreció del virus, respectivament, tant en perdius inoculades amb el VIAAP com contactes. No es van observar ni signes clínics ni troballes histopatològiques a les perdius infectades pel VIABP. A més, a algunes perdius inoculades amb el VIABP, només es va detectar l'excreció del virus a curt termini, juntament amb seroconversió. L'**Estudi I** va posar de manifest que la perdiu roja presenta una elevada susceptibilitat a la soca del VIAAP H7N1 que es va utilitzar, la qual va causar greu malaltia clínica, alta mortalitat i excreció vírica abundant. Per consegüent, aquesta au podria contribuir a la propagació d'un possible brot local del virus. En canvi, els resultats que es van observar pel VIABP H7N9 indicarien que la perdiu roja no és un reservori d'aquest virus.

A l'**Estudi II**, es van observar greus signes neurològics i una taxa de mortalitat del 67 % (H7N1/AP) i del 92 % (H5N1/AP) a les guatlles europees infectades pel VIAAP. Tot i que van aparèixer troballes histopatològiques en ambdós grups infectats pel VIAAP, les guatlles infectades amb VIAAP H5N1 van mostrar una distribució d'antigen víric més àmplia i lesions microscòpiques més intenses. No es va observar afectació clínica o patològica a les guatlles infectades pel VIABP. Pels tres VIA investigats es va demostrar una excreció vírica consistent i de llarg termini i una transmissió efectiva a les guatlles susceptibles. L'aigua va sorgir com a possible ruta de transmissió, i les plomes com a possible font de propagació de VIAAP. L'**Estudi II** posa en relleu que la guatlla europea pot tenir una funció important en l'epidemiologia de la IA, la qual cosa fa ressaltar la necessitat de conèixer amb més profunditat la seva possible funció com a hoste intermediari en el cas dels virus recombinants d'aus/mamífers.

A l'**Estudi III**, els falcons van mostrar una dinàmica d'infecció similar tot i les diferents rutes d'exposició (és a dir, ruta nasocoanal o a partir de la ingestió de preses infectades), fet que va demostrar l'efectivitat de la ruta d'alimentació *in vivo*. Els falcons infectats pel VIAAP H5N1 mostraven greus signes neurològics aguts i van morir, o van ser sacrificats, entre 5 i 7 dpi. Mitjançant IHQ i RRT-PCR es va confirmar la presència de l'antigen víric a diferents teixits. Aquest antigen

s'associava normalment a lesions microscòpiques, sobretot a l'encèfal. No es van observar ni signes clínics ni lesions histopatològiques a cap dels falcons infectats pel VIABP H7N2, encara que tots els espècimens havien seroconvertit a 11 dpi. Es va detectar forta presència de receptors aviaris al tracte respiratori superior dels falcons, en concordança amb l'excreció vírica detectada per RRT-PCR tant als falcons infectats per VIABP com per VIABP. L'**Estudi III** mostra que falcó híbrid sacre-grifó presenta elevada susceptibilitat a la infecció pel VIAAP H5N1, tal com s'havia observat prèviament, i que podria jugar un paper rellevant en la propagació tant de VIAAP com de VIABP. Per primera vegada en el cas dels rapinyaries, es va reproduir amb èxit la infecció natural a partir de la ingestió de preses infectades. L'ús d'aus de presa a la falconeria i a centres de recuperació de fauna podria posar en risc rapinyaires de gran valor i humans i, per tant, seria convenient realitzar un seguiment intensiu d'aquesta pràctica.

La present tesi posa en relleu la importància d'estudiar la susceptibilitat, la dinàmica d'infecció i la probabilitat de transmissió del VIA en hostes aviaris de la interfície domèstic-salvatge. Adquirir aquest coneixement és vital per realitzar tasques de vigilància eficaces, desenvolupar mesures preventives adequades i gestionar amb èxit els brots d'IA quan es veuen afectades diferents espècies aviàries.

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Part I

General Introduction and Objectives

Chapter 1

General Introduction

1.1. AVIAN INFLUENZA INFECTION

1.1.1. HISTORY OF AVIAN INFLUENZA

Avian influenza (AI) was first identified as a distinct disease entity of poultry in 1878, in Italy. It was called “fowl plague” and was defined as a highly lethal, systemic disease of chickens [177]. From the 1870s into the early 1900s, fowl plague spread from Northern Italy into the rest of Europe, and by the 1930s it was endemic in parts of Europe and Africa [194,226]. Likewise, in the United States the disease was reported in 1924-1925 and 1929 [226]. Into the mid-twentieth century, fowl plague had been diagnosed in North Africa, South America, North America, and much of Europe [4,226,245]. Over that time, in 1933, the agent causing the human influenza was discovered, together with the successful isolation of the swine influenza virus, in 1931 [200,208]. Prior to that, one of the most devastating influenza pandemics in human history, the “Spanish Flu” (H1N1 subtype), hit the population in 1918, causing thousands of deaths [253].

Even if Centanni and Savonuzzi [41] had already demonstrated the existence of a filter-passing agent, the viral etiology of fowl plague was unknown until 1955, when the disease was determined to be caused by influenza A virus [195]. It is worth highlighting that the original AI infections in poultry were only associated with severe clinical disease, and they were serologically linked to two subtypes defined by their hemagglutinin (HA): H5 and H7 highly pathogenic AI viruses (AIVs) (HPAIVs) [56,229]. Nevertheless, mild clinical forms of AI, characterized by respiratory disease and drops in egg production, were first recognized in 1949 in chickens and, subsequently, in several domestic poultry species [57]. These forms of AI were designated at the Fifth International Symposium on Avian Influenza as low pathogenicity AI (LPAI) [62]. Therefore, since 1971, H5 and H7 viruses have been isolated and characterized not only as HPAIVs but also as LPAIVs [16,57,209].

Although wild birds were already suspected to participate in fowl plague

transmission, it was not until 1961 that the first proof of AIV infection in wild birds arose, in an outbreak in South Africa affecting common terns (*Sterna hirundo*) with high mortality [17]. Since then, and particularly during the recent past decades, numerous surveys have been conducted in migratory waterfowl, confirming asymptomatic infection by AIV in healthy wild aquatic birds, especially in the orders Anseriformes and Charadriiformes [83,220]. These surveys have demonstrated the presence of thousands of LPAIVs of all 16 HA and 9 neuraminidase (NA) subtypes from asymptomatic wild birds [158,229], as well as several HPAIVs [17,131].

Since the 1950s, when consistent diagnostic and control strategies were developed, 26 epidemics or limited outbreaks of HPAIV (either of H5 or H7 subtypes) in birds have been documented worldwide [229]. It was not until 1997 that AI became considered a disease not only of birds, when the occurrence of fatal disease in poultry and humans in Hong Kong was associated with the H5N1 strain of HPAIV [48,277]. This episode increased the international interest in HPAIV among the veterinary community and public health, because it was the first indication that H5N1 AIVs could potentially be the precursor to a human pandemic virus [203]. Indeed, over the next decade, H5N1 HPAIV in poultry spread across three different continents with unprecedented socioeconomic consequences. These concerns were amplified because of the potential reassortment with a human influenza A virus, which could create a new virus capable to produce the next human influenza pandemic [229].

1.1.2. ETIOLOGY

1.1.2.1. Classification

The AIV belongs to the *Orthomyxoviridae* family of segmented negative-sense RNA viruses. Viruses of this family are divided into five different genera, including influenza types A, B, and C, Isavirus, and Thogotovirus [229]. Avian influenza viruses are classified within type A, which widely infects bird and mammalian species, including humans.

Types B and C influenza viruses are human pathogens that rarely infect other species [161].

Besides, AIVs are categorized based on serological typing of the two surface glycoproteins, being 16 HA and 9 NA different subtypes described so far [65,245,276]. In addition, AIVs can be further classified into two different pathotypes (LP and HP) based on the ability to produce disease and death in the major domestic poultry species, the chicken (*Gallus domesticus*) [248].

1.1.2.2. Morphology and Molecular Organization

All influenza A viruses are spherical to pleomorphic enveloped viruses of approximately 100 nm in diameter [66]. Influenza A virus has eight different gene segments that encode at least 10 different viral proteins, which can be divided into surface proteins, internal proteins, and nonstructural proteins (Figure 1). The surface proteins include the HA, NA, and membrane ion channel (M2) proteins. The internal proteins include the nucleoprotein (NP), the matrix protein (M1), and the polymerase complex composed of the polymerase basic proteins 1 (PB1) and 2 (PB2), and polymerase acidic protein (PA) [161]. The nonstructural proteins 1 (NS1) and 2 (NS2) are also known as the nuclear export proteins (NEPs) [160]. One protein that is not present in all type A influenza viruses is the PB1-F2 protein, which is thought to be involved in apoptosis of host cells, but its role in pathogenesis is still being defined [45].

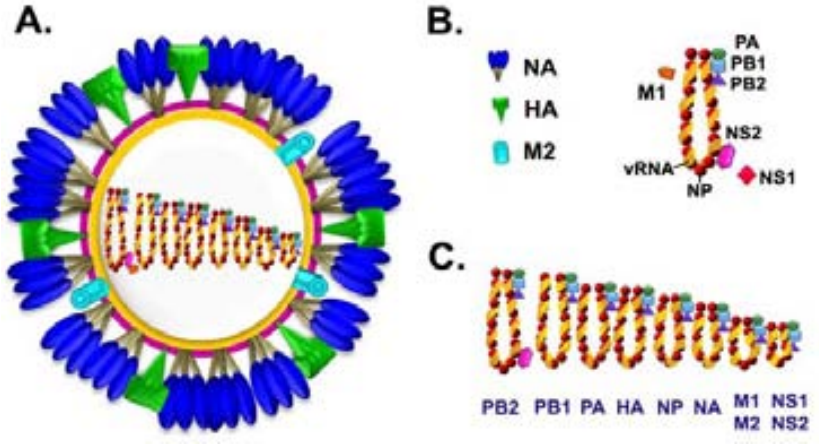


Figure 1. A. Structure of influenza A virus. B. Surface proteins are: HA (consisting of trimmers), NA (which forms tetramers), and M2 (a tetramer which forms ion-channels). The M1 protein is found inside the viral membrane and the viral genome which consists of eight negative-stranded RNA segments that are packaged into the viral particle as a vRNP in complex with NP and the viral polymerases PA, PB1, and PB2. **C.** The eight segments of influenza A virus codify 11 proteins: segment 1 (PB2), segment 2 (PB1 and PB1-F2), segment 3 (PA), segment 4 (HA), segment 5 (NP), segment 6 (NA), segment 7 (M1 and M2), and segment 8 (NS1 and NS2) (Illustration modified from [101]).

1.1.2.3. Virus Life Cycle

Virus replication cycle is schematized in Figure 2. The first step in viral infection is the attachment of the viral HA protein to the host cell receptor sialic acid. Sialic acid molecules are often classified as to how they are linked to the underlying sugars by the α-2 carbon, being α-2,3 and α-2,6 the most common linkages [235]. These different sialic acid linkages result in different conformations of the host receptor that affects virus binding. The viral HA, based on amino acid sequence, has strong specificity for either the α-2,3 or α-2,6 linkage, likely being a factor in host specificity. Thus, the α-2,3 sialic acid is typically expressed in avian species, whereas the α-2-6 sialic acid is classically expressed in humans [49,188,189]. However, human and at least some avian hosts express both types of sialic acid, although with different tissue distributions [50].

Once viral attachment has occurred, the virus is endocytosed. The acidification of the endosome triggers the fusion domain of the HA protein to become active, and the viral RNA is released into the cytoplasm [224]. The M2 protein plays a key role in this triggering process; it is an integral membrane protein that allows H^+ ions to enter into

the virion, which causes a conformational change of the HA at the lower pH to allow the fusion domain to become active [180]. The fusion of the viral membrane and the endosomal membrane allows the release of the viral RNA-polymerase complex into the cytoplasm, which is then actively transported to the nucleus by nuclear localization signals [159]. Once in the nucleus, the negative-sense viral RNA is copied by the polymerase complex (PB1, PB2, PA, and NP proteins) into two positive-sense RNA species: a messenger RNA (mRNA) and a complementary RNA (cRNA). The cRNA serves as template from which the polymerase transcribes more copies of negative-sense, genomic viral RNA (vRNA) that will form the virions [22]. The mRNA has to be polyadenylated and capped in order to be translated into proteins in a process known as "cap snatching", where the viral PB2 protein steal a 5' capped primer from host pre-mRNA transcripts [112]. The positive-sense viral mRNA then migrates from the nucleus to begin viral protein translation in the cytoplasm using the host cell machinery [229].

It is believed that M1 and NEP are crucial for trafficking of viral proteins to and from the nucleus [21]. Some of the newly synthesized viral proteins are transported to the nucleus, where they bind to vRNA to form ribonucleoproteins (RNPs); others are processed in the endoplasmic reticulum, where glycosylation of the three integral membrane proteins (HA, NA, and M2 proteins) occurs [13]. These modified proteins are transported to the cell membrane, where they stick in the lipid bilayer. Part of the viral mRNA is spliced by cellular enzymes so that finally viral proteins, such as M1 and NS2, can be synthesized without any further cleavage [22]. At this point, M1-NS2 compound functions as a bridge between the lipid membrane and the viral core of NP, vRNA, and the polymerase complex. Therefore, the nuclear localization of M1 and NS2 proteins is essential for the migration of the vRNP out of the nucleus and assembly into progeny viral particles in the cytoplasm [160]. Also, HA, NA, and M2 proteins are important for virus assembly [13]. The viral assembly is a highly inefficient process, where more than 90% of the viral particles are noninfectious because they are defective of viral gene segments [55,60]. Finally, the NA protein removes sialic acid from the surface HA proteins, which prevents aggregation of new viruses at the cell surface [140,198].

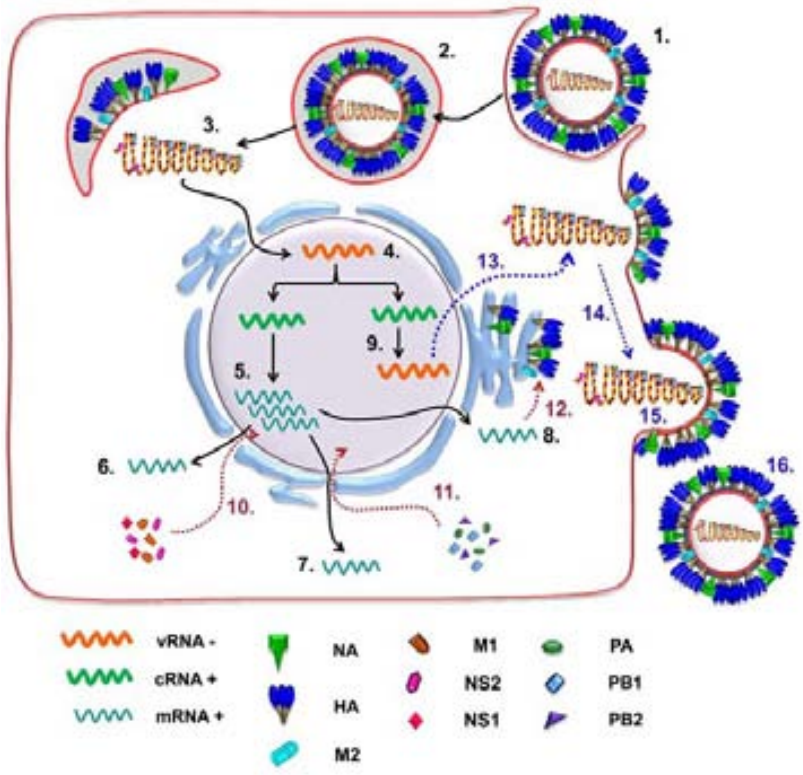


Figure 2. Influenza virus cycle. 1. Binding to Sia receptors and entry into the host cell. **2.** Endocytosis. **3.** Fusion after acidification of the endocytic vesicle. **4.** Entry of vRNPs into the nucleus and release of vRNP complexes. **5.** The (-) stranded vRNA is copied by virion RNA polymerase into mRNA. **6, 7.** Export of mRNA from the nucleus to the cytoplasm for translation and production of viral proteins. **8.** The mRNA codifying for the viral surface proteins are translated by ribosomes bound to the endoplasmic reticulum, **12.** where they experience glycosylation. **10.** The PA, PB1, PB2 and NP proteins are imported into the nucleus, **9.** where they participate in the synthesis of full length (+) strand RNAs and (-) strand RNA. **11.** The M1 and NS2 are also transported to the nucleus. **13.** The M1 binds to the new synthesized (-) strand vRNA, shutting down the viral mRNA synthesis. M1 and NS2 induce the export of progeny nucleocapsids to the cytoplasm **14, 15.** and then to the cell surface in regions of the cell membrane containing NA and HA proteins. **16.** When the assembly is complete, the new virions are bud from the plasma membrane (Illustration modified from [63,101]).

1.1.2.4. Antigenic Diversity

Influenza viruses have two primary mechanisms of diversity within the viral population: a high mutation rate (antigenic drift) and the ability to reassort gene segments (antigenic shift). Both methods provide an opportunity for the virus to rapidly change and adapt, which contributes to the ability of AIV to establish infections in new host species [229].

Antigenic drift

It is generally accepted that the highly error-prone RNA polymerase of influenza viruses may introduce mutations during replication [166,223], especially in the HA antigenic sites [229]. One of the primary selective factors on the HA protein is believed to be the antibody pressure from the host, either from previous exposure to the virus or by vaccination [182]. In practice, these changes in the antibody specificity can result in a better ability of the virus to escape from the immune system, and thus, in a reduction of the protection induced by influenza vaccines over time [229]. The antigenic drift is clearly observed in humans, as a consequence of the selective pressure implemented by the use of vaccines to protect the population [272]. For this reason, human vaccines need to be evaluated yearly to determine if they produce neutralizing antibodies to the currently circulating field strains [229]. Antigenic drift is much more complicated to take place in poultry species [229], yet it is believed to occur in countries where AI immunization has been continuously and extensively implemented [249], as in Mexico [123]. Regardless of vaccination, viruses that are circulating for long time in the field evolve increasing their adaption and virulence through mutations in the viral HA by diverse mechanisms such as site mutations, nucleotide insertions, and duplications [169] (Table 1). As a consequence of these mutations while circulating in poultry, LPAIVs can become HPAIVs [229].

Table 1. Examples of antigenic drift events observed in poultry and proposed genetic changes in the HA cleavage site [169].

HPAIV	LPAIV precursor	Proposed genetic change in the HA cleavage site	Likely molecular mechanism
A/chicken/Pennsylvania/1370/83 H5N2	A/chicken/Pennsylvania/21525/83 H5N2	(PQRETR*G) → PQRKKR*G (+ loss of CHO)	Site mutation
A/turkey/Ontario/7732/66 H5N9	A/turkey/Ontario/6213/66 H5N9	(PQRETR*G) → PQRRKKR*G	Accumulated single nucleotide insertions
A/chicken/Jalisco/14588-660/94 H5N2	A/chicken/Quetaro/7653/95 H5N2	(PQRETR*G) → PQRKRKRKR*G	Tandem duplication/insertion
A/chicken/Chile/176822/02 H7N3	A/chicken/Chile/4977/02 H7N3	(PEKPKTR*G) → PEKPKTCSPLSRCRETR*G	RNA/RNA recombinant

Antigenic shift

Having eight different gene segments as genome confers to influenza viruses the capability to reassort genes from two different influenza virus subtypes infecting a single cell, which results in a virus with new antigenic proteins [22]. Other situations, like the transfer of a whole virus from one host species to another and the reemergence of previously non circulating virus strains, are also considered antigenic shift. The lack of protective immunity allows the new virus to rapidly spread within the new host population. Interestingly, the short production life of most commercial poultry has prevented antigenic shift to a large degree [229]. Regardless, there are land-based poultry species that harbor both avian (α-2,3) and mammal (α-2,6) influenza receptors [50], and thus, might reflect a potentially decisive factor in the emergence of reassortant viral strains. Previously, Guan *et al*. [71] suggested that the H5N1 influenza viruses isolated from mammals (including humans) and poultry in Hong Kong in 1997 possessed internal genes phylogenetically related to those of the quail influenza virus quail/Hong Kong/G1/97 (H9N2). Experimentally, Japanese quail (*Coturnix c. japonica*) were confirmed to support efficient replication and transmission of H9N2 avian/swine reassortant viruses [173].

1.1.2.5. Determinants of Virulence

Virus factors

As previously mentioned, AIVs can be categorized as LP or HP according to their ability to produce disease and death in chickens [248]. The pathogenicity of AIV is clearly due to a polygenic effect [269]. One of the main determinants of virulence is the sequence of the HA cleavage site. Influenza viruses must cleave the HA protein into the HA1 and HA2 subunits in order to become infectious. Normally, trypsin-like proteases cleave the HA protein by recognizing a single arginine, which defines LPAIVs [69,121]. Typically, these trypsin-like proteases are present in the respiratory and enteric tracts, greatly limiting LPAIV distribution in the host within these organs [108]. However, when

multiple basic amino acids (lysine and arginine) are present at the HA cleavage site, which is characteristic of HPAIVs, the cleavage site becomes accessible to furin or other ubiquitious proteases that are found in most cells of the body [225] (Table 1). This allows HPAIVs to highly replicate in a number of different cell types, including cells in critical organs such as the brain, heart, and pancreas, causing damage and leading to the death of the host [176,241]. In addition to the HA cleavage site, diverse studies in chickens and ducks indicate that the NS1, PB1, PB2, NP, and M2 proteins are important contributing factors in the increased virulence of AIVs in chickens [40,92,129,131,269].

Host factors

Host variables such as the age and species can affect the AIV virulence [40,247]. Increased virulence in younger animals may be due to the immaturity of the immune response, although virulence can be greater in older birds or birds in egg-production [84,120,163,233]. Besides, HPAIVs by definition cause high mortality in chickens [156], but that does not necessarily provide a predictor for disease in other species. The species factor is discussed in more detail in section 1.1.3.4. (Species Susceptibility).

1.1.2.6. Viral Pathotypes

For reasons that are not readily apparent, HPAIVs have been restricted so far to the H5 and H7 subtypes, even if most H5 and H7 AIVs are of LP [229]. Typically, HPAIVs arise from H5 and H7 LPAIVs after having circulated in gallinaceous poultry for extended periods of time. Thus, the replication of LPAIV in gamefowl birds like chickens, turkeys (*Meleagris gallopavo*), and quail is a critical part of the process [36,90].

Bearing in mind this fact, all AIVs of H5 and H7 subtypes (both LPAIV and HPAIV) are classified as notifiable AIVs (NAIVs) by the World Organization for Animal Health (OIE) [156]. The OIE has dictated two official criteria to confirm the detection of NAIVs: 1) an *in vivo* intravenous pathogenicity index (IVPI) greater than 1.2, which defines an AIV as HP if the inoculation of a minimum of eight 4- to 8-week-old chickens results in

more than 75% mortality within 10 days; and/or 2) a sequence analysis of the HA cleavage site being characteristic of HPAIV. All HPAIVs accomplishing these criteria are identified as notifiable HPAIVs (HPNAIVs). All H5 and H7 isolates that are not pathogenic for chickens and do not have multiple basic amino acids in the HA cleavage site are identified as notifiable LPAIVs (LPNAIVs) [156].

1.1.3. EPIDEMIOLOGY

1.1.3.1. Wild Bird Reservoir

A species is considered a natural reservoir if infection can be maintained within the species population without needing periodic reintroduction [217]. Wild birds, particularly those belonging to the orders Anseriformes (waterfowl, i.e., ducks, geese, and swans) and Charadriiformes (gulls and shorebirds), have long been recognized as the natural host and reservoir for all type A influenza viruses [3,102,207]. Since their first isolation from wild birds in 1961 [17], influenza A viruses of all 16 HA and 9 NA have been isolated from more than 100 wild bird species belonging to more than 25 families [7], which confirms that AIV have a global distribution within free-living avian populations. However, complex relationships between species and viruses have been further defined. For instance, migration connects many wild bird populations in space and time at common breeding and wintering areas, during migration or at stopovers. Thereby, Anseriformes species' assemblages may define more realistically the reservoir rather than a concrete duck species alone [217]. Another example of the AI reservoir complexity is the unique prevalence of H13 and H16 subtypes evidenced in gulls and terns, which has lead these viruses to evolve into a separate lineage of LPAIVs [65,102].

HPAIVs are not normally present in the wild bird host reservoir [190]. However, during the past years such epidemiologic situation has changed due to the emergence of the panzootic H5N1 HPAIV. This virus was first detected in geese in Guandong (China) in 1996 [277] and then spread and evolved in poultry populations in several Southeast countries from 1997 [48,70,202,234] to 2003/2004 [61,89,128,259]. The presence and

spread of H5N1 in Southeast Asia is believed to be the result of the virus becoming endemic in domestic Pekin ducks (*Anas platyrhinchos domestica*), with their rearing in open-range paddy fields as a possible contributing factor [93,228]. In 2005, a large number of migratory aquatic birds died in Qinghai lake (Northeast China) due to H5N1 HPAIV infection [43,44,131]. Since then, continuous outbreaks among wild birds have been reported in Asia and Europe [93,117,126,138,228]. Furthermore, such evolved viruses are highly pathogenic in domestic and wild Anseriformes under experimental conditions [133,163], which is unusual for HPAIV [5,6]. It is worth mentioning that these viruses have been able to infect humans, causing severe and fatal infections [48].

LPAIVs can be found in numerous other bird species [148], but it is unclear in which of these species LPAIVs are endemic and in which the virus is a transient pathogen. Whether there are other avian reservoirs outside of the Anseriformes and Charadriiformes is a recurrent question that may help understand the role that other species could play in the epidemiology of AI. Indeed, surveillance data reveals that a wild bird reservoir for H5N1 AIV lineage has not been defined so far.

1.1.3.2. Maintenance and Transmission

It is believed that AIV maintenance in nature relies on the combined effects of: 1) continual bird-to-bird transmission (involving multiple species and different species' assemblages on breeding and wintering habitats), which requires significant viral shedding and low infective dose; and 2) environmental persistence [52,217,222].

In **waterfowl**, replication of LPAIVs predominantly takes place in the epithelial cells of the gastrointestinal tract, with high amounts of infectious virus shed in feces during prolonged periods. It has been reported that experimentally infected Muscovy ducks (*Cairina moschata*) can shed an estimated $1x10^{10}$ mean egg infective dose (EID_{50}) of AIV within a 24-hour period [274]. Prolonged viral shedding has also been demonstrated in domestic Pekin ducks, which were able to shed virus for more than 28 days [85]. In contrast to LPAIVs, the H5N1 HPAI viral shedding pattern in waterfowl seems relatively

inefficient; it is predominantly oral, involves low viral titers, and is of short duration [27,217,228]. Overall, contaminated lake or drinking water may result in infection of aquatic bird populations by the fecal-oral route, or possibly by the fecal-cloacal route as a result of "cloacal drinking" [7,86]. Thus, presence and persistence of AIV in the environment, particularly in water surfaces, has been a main subject of discussion so far.

It is generally thought that AIV shed by birds prior to fall migration could be preserved in the water over winter, thereby providing a source of infection to birds returning during the following spring [222,270]. Previously, AIVs have been isolated from surface water in various environments, especially in Northern latitudes [85,97]. Initially, wild-type LPAIVs were proved to remain infective for extended periods of time (months or years) in water at different temperatures [222,274]. Later, the duration of infectivity was seen to be dependent on temperature, pH, and salinity, being the longest persistence in cold freshwater (17ºC, 0 ppt) with slightly elevated pH (8.2) [221]. However, responses to these variables are seen to vary between individual isolates [26]. In addition, although water assessments are limited to two Asian H5N1 HPAIVs, it is believed that persistence of infectivity of H5N1 HPAIV is lower than that of wild-type LPAIVs [30]. Interestingly, even if H5N1 HPAIVs seem to fall short concerning viral shedding and environmental persistence, these factors may be offset by transmission via a low infective dose [217].

While fecal-oral is the primary route described for transmission in aquatic birds, non-reservoir birds may have prevalently other routes of AIV exposure. Experimental studies in **chickens** have evidenced that LPAIVs are shed predominantly from the oropharynx and in slightly lower quantity from the cloaca [242]. Similar dynamics occur for HPAIV, although in higher amounts than LPAIV [242,244]. Consequently, higher shedding titers for HPAIVs produce greater environmental contamination and greater transmissibility than comparable LPAIVs [263], which may compensate the lower environmental persistence that HPAIVs have compared to LPAIVs. Similarly to chickens, wild **gamefowl** species have shown viral shedding primarily associated with the respiratory rather than the alimentary tract infections. This seems to be the general

shedding pattern of the gallinaceous species, like the chukar partridge (*Alectoris chukar*), Japanese quail or ring-necked pheasant (*Phasianus colchicus*), some of them recognized as efficient shedders of HPAIV [192] and LPAIV [94,136].

Oral and cloacal secretions are the major environmental viral sources responsible for transmission, but infected carcasses may also contribute to the contamination of the environment and susceptible birds [31], either through feather picking of dead individuals or, in certain species, through predation or cannibalization. Furthermore, feathers have been identified as a location for viral replication and potential origin of dissemination in H5N1 HPAIV infection in waterfowl [278,279,282] and H7N1 HPAIV infection in chickens [31].

Despite the existence of such data, further studies are needed to identify the most efficient routes of transmission for a variety of still-unknown bird species.

1.1.3.3. Host Range and Species Adaptation

The AIV can infect and replicate in a wide variety of host species other than their reservoirs, including mammals and human beings [217,270] (Figure 3). However, the infective dose for each host can vary greatly depending on the host adaptation of the virus [260]. Interestingly, as it becomes fully adapted to a new host, the virus becomes more species-specific and less efficient to replicate in the original host species [229].

Interspecies transmission and adaptation of AIV is a multistep process proportionally complex to the grade of species relatedness [229]. In this way, the transfer of AIVs from free-living waterfowl to domestic ducks and geese is much easier than to gallinaceous poultry, requiring minimal adaptation because of: 1) the close genetic relationship of the wild and domestic duck species; and 2) the more frequent contact between outdoor-reared domestic ducks and geese, and free-living aquatic birds [79,171]. However, transfer of AIV between some wild aquatic bird species may require

multiple steps for adaptation, such as from free-living shorebirds and gulls (order Charadriiformes) to domestic ducks (order Anseriformes) [216,270].

The mechanism of species adaptation and virulence is poorly understood, but is believed to be the result of optimized molecular interactions between viral proteins and cellular factors [139], such as viral release efficiency by NA and efficiency of AI viral polymerase genes to express and take over the host metabolic machinery to produce new viruses [113]. Yet, several studies have identified HA receptor binding affinity as one of the most important factors in species adaptation [139]. In this way, turkeys [179,212] and Japanese quail [172,173,257] are believed to act as intermediate host species that can be readily infected with AIVs of diverse host origins and act as source of infection for other land-based poultry or even mammals.

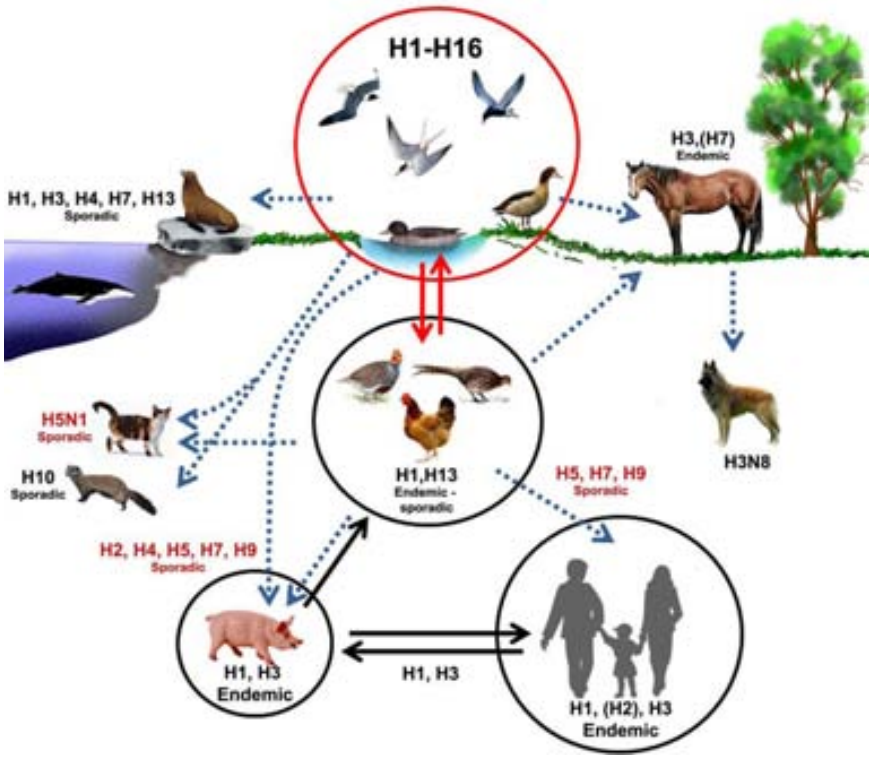


Figure 3. Host range influenza viruses. Wild aquatic birds are the reservoir for all influenza A virus, which are transmitted to poultry species, and occasionally to pigs, horses, and sea mammals. Rarely, wild or domestic birds transmit influenza A viruses to cats or minks. Pig viruses could be transmitted to humans (and vice versa), and sporadically poultry species transmit influenza viruses to humans (dashed lines indicate sporadic and rare transmission of influenza A virus between species) (Illustration modified from [238]).

1.1.3.4. Species Susceptibility

LPAIVs have been demonstrated to infect a broad diversity of taxonomic groups, although species-related differences exist for individual AIV strains [5,6,206,275]. This is in agreement with several reported AIV isolations from species in taxonomic groups that are not usually associated with natural infections, such as Passeriformes, Psittaciformes, and Galliformes [5]. Similarly to LPAIVs, **HPAIVs** can infect a wide wild host range, which increased in the past decades as a result of the H5N1 HPAIV outbreak in Eurasia. Since 2002, iolations of H5N1 HPAIV have been reported from more than 50 species of wild birds in both Asia and Eastern Europe, suggesting that these viruses may have been transported throughout Eurasia during wild bird migrations [217] (Table 2). Interestingly, most of the wild bird species from which H5N1 HPAIVs have been isolated belong to three main groups: 1) Anseriformes birds, which is consistent with the known natural history of AIV; 2) bridge species (both migratory and non-migratory), that may transport the disease among poultry and wildlife; and 3) raptors and other species, which potentially either predate or scavenge other birds (wild or domestic) and may be more exposed to H5N1 HPAIV in a higher incidence context of AI among domestic and wild bird populations [61,217]. There is strong evidence that clinical response for a given species is related to the lineage of the HPAIV, but differences in susceptibility can also be observed even between taxonomically closely related species [27,227].

Several **Galliformes** may be considered as bridge species between poultry and wildlife since they are ranged in outdoor operations and, at the same time, they belong to the autochthonous fauna. Some studies suggest that certain gallinaceous species like turkeys, ring-necked pheasants, and Japanese quail are more susceptible to LPAIVs from free-living aquatic birds than chickens are [94,172,196,212,245]. Humberd *et al*. [94] indicated that chukar partridges may not be a reservoir for LPAIVs, since this species is a short-term shedder of this kind of AIVs and replication might be limited to the respiratory tract. On the contrary, Japanese quail may support the replication (predominantly in the respiratory tract) of almost all LPAIVs representing subtypes H1 to H15 [136]. More recently, Cilloni *et al*. [47] suggested that multiple *in vivo* passages in

Japanese quail facilitate the adaptation of duck AIVs to chicken. Abundant studies have also proven that HPAIVs (H7N1, but principally Eurasian H5N1) are able to infect and cause lesions and death in a large number of wild game fowl under experimental conditions [165,174-176]. Some experimental infections suggest that H5N1 HPAIVs are highly pathogenic to chukar partridges, being able to cause clinical signs, lesions, and death similar to what is described for chickens [174,176]. Besides, numerous studies in Japanese quail have reported either higher, similar or lower susceptibilities than chickens to H5 HPAIV [98,176,192,252,271].

Table 2. Free-living species from which H5N1 HPAIVs have been isolated [217].

Taxonomic group	Species
Anseriformes	Bar-headed goose (*Anser indicus*), Common pochard (*Aythya ferina*), Gadwall (*Anas strepera*), Greater white-fronted goose (*Anser albicans*), Greylag goose (*Anser anser*), Mute swan (*Cygnus olor*), Red-breasted goose (*Branta ruficollis*), Ruddy shelduck (*Tadorna ferruginea*), Smew (*Mergus albellus*), Tufted duck (*Aythya fuligula*), Whooper swan (*Cygnus cygnus*)
Charadriiformes	Black-headed gull (*Chroicocephalus ridibundus*), Brown- headed gull (*Larus atricilla*), Great black-backed gull (*Larus marinus*), Green sandpiper (*Tringa ochropus*)
Ciconiiformes	Chinese pond heron (*Ardeola bacchus*), Grey heron (*Ardea cinerea*), Little egret (*Egretta garzetta*), Open-billed stork (*Anastomus oscitans*), White stork, (*Ciconia ciconia*)
Columbiformes	Red-collared dove (*Streptopelia tranquebarica*), Rock pigeon (*Columba livia*)
Falconiformes	Crested hawk-eagle (*Spizaetus nipalensis*), Eurasian buzzard (*Buteo buteo*), Northern Goshawk (*Accipiter gentilis*), Peregrine falcon (*Falco peregrinus*), Rough-legged buzzard (*Buteo lagopus*)
Galliformes	Kalij pheasant (*Lophura leucomelanos*), White Indian peafowl (*Pavo cristatus*)
Gruiformes	Brown crake (*Amaurornis akool*), Common moorhen (*Gallinula chloropus*), Eurasian coot (*Fulica atra*), Purple swamphen (*Porphyrio porphyrio*)
Passeriformes	Black drongo (*Dicrurus macrocercus*), Crested Mynah (*Acidotheres cristatellus*), Eurasian tree sparrow (*Passer domesticus*), House crow (*Corvus splendens*), Japanese white-eye (*Zosterops japonicas*), Jungle crow (*Corvus macrorhynchos*), Korean magpie (*Pica pica sericea*), Oriental magpie robin (*Copsychus saularis*), Scaly-breasted munia (*Oriolus chinensis*), White-rumped munia (*Lonchura stiata*)
Pelecaniformes	Great cormorant (*Phalacrocorax carbo*), Little cormorant (*Phalocrocorax niger*)
Podicipediformes	Great crested grebe (*Podiceps cristatus*), Little grebe (*Tachybaptus ruficollis*)

In the past, HPAIVs were rarely found in **birds of prey** and were restricted to only a few isolated cases [134,137]. However, during recent H5N1 outbreaks, increasing number of infected raptors have been reported, probably as a result of improvements in sampling and diagnostic tools [46,88,105,181,183,193,261]. Even though the number of AI natural cases in raptor species has gradually increased, only some countries have

performed active AI surveillance on these birds [72,155]. In addition, just two experimental infections have been performed in birds of prey so far, both of them confirming that these birds are extremely susceptible to H5N1 HPAIVs [77,130].

1.1.3.5. Epidemiology of AI in Man-made Systems

Through captivity, domestication, and commerce, humankind have created new niches that have favored transmission, adaptation, and maintenance of AIVs outside the free-living aquatic bird reservoirs to other bird species, including Galliformes poultry, which are not naturally infected by AIV [232,240]. Swayne [240] proposed a classification of the various man-made systems that could affect AIV ecology and epidemiology:

1. Bird collection and trading systems, including captive wild birds and zoological collections.
2. Village, backyard, and hobby flocks, including fighting cocks and exhibition poultry.
3. Live poultry market (LPM) systems with rural-to-urban movement of poultry for sale and slaughter.
4. Outdoor-raised commercial poultry, including organic poultry, free-range turkeys, and game birds.
5. Integrated indoor commercial poultry.

Nowadays, major avian species like chickens, turkeys, and domestic ducks constitute the primary source of avian meat and eggs in both developed and developing countries [144,238]. Besides, several other species of poultry are raised for meat, eggs, feathers, and hides throughout the world, although they are minor contributors to agricultural production. However, in some countries with wet market systems, minor species have been very important contributors to livelihoods. Such minor species include ratites (ostrich [*Struthio camelus*] and emu [*Dromaius novaehollandiae*]), Japanese quail, bobwhite quail (*Colinus virginianus*), ring-necked pheasant, chukar partridge, guinea

fowl (*Numida meleagris*), and pigeons (*Columba livia*) [238]. These species are normally raised in outdoor operations or found in LPM systems [197], fact that highlights their interest from the transmission and biosecurity points of view. Outdoor-raised systems have higher AIV infection rates than intensive industrial poultry [201], and they have been identified as one of the factors contributing to the increase of AIV outbreaks and their impact [7].

As previously mentioned, LPAIVs from free-living aquatic birds have been introduced into domesticated gallinaceous poultry and waterfowl (mostly ducks and geese), resulting in infections through a two-step process: 1) exposure to an infected host; and 2) adaptation to the new host [239] (Figure 4). Several routes of exposure of waterfowl viruses to poultry have been documented [229]:

- Direct exposure to wild birds. It is the most likely method in outdoor-raised poultry [80,251].
- LPM system. This marketing system offers a variety of live bird species (including domestic ducks and a number of gallinaceous species like quails, partridges, and pheasants) for consumption, providing an ideal environment to introduce and maintain AIVs in the poultry population [115,230].
- Birds' drinking water. If the drinking water is not properly purified, AIVs from wild birds can be introduced to the poultry flock. The use of untreated AIV-contaminated surface water from ponds or lakes occupied by AIV-infected wild aquatic birds could introduce AIVs to the poultry flock [86,204]. This could be mitigated by treating the water or by using untreated water from deep wells [238].
- Exposure to pigs infected with the swine influenza virus, which has been demonstrated, so far, in turkeys [84,233].

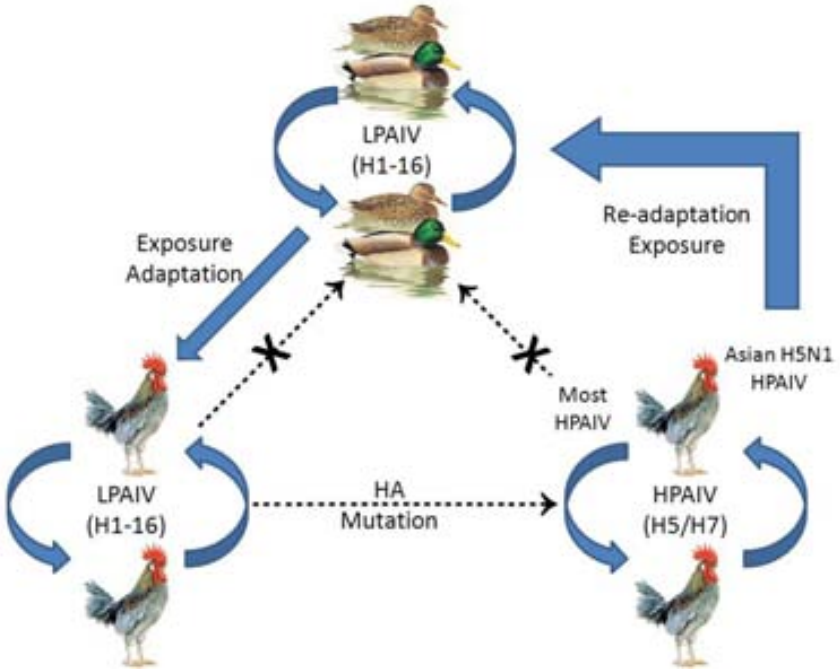


Figure 4. Epidemiology of LPAIVs and HPAIVs between free-living aquatic birds and poultry (Illustration modified from [238]).

Adaptation of the LPAIV to the new host is essential for its maintenance to the new population. If so happens, LPAIVs will lose their potential to transmit back into a free-living aquatic bird population, preventing them to have any role in secondary dissemination or farm-to-farm spread [82]. When circulating in gallinaceous poultry, H5 and H7 LPAIVs can mutate and become HPAIVs [170], and as already adapted to gallinaceous poultry, these new HPAIVs have typically not gone back into free-living bird or domestic duck HPAIVs [239]. Nonetheless, such ecological situation may be different with the Asian H5N1 strain. Indeed, infection and mortality have been reported in captive and free-living aquatic birds following the poultry outbreaks, suggesting that the H5N1 HPAIV readapted back to some free-living and domestic aquatic bird species [175,239].

1.1.4. PATHOBIOLOGY

Influenza A virus infections can be divided into two distinct groups based on their ability to cause disease in chickens [248]. LPAIVs can be asymptomatic, but they typically cause mild to severe respiratory disease, often accompanied by a decrease of water or feed consumption, and drops in egg production. In contrast, HPAIVs cause severe systemic disease with very high mortality in gallinaceous poultry. Particularly, Eurasian H5N1 HPAIV have been shown to own a unique pathobiology [248] (Table 3).

Clinical signs are extremely variable depending on host species, virus strain, age, sex, concurrent infections, acquired immunity, and environmental factors. Besides, the official pathotype classification (LPAI and HPAI) is only specific for the chickens, even if it has pathobiological application to related Galliformes species [156]. However, it is not predictive of AIV pathogenicity in different orders of birds, other mammals or humans [238].

Table 3. Summary data obtained from the intranasal inoculation of multiple avian species with early Eurasian-African H5N1 lineage HPAIV (A/chicken/Hong Kong/220/97) [164,174].

Species	Pathobiol. group	Morbidity[a]	Mortality[a]	Gross lesions[b]	Histological lesions[b]	Viral antigen[c]	Virus reisolation[d]
Chickens, turkeys, quails, guineafowls, pheasants, partridges, z. finches	1	+++	+++	+++	+++	+++	+++
Domestic geese, emus, h. finches, budgerigars	2	++/+++	–/++	–/+	+/++	+/+++	++
Domestic ducks, h. sparrows, gulls	3	–/+	–	–	–/+	–/+	+/–
Starlings, pigeons	4	–	–	–	–	–	+/–

Pathobiol. group, pathobiology group.
[a]Morbidity and mortality: +++ = ≥ 75%; ++ = 50 to 74%; + = less than 50%; – = none.
[b]Gross and histological lesions: +++ = common and in multiple organs; ++ = sporadic and in few organs; + = infrequent; ± = rare and mild; – = no lesions observed.
[c]Viral antigen: +++ = widespread; ++ = multifocal; + = infrequent; ± = rare; – = no viral antigen.
[d]Virus reisolation: +++ = high viral titers (≥$10^{5.0}$ ELD_{50}/g tissue) obtained consistently from all brain, lung, and kidney; ++ = high viral titers (≥$10^{5.0}$ ELD_{50}/g tissue) obtained primarily from brain; + = low to moderate viral titers (≤$10^{4.1}$ ELD_{50}/g tissue) obtained from lung and/or kidney, negative reisolation from brain; ± = virus reisolated at low titers from only lung and/or kidney (≤$10^{1.9}$ ELD_{50}/g tissue); – = virus not reisolated from brain, lung, or kidney.

1.1.4.1. Pathogenesis

Gallinaceous species

The predominant initial site of **LPAIV** replication in Galliformes is the nasal cavity (and to a much lesser extent, the intestinal epithelium), with the resulting release of virions and infection of other cells in the respiratory and intestinal tracts. Illness or death is most often from respiratory damage, especially if accompanied by secondary infections [239,245].

In contrast, **HPAIVs** in gallinaceous poultry have initial replication in nasal epithelium, with visualization of AI viral antigens in respiratory epithelium by 16 hours post-inoculation (hpi) [239]. By 24 hpi, the nasal epithelium is ulcerated and inflamed with virus in submucosal macrophages, heterophils, and capillary endothelial cells [239,245]. Macrophages and heterophils play a key role in this initial replication and dissemination of HPAIVs [248]. The virus replicates within endothelial cells and spreads via the vascular or lymphatic systems to infect and replicate in a variety of cell types within visceral organs, brain, and skin [248]. Such initial visceral replication may be seen as early as 24 hpi, and by 48 hpi high viral loads and severe lesions are present [248]. However, with some HPAIVs, the viremia may occur without extensive replication in vascular endothelial cells and with more extensive replication in parenchymal cells of visceral organs [248]. Clinical signs and death are due to multiorgan failure. Damage caused by AIV is the result of one of the three processes: 1) direct virus replication in cells (which cause either necrosis or apoptosis), tissues, and organs; 2) indirect effects from production of cellular mediators, such as cytokines; and 3) ischemia from vascular thrombosis [245].

Non-gallinaceous species

Pathogenesis of the infection process is less well-understood in non-gallinaceous birds, and particularly that of **LPAIV** among waterbird species has only been studied in domestic duck [245]. Ingestion of contaminated water is considered the main route of

LPAIV infection in waterbirds [270]. Therefore, LPAIVs are believed to primarily replicate in epithelial cells of the intestinal tract and the bursa of Fabricius, resulting in high amounts of virus in feces [85,274], and to a lesser extent in epithelial cells of the respiratory tract [106,205,274]. Hence, LPAIVs are typically restricted to the digestive tract and to a lesser extent the respiratory tract, thus do not involve other organs. If ingested, LPAIVs must pass through and survive the acidic environment of the proventriculus of ducks so as to reach their intestinal target site of replication. This may be partially accomplished because uncleaved HA of LPAIVs appears more resistant to acidic pH than that of mammalian influenza viruses [11].

The infection route of H5N1 **HPAIV** in aquatic birds may be inhalation of respiratory secretions or ingestion of water contaminated by respiratory secretions and carcasses [185]. The first site of H5N1 HPAIV replication appears to be the respiratory epithelium. In contrast to LPAIV infection, intestinal epithelial cells are rarely reported to be infected by H5N1 HPAIV possibly due to its inability to survive the acid barrier of the proventriculus [270]. The 1997-2001 H5N1 HPAIV infections in Pekin ducks resulted in either no or limited virus replication and few clinical signs [106,205,274]. In contrast, post-2001 Eurasian-African lineage H5N1 HPAIVs are able to replicate in the respiratory tract of domestic ducks and later systemically, with a pantropic potential akin to that described in chickens [59,93,107,126,163,165,227,228]. In contrast to H5N1 HPAIV infection in chickens, such virus rarely infects endothelial cells in most wild bird species [165]. The only exceptions are mute (*Cygnus olor*) and whooper (*C. cygnus*) swans [100,254] and tufted ducks (*Aythya fuligula*) [24], in which H5N1 HPAIV shows a clear endotheliotropism.

1.1.4.2. Clinical Signs

Gallinaceous species

Typically, **LPAIVs** in gallinaceous poultry produce high morbidity (>50%) and low mortality rates (<5%), although the latter can be greater if accompanied by secondary

pathogens or if the disease occurs in young birds. In gallinaceous poultry, clinical signs reflect pathophysiological changes in the respiratory, digestive, urinary, and reproductive systems [245]. In this way, LPAIV infections frequently present mild to severe respiratory signs such as coughing, sneezing, rales, rattles, and excessive lacrimation. In mature layers and breeders, increased broodiness and decreased egg production may be present. General clinical signs like huddling, ruffled feathers, listlessness, decreased activity, decreased feed and water consumption, and occasionally diarrhea may be present [248].

With **HPAIV** in chickens, the incubation period ranges from a few hours (intravenous inoculation) to 24 hours (intranasal inoculation) to 3 days (natural infection) [238]. HPAIV infections in chickens cause high morbidity and mortality, being able to affect 100% of the flock [239]. Perkins and Swayne [176] demonstrated that the same H5N1 HPAIV strain caused high mortality in all of the gallinaceous species tested, including chickens, turkeys, quail, and pheasants, although differences in mean death time (MDT) were observed among species.

The clinical presentation mainly depends on the HPAIV strain and species of bird affected, and reflects the extent of HPAIV replication and damage to major organ systems. In the peracute stage, which is observed in the first hpi, birds are found dead without apparent clinical signs. Birds that survive for 3 to 7 days post-inoculation (dpi) experience a second or acute stage, and may exhibit severe neurological signs such as tremors of head and neck, incoordination, inability to stand, loss of balance and recumbency with pedaling movements, paresis, paralysis of the legs and wings, shaking of the head with abnormal gait, torticollis, opisthotonus, nystagmus, excitation, convulsions, rolling and circling movements, flapping movements of the wings, and unusual position of the head and wings [248]. Presence of neurological signs will vary with the species of bird, ranging from 8% in Japanese quail to 28% in chukar partridges or 41% in turkeys infected with a Eurasian H5N1 HPAIV [176]. Besides, respiratory signs such as rales, sneezing, coughing, and excessive lacrimation can be observed in this

acute phase, as well as diarrhea, and bile and urate loose droppings intermixed with mucus [248].

Non-gallinaceous species

In wild birds, especially waterfowl, neither **LPAIVs** nor **HPAIVs** normally produce morbidity or mortality [164]. However, since 2002, H5N1 HPAIV infections began to show wide variations in lesions and lethality [59,93,107,126,163,165,227,228]. In particular, respiratory and neurological fatal diseases have repeatedly been observed [29,103,107,165]. Regarding birds of prey, experimental infections with H5N1 HPAIV induced nonspecific and neurologic clinical signs associated with high mortality rates and late MDTs as compared to chickens [77,130].

Infectious period, incubation time, and clinical signs in minor gallinaceous and other wild species are summarized in Table 4 (LPAIV infections) and Table 5 (HPAIV infections).

1.1.4.3. Gross and Microscopic Lesions

Gallinaceous species

In **LPAIV** infected poultry, the infraorbital sinuses may be swollen with accompanying mucoid to mucopurulent nasal discharge. The most frequent lesions are rhinitis and sinusitis, which can vary between catarrhal, fibrinous, mucopurulent, and fibrinopurulent (the latter often accompanied by secondary bacterial infections). The trachea may show edema, congestion, occasionally hemorrhages, and presence of serous to caseous exudates in the lumen [248]. When associated with bacterial infection, fibrinopurulent bronchopneumonia could be present. Catarrhal to fibrinopurulent air sacculitis or celomitis could be found. Lesions in the reproductive tract in adult birds include regression of the ovaries, with presence of hemorrhages in the large follicles progressing to colliquation and egg yolk peritonitis. Besides, catarrhal

to fibrinous salpingitis may be observed before involution of the oviduct. The eggs are fragile, misshapened, and have loss of pigment [248].

Regarding microscopic lesions, LPAIVs often produce heterophilic to lymphocytic tracheitis, bronchitis and ventromedial fibrinocellular to peribronchiolar lymphocytic pneumonia [248]. Rarely, pancreatic acinar necrosis is observed, as reported in turkeys infected with an H7N1 LPAIV in Italy during the 1999 outbreak [37,150]. Birds that die from LPAI have lymphocyte depletion and necrosis of lymphocytes in the bursa of Fabricius, thymus, spleen, and lymphoid associated tissues [248]. In intranasally inoculated birds, viral antigen is commonly detected in upper respiratory epithelial cells [247], whereas intravenous inoculation demonstrates viral antigen in renal tubule epithelium, pancreatic acinar epithelium, intestinal epithelium, and rarely in lymphocytes [248].

Lesions observed in **HPAIV** infected gallinaceous birds reflect the clinical presentation of the disease. Consequently, birds that die in the peracute phase may not show gross lesions [248]. On the contrary, birds in the acute stage generally show ruffled feathers, sinusitis, edema of the head, face, upper neck, and legs, which may be associated with petechial to ecchymotic haemorrhages in the unfeathered skin of the comb, wattles and legs [245]. The presence of cyanosis, petechial to ecchymotic haemorrhages and necrotic foci in the comb and wattles is considered syndromic of a HPAIV infection. Lesions in internal organs vary with the strain, but generally include hemorrhages in the coronary fat and epicardium, pectoral muscle, and on the serosa and mucosa of the proventriculus and ventriculus. More rarely, hemorrhages are found on the inner surface of the sternum, cecal tonsils, and Meckel's diverticulum [245]. The pancreas may have multifocal areas of necrosis [99,241]. Necrotic foci have been frequently reported in the heart, kidney, and liver of chickens, pheasants, chukar partridges, and turkeys [176]. Urate deposition in the kidneys has been observed alongside kidney lesions. The lungs may show congestion and hemorrhage [99,176,239]. In young birds, the thymus and the bursa of Fabricius may be atrophic and hemorrhagic

[99]. Additionally, the spleen may become enlarged, showing parenchymal pale necrotic foci [248].

Microscopic lesions in gallinaceous poultry are generally more consistent than are gross lesions in HPAI cases. Histopathological findings vary with individual virus strains, breed of the chicken, inoculum dose, route of inoculation, and passage history of the virus. Typically, histological lesions consist of necrosis and/or inflammatory changes in multiple organs, most consistently and severely in skin, brain, heart, pancreas, adrenal glands, lungs, and primary and secondary lymphoid organs [176,231,239,245,248]. In agreement with the clinical presentation and gross lesions, birds in the peracute stage may only show mild and multifocal presence of viral antigen in vascular endothelial cells and cardiac myocytes by immunohistochemistry (IHC) without microscopic lesions. On the contrary, in the acute phase lesions in multiple organs appear, predominantly necrosis and, to a lesser extent, apoptotic cell death with associated inflammation, hemorrhage, and edema, together with presence of antigen by IHC. The longer the birds survive, the less prominent are the necrosis and apoptosis, and the more prominent is the inflammation [248]. The HPAIVs, especially the Eurasian H5N1 HPAIVs, are generally defined as neurotropic [99,176,248] and are thought to reach the central nervous system via the hematogenous route [42]. Common lesions in the brain are lymphocytic meningoencephalitis with neuronal necrosis, neuronophagia, and focal gliosis, occasionally with edema and hemorrhage [248].

Non-gallinaceous species

LPAIV infection in wild birds generally does not cause gross or microscopic lesions. However, mild epithelial degeneration of the intestinal and bursal mucosa was observed microscopically in experimentally infected domestic ducks [106,205,274].

In contrast to LPAIV, H5N1 **HPAIV** infected waterfowl may show severe necrosis and inflammation in multiple organs [29,100]. Surprisingly, this is not often reflected grossly. H5N1 **HPAIV** infected wild birds may show multifocal pulmonary congestion and

consolidation with mild air sacculitis as most striking gross lesions [29,103]. Mild to moderate multifocal pancreatic necrosis can also be observed [100,103]. As for Galliformes, virus localization in the nasal cavity, pancreas, brain, adrenal glands, and myocardium has been reported in wild birds, including birds of prey, with similar histological findings [103,165]. However, there is no evidence of vascular damage, neither viral antigen on endothelial cells in wild birds [165], except in mute and whooper swans [100,254] and tufted ducks [24], where multi-organ hemorrhages can be observed.

Gross pathology, histological lesions, and distribution of AIV antigen in minor gallinaceous and other wild species following HPAIV infections are summarized in Table 6.

Table 4. Infectious period, incubation time, and clinical signs following natural or experimental LPAIV infections in minor gallinaceous and other wild species.

Species	AI virus	Type of infection	Oral shedding (duration; N/total)*	Cloacal shedding (duration; N/total)*	Clinical signs (duration; N/total or %)†	Mean death time (N/total or %)†	Transmission confirmation	References
Ring-necked pheasant (*Phasianus colchicus*)	LPAIV (various subtypes)	Experimental	5.5 (≤10 days; 0-3/3) 5.5 (≥14 days; 0-3/3)	5.5 (≤10 days; 0-3/3) 4.5 (≥14 days; 0-3/3)	None	None	Yes	[94]
Chukar partridge (*Alectoris chukar*)	LPAIV (various subtypes)	Experimental	6.25 (≤10 days; 0-3/3)	3.5 (≤10 days; 0-3/3)	None	None	Yes	[94]
Japanese quail (*Coturnix c. japonica*)	LPAIV H9N2	Experimental	ND (3-11 days; 2-10/10)	ND	Ruffled feathers, excess mucus, diarrhea, cyanosis of the tongue, egg dropping (10/10)	None	ND	[120]
J. quail	LPAIV (various subtypes)	Experimental	6.8 (3-5 days; 3/3)	3.8 (3-5 days; 1/3)	Nonspecific signs (H10N4)	ND (2/6 of H10N4)	No	[136]
J. quail	LPAIV swine H3N2	Experimental	2.0 (1-3 days; 2/3)	None	None	None	No	[173]
J. quail	LPAIV H9N2 reassortant avian/swine	Experimental	4.0 (1-7 days; 3/3)	None	None	None	Yes	[173]
J. quail	LPAIV H9N2 and H9N6	Experimental	6.0 (1-7 days; 3/3)	Occasionally	None	None	Yes	[172]
Pearl guineafowl (*Numida meleagris*)	LPAIV H7N1	Natural	ND	ND	Conjunctivitis, respiratory signs, neurologic signs	ND (30%)	ND	[150]

ND: not determined.

*Maximum oral and cloacal shedding titers are expressed as $\log_{10}$ of mean tissue culture infectious dose ($TCID_{50}$) or EID_{50}. N/total is the number of infected birds shedding virus over the total number of infected birds.

† N/total or percentage of infected birds exhibiting clinical signs or dying.

Table 5. Infectious period, incubation time, and clinical signs following natural or experimental HPAIV infections in minor gallinaceous and other wild species.

Species	AI virus	Type of infection	Oral shedding (duration; N/total)*	Cloacal shedding (duration; N/total)*	Clinical signs (duration; N/total or %)*	Mean death time (N/total or %)*	Transmission confirmation	References
Japanese quail (*Coturnix c. japonica*)	HPAIV H5N1	Experimental	ND	ND	Depression (100%), increased fecal fluids (1 day, 100%), neurologic signs (2 days; 8%)	2 days (100%)	ND	[176]
J. quail	HPAIV H5N1	Experimental	5.0 (2-6 days)	2.5 (2-6 days)	Depression (100%)	4.8 days (100%)	Yes	[98]
J. quail	HPAIV H5N1	Experimental	2.9 (1-4 days; 3/5)	None	Sudden death (100%)	1-3.4 days (100%)	ND	[192]
J. quail	HPAIV H7N1	Natural	ND	ND	Anorexia, depression	ND (100%)	ND	[150]
Bobwhite quail (*Colinus virginianus*)	HPAIV H5N1	Experimental	ND	ND	Depression (100%), increased fecal fluids (1 day, 100%), neurologic signs (3 days; 14%)	2.25 days (100%)	ND	[176]
Pearl guineafowl (*Numida meleagris*)	HPAIV H5N1	Experimental	ND	ND	Depression (100%), increased fecal fluids (1 day, 100%), neurologic signs	2.5 days (100%)	ND	[176]
P. guineafowl	HPAIV H7N1	Natural	ND	ND	Anorexia, depression, neurologic signs	ND (100%)	ND	[150]
Ring-necked pheasant (*Phasianus colchicus*)	HPAIV H5N1	Experimental	ND	ND	Depression (100%), increased fecal fluids (1 day, 100%), neurologic signs (3 days; 13%)	3.25 days (100%)	ND	[176]
R. pheasant	HPAIV H7N1	Natural	ND	ND	Anorexia, depression, neurologic signs	ND (100%)	ND	[150]
Chukar partridge (*Alectoris chukar*)	HPAIV H5N1	Experimental	ND	ND	Depression (100%), increased fecal fluids (1 day, 100%), neurologic signs (4 days; 28%)	4.5 days (75%)	ND	[176]
Zebra finch (*Taeniopygia guttata*)	HPAIV H5N1	Experimental	ND	ND	Depression, neurologic signs (3-5 days; 100%)	4 days (100%)	ND	[176]
House finch (*Carpodacus mexicanus*)	HPAIV H5N1	Experimental	ND	ND	Neurologic signs (4-13 days; 7/9)	9.5 days (4/7)	ND	[174]
Budgerigar (*Melopsittacus undulates*)	HPAIV H5N1	Experimental	ND	ND	Neurologic signs (5-9 days; 7/8)	7 days (6/8)	ND	[174]
Emu (*Dramaius novaehollandiae*)	HPAIV H5N1	Experimental	ND	ND	Neurologic signs (8-14 days; 50%)	None	ND	[174]
Ostrich (*Struthio camelus*)	HPAIV H7N1	Natural	ND	ND	Anorexia, depression, swollen throat and neck, increased fecal fluids, neurologic signs	ND (30%)	ND	[150]
European starling (*Sturnus vulgaris*)	HPAIV H5N1	Experimental	ND	ND	None	None	ND	[174]

House sparrow (*Passer domesticus*)	HPAIV H5N1	Experimental	ND	ND	Mild clinical signs (4-7 days; 3/7)	None	ND	[174]
H. sparrow	HPAIV H5N1	Experimental	5.0 (4-9 days; 13/15)	4.3 (4-9 days; 13/15)	Lethargy, ruffled feathers, neurologic signs, sudden death (4-7 days; rare)	6.5 days (13/15)	ND	[28]
Rock pigeon (*Columba livia*)	HPAIV H5N1	Experimental	ND	ND	None	None	ND	[174]
R. pigeon	HPAIV H5N1	Experimental	<1.90 (1-2 days; 3/15)	<1.90 (1-2 days; 3/15)	Lethargy, neurologic signs, sudden death (5-6 days; 3/15)	7.5 days (2/15)	ND	[28]
Gyr-saker hybrid falcon (*Falco rusticolus* x *F. cherrug*)	HPAIV H5N1	Experimental	5.4 (1-4; 5/5)	3.0 (2-4; 5/5)	Anorexia, bloody tracheal exudate (1-5 days; 4/5)	4 days (5/5)	ND	[130]
American kestrel (*Falco sparverius*)	HPAIV H5N1	Experimental	19.7 Ct[§] (1-7; 16/16)	21.9 Ct[§] (2-6; 16/16)	Ruffled feathers, anorexia, neurologic signs (3-7; 16/16)	4.5 days (16/16)	ND	[77]

ND: not determined.

*Maximum oral and cloacal shedding titers are expressed as $\log_{10}$ of $TCID_{50}$ or EID_{50}. N/total is the number of infected birds shedding virus over the total number of infected birds.

[†] N/total or percentage of infected birds exhibiting clinical signs or dying.

[§] Results are expressed as cycle threshold (Ct).

Table 6. Gross pathology, histological lesions, and distribution of AIV antigen in minor gallinaceous and other wild species following natural or experimental HPAIV infections.

Species	AI virus	Type of infection	Gross pathology	Histological lesions	Distribution of AI virus antigen	References
Japanese quail (*Coturnix c. japonica*)	HPAIV H5N1	Experimental	Pulmonary edema and congestion Splenomegaly Renomegaly Hemorrhages in enteric lymphoid areas Hemorrhages on epicardium	Nasal epithelial necrosis, rhinitis, sinusitis Interstitial pneumonia Enteritis, enteric lymphoid depletion Enteric epithelial necrosis Pancreatic necrosis Adrenal necrosis Bone marrow cellular depletion and necrosis Spleen, bursa, thymus lymphoid depletion Myocyte necrosis Myocarditis Neuronal necrosis, gliosis	Nasal, sinus epithelium Pulmonary endothelial cells Pancreatic acinar epithelium Splenic phagocytes, endothelium Corticotrophic and cortical cells Myeloid cells Bursal phagocytic leukocytes Thymic epithelium, phagocytes Cardiomyocytes Brain neurons, glial cells, ependymal and choroid epithelium Epithelial cells of the feather follicles Thecal cells	[176]
J. quail	HPAIV H5N1	Natural	ND	ND	Epithelium and muscular layer of the trachea Pulmonary endothelial cells of the septum Mucosal epithelium of the rectum Endothelial cell lining of the spleen Renal tubular epithelium Cardiomyocytes Oviduct epithelium	[8]
J. quail	HPAIV H7N1	Natural	Pancreatic necrosis	Pancreatic necrosis	Pancreatic acinar epithelium Brain neurons	[150]
Bobwhite quail (*Colinus virginianus*)	HPAIV H5N1	Experimental	Splenomegaly Renomegaly Pulmonary edema and congestion Hemorrhages in cecal tonsil Hemorrhages on epicardium Pancreatic necrosis	Nasal epithelial necrosis Rhinitis, sinusitis Interstitial pneumonia Enteritis, enteric lymphoid depletion Enteric epithelial necrosis Pancreatic necrosis Adrenal necrosis Bone marrow cellular depletion and necrosis Spleen, bursa, thymus lymphoid depletion	Nasal, sinus epithelium Pulmonary endothelial cells Pancreatic acinar epithelium Splenic phagocytes, endothelium Corticotrophic and cortical cells Myeloid cells Bursal phagocytic leukocytes Thymic epithelium, phagocytes Cardiomyocytes Brain neurons, glial cells, ependymal and choroid epithelium	[176]

				Myocyte necrosis Neuronal necrosis, gliosis	Epithelial cells of the feather follicles Thecal cells	
Pearl guineafowl (*Numida meleagris*)	HPAIV H5N1	Experimental	Splenomegaly Renomegaly Pulmonary edema and congestion Hemorrhages in lymphoid areas of intestinal tract Hemorrhage in bursa Hemorrhage in submucosal lymphoid tissue of the palate Hemorrhage in skeletal muscle Hemorrhages on epicardium	Nasal epithelial necrosis Rhinitis, sinusitis Interstitial pneumonia Enteritis, enteric lymphoid depletion Enteric epithelial necrosis Pancreatic necrosis Adrenal necrosis Bone marrow cellular depletion and necrosis Spleen, bursa, thymus lymphoid depletion Myocyte necrosis Myocarditis Neuronal necrosis, gliosis Subcutaneous edema	Nasal, sinus epithelium Pulmonary endothelial cells Pancreatic acinar epithelium Splenic phagocytes, endothelium Corticotrophic and cortical cells Myeloid cells Bursal phagocytic leukocytes Thymic epithelium, phagocytes Cardiomyocytes Brain neurons, glial cells, ependymal and choroid epithelium Epithelial cells of the feather follicles Thecal cells	[176]
Ring-necked pheasant (*Phasianus colchicus*)	HPAIV H5N1	Experimental	Splenomegaly Renomegaly Pulmonary edema and congestion Hemorrhages in cecal tonsil Hemorrhages in skeletal muscle Hemorrhages on the epicardium	Nasal epithelial necrosis Rhinitis, sinusitis Interstitial pneumonia Lymphoid depletion at the enteric tract Enteritis Adrenal necrosis Bone marrow cellular depletion and necrosis Spleen, bursa, thymus lymphoid depletion Myocyte necrosis Myocarditis Neuronal necrosis, gliosis	Nasal, sinus epithelium Pulmonary endothelial cells Pancreatic acinar epithelium Splenic phagocytes, endothelium Corticotrophic and cortical cells Myeloid cells Bursal phagocytic leukocytes Thymic epithelium, phagocytes Cardiomyocytes Brain neurons, glial cells, ependymal and choroid epithelium Epithelial cells of the feather follicles Thecal cells	[176]
Chukar partridge (*Alectoris chukar*)	HPAIV H5N1	Experimental	Splenomegaly Renomegaly Pulmonary edema and congestion Hemorrhages in cecal tonsil Hemorrhages on epicardium Pancreatic necrosis	Nasal epithelial necrosis, rhinitis, sinusitis Interstitial pneumonia Enteritis, enteric lymphoid depletion Enteric epithelial necrosis Pancreatic necrosis Adrenal necrosis Bone marrow cellular depletion and necrosis Spleen, bursa, thymus lymphoid depletion	Nasal, sinus epithelium Pulmonary endothelial cells Pancreatic acinar epithelium Splenic phagocytes, endothelium Corticotrophic and cortical cells Myeloid cells Bursal phagocytic leukocytes Thymic epithelium, phagocytes Cardiomyocytes Brain neurons, glial cells, ependymal and choroid epithelium	[176]

				Myocyte necrosis Myocarditis Neuronal necrosis, gliosis	Epithelial cells of the feather follicles	
Zebra finch (*Taeniopygia guttata*)	HPAIV H5N1	Experimental	Minimal	Neuronal necrosis, gliosis	Pancreatic acinar epithelium Corticotrophic and cortical cells	[176]
Emu (*Dramaius novaehollandiae*)	HPAIV H5N1	Experimental	Splenomegaly Mottling of the pancreas	Pancreatic necrosis Myocarditis Neuronal necrosis, gliosis	Cardiomyocytes Brain neurons, glial cells	[174]
Ostrich (*Struthio camelus*)	HPAIV H7N1	Natural	Edema of the head and neck Severe hemorrhagic enteritis Enlarged mottled liver Enlarged, hemorrhagic pancreas Renomeglay Splenomegaly Pulmonary and tracheal congestion Hemorrhages on the epicardium	Pancreatic necrosis Splenic necrosis Renal necrosis Hepatic necrosis Neuronal necrosis	Pancreatic acinar epithelium Splenic phagocytes, endothelium Kidney tubular epithelial cells Hepatocytes and Kupffer cells Brain neurons, glial cells	[150]
House finch (*Carpodacus mexicanus*)	HPAIV H5N1	Experimental	Splenomegaly Mottling of the pancreas	Pancreatic necrosis Myocarditis Neuronal necrosis, gliosis	Pancreatic acinar epithelium Cardiomyocytes Brain neurons, glial cells	[174]
Budgerigar (*Melopsittacus undulates*)	HPAIV H5N1	Experimental	Splenomegaly	Myocarditis Neuronal necrosis, gliosis	Brain neurons, glial cells	[174]
European starling (*Sturnus vulgaris*)	HPAIV H5N1	Experimental	None	None	None	[174]
House sparrow (*Passer domesticus*)	HPAIV H5N1	Experimental	Mild splenomegaly Air sacculitis	Lymphohistiocytic myocarditis Testicular degeneration	Cardiomyocytes Sertoli cells, testis	[174]
H. sparrow	HPAIV H5N1	Experimental	Pulmonary edema and congestion Pancreatic necrosis Splenomegaly	Lymphocytic air sacculitis Pancreatic necrosis Necrotizing adrenalitis Lymphocytic and heterophilic nephritis Lymphocytic ganglioneuritis Myocardial necrosis and myocarditis Necrotizing myositis Lymphoplasmacytic and necrotizing encephalitis Lymphocytic orchitis Heterophilic oophoritis	Pancreatic cells Kidney tubular epithelial cells Adrenocortical cells Hepatocytes and Kupffer cells Cardiomyocytes Myocytes Brain neurons, glial cells Thecal epithelial cells, sertoli cells, testis	[28]
Rock pigeon (*Columba livia*)	HPAIV H5N1	Experimental	None	None	None	[174]

R. pigeon	HPAIV H5N1	Experimental	Pancreatic necrosis	Hepatic necrosis Lymphocytic encephalitis and meningoencephalitis	Hepatocytes Brain neurons, glial cells Thecal epithelial cells	[28]
Magpie (*Pica pica sericea*)	HPAIV H5N1	Natural	Pancreatic necrosis Liver enlarged, congested Splenomegaly	Heterophilic pancreatic necrosis Heterophilic infiltrations in spleen, adrenal gland, testicle Malacia, gliosis, mononuclear perivascular cuffing	Pulmonary endothelial cells Small intestinal epithelium Pancreatic acinar epithelium Renal tubular epithelial cells Corticotrophic cells Cardiomyocytes Brain neurons, Purkinje cells, ependymal cells Interstitial cells of testicle	[116]
Gyr-saker hybrid falcon (*Falco rusticolus* x *F. cherrug*)	HPAIV H5N1	Experimental	Pancreatic necrosis Splenic hyperplasia	Heterophilic pancreatic necrosis Heterophilic splenic necrosis Heterophilic renal necrosis Malacia in cerebellum, cerebrum, spinal cord with heterophilic infiltrate	Nasal, tracheal, bronchial epithelium Gastrointestinal tract Pancreatic acinar epithelium Renal tubular epithelial cells Brain and spinal cord neurons	[130]
American kestrel (*Falco sparverius*)	HPAIV H5N1	Experimental	Liver enlarged Splenomegaly Congestion of the serosal surface of the duodenum and jejunum	Pancreatic necrosis Lymphoplasmacytic periportal inflamation and hepatocellular vacuolation with heterophilic infiltrate Meningitis and encephalitis with heterophilic infiltrate	Tracheal epithelium Pancreatic acinar epithelium Renal tubular epithelial cells Kupffer cells Corticotrophic and cortical cells Cardiomyocytes Brain neurons	[77]

ND: not determined.

1.2. DIAGNOSIS, CONTROL, AND PREVENTION

Different aims are meant to be achieved for the control of AI. If a country or region is free from AI, the goal should be the **prevention** of introduction either from a wild bird reservoir or from infected poultry. If the country has infected flocks, the goal should be to improve the **management** of the disease so as to achieve **eradication** [237]. These goals are accomplished by applying five inclusive strategies: 1) education; 2) biosecurity; 3) diagnostics and surveillance; 4) stamping out; and 5) vaccination [251]. Besides, other factors such as the pathogenicity of the virus, species of birds at risk or infected, and financial resources available, among others, further define the control strategies to be implemented [237].

1.2.1. DIAGNOSTIC TESTS

Because pathological lesions of AI are not definitive, several tests have been developed for an accurate diagnose when AIV infection is suspected and for surveillance programs. Such tests are based either on the detection of the virus or the detection of the host immune response. Besides, a series of techniques are used to characterize the virus and study the pathogenesis of the disease [156,214]. Practical considerations regarding diagnosis and surveillance protocols are discussed in more detail in section 1.2.5. (Practical Considerations).

1.2.1.1. Identification of the Agent

When live or dead birds are suggestive of AIV infection, the diagnosis must be confirmed by isolating virus or demonstrating AIV nucleic acid or antigen within infected tissues [219]. The most frequently used tests for the detection of AIV are presented below.

- Virus isolation (VI)

It is the reference standard method to diagnose AI in both poultry [214] and wild birds [219]. However, it is not utilized for routine diagnosis because of being expensive and time-consuming [214]. Embryonating chicken eggs are widely used for VI, although cell cultures, e.g., Madin-Darby canine kidney (MDCK), may also be employed. Virus isolation is considered the most sensitive method for the isolation and diagnosis of poultry-adapted AIVs. However, it is not highly specific since other agents that may be present in a poultry-origin specimen will readily grow in eggs and cell cultures (e.g., avian paramyxoviruses) [214]. For this reason, additional tests may be required to confirm presence of AIV; some of them are described below.

- Molecular techniques

In recent years, molecular methods have become an essential tool for the diagnosis and surveillance of AIV [214,219]. Usually, an initial detection of the viral RNA is accomplished by NP- or M1-specific conserved primers [191]. This may be followed by the detection of H5 or H7 subtype influenza virus using H5- or H7-specific primers [191], like Spackman's widely used assay [213]. Molecular methods have the advantage of speed and increased sensitivity [214], although reduction of the latter may occur in cloacal swabs (CS) due to PCR inhibitors [219]. Additionally, real time RT-PCR (RRT-PCR) enables the quantification of viral RNA amounts, is the most rapid molecular test available, and potential of cross-contamination is reduced [214]. Such type of RT-PCR assay has been successfully used for AI surveillance of wild birds in Europe [64] and is being implemented for HPAIV H5N1 in the United States [219]. Further sequencing of the HA proteolytic cleavage site of the H5 and H7 subtypes is critical for quickly predicting the pathotype of the virus and eventually develop appropriate control strategies [214]. Moreover, sequence analysis allows performing invaluable molecular epidemiological studies [214].

- Immunohistochemistry (IHC) and immunofluorescence (IF) staining

These techniques are based on the demonstration of the presence of influenza A viral antigen in tissue samples using polyclonal and monoclonal antibodies. The reaction is visualized by the deposition of a chromogen (in IHC) or by detecting the presence of fluorochrome (in IF) in infected cells [214]. The IHC directed at the NP-antigen detection is widely used for AI pathogenesis studies by permitting the identification of the replication sites in the infected tissues [76,162,165,175,250]. Moreover, the IHC enables to correlate the presence of the virus with the histopathological findings caused during the infection [162].

1.2.1.2. Serological Tests

Diagnosis of AI can be further confirmed by demonstrating seroconversion in live birds [219]. Furthermore, evidence of previous AIV infection can be obtained by detecting the host immune response specific antibodies. Although serologic testing has potential application for wildlife studies, there are many unknowns related to sensitivity and specificity, the duration of a detectable antibody response, and species differences [219]. Hence, serologic testing of wild birds has not been routinely used for AIV surveillance [219].

- Agar gel immunodiffusion (AGID) test

It is based on the visualization of the immunoprecipitation reaction of NP-AIV antibody and antigen after diffusion in an agar matrix [214]. This assay is used to detect both antibody (using a reference antigen) and antigen (using a reference antibody) such as to confirm results of VI and HA assays. The AGID test has the advantage of being influenza-A specific, but results have proven inconsistent when applied to ducks [206] and are generally not recommended for wild birds [219]. The main disadvantage of the AGID test is the low sensitivity [15].

- Hemagglutination (HI) and neuraminidase (NI) inhibition assays

Viral subtype testing through HI and NI is generally deferred to national or international AIV reference laboratories. The HI can be used as a confirmatory test for the presence of subtype-specific AIV in hemagglutinating egg fluids, to further characterize AIV isolates by identifying the HA subtype or to identify subtype-specific antibodies to AIV in serum, plasma or egg yolk [256]. The HI is normally performed using subtype specific antibodies (polyclonal antisera) against the 16 HA subtypes. Instead, NI assay is frequently used to identify the NA subtype of a newly isolated influenza virus using specific antibodies against the 9 NA subtypes [168]. Both HI and NI are inexpensive and fast, although they require an extensive number of reference reagents [167]. As for AGID, HI results have also proven inconsistent because: 1) antibodies are often not detectable against killed intact virus [106]; 2) nonspecific inhibitors may be present in serum samples; and 3) serum may cause nonspecific agglutination of chicken erythrocytes. Some of these problems can be solved using different methods such as pretreatment of the serum with chicken erythrocytes [245].

- Enzyme-linked immunosorbent assay (ELISA) antibody testing

Antibody detection is a common method of surveillance for detecting prior exposure of poultry flocks to AIV. It is fast and highly sensitive, but it may give false-positive results due to poorer specificity [245]. Several commercial ELISA kits are available for the antibody detection in serum, plasma, and egg yolk from poultry. Especially common is the use of competitive ELISA (C-ELISA) tests, which are directed at NP-antibodies [199] and have been used for wildlife surveillance [52]. Compared to traditional ELISA test, C-ELISA assays have increased sensitivity and are not species specific [219].

1.2.2. BIOSECURITY

Infected domestic animals, rather than wild animals, may represent the most likely source of human infection. Because there are no realistic options for reducing AIV prevalence in wild bird populations, biosecurity highlighted on the wild-domestic animal interface should be the primary defense to restrict AIV exposure [217]. Birds that could potentially act as AI-bridge species between wild and domestic animals may: 1) be highly susceptible of getting infected and of shedding HPAIV; and 2) have high chances of coming into direct or indirect contact with domestic birds [9].

In preventing primary introduction of LPAIVs from wild aquatic birds, poultry should be raised in confinement. Otherwise, if raised in outdoor operations, domestic birds should be confined or separated during specific migration periods of potentially infected wild aquatic birds, or by an outdoor access in specially constructed areas where wild birds are excluded through netting [237,238]. In some countries, the village/rural poultry sector and associated LPM systems have become an important entry point for LPAIVs from wild aquatic birds into agricultural systems and have served as the major reservoir for LPAIV in agricultural systems of many developed countries [84,122,233]. If biosecurity is lax and the poultry density is high, AIV can spread to the commercial industry and rapidly move within the integrated commercial system resulting in epidemics of HPAI or LPAI [237].

1.2.3. SURVEILLANCE

Surveillance is the second critical component for reducing potential domestic animal and public health impacts. Effective surveillance supports efficient disease control through early detection of the disease, definition of risk factors, better assessment of vaccination programs, improved understanding of genetic evolution of the virus, and clearer elucidation of the epidemiology of the disease. Therefore, surveillance activities should focus on specific geographical sampling sites and periods, and targeted to pre-defined species of high risk of exposure [191]. However, a better

understanding of the wild-domestic interface and AIV exposure risk, transmission and pathogenesis of HPAIV in wild birds, and potential wild avian reservoirs is still needed globally [158,217]. For all these reasons, both **active/scanning surveillance** and **passive/diagnostic surveillance** are required.

Since the spread of H5N1 HPAIV in 2005, many surveillance programs have been initiated worldwide [9,148,158]. National [143,145] and international [156,191] guidelines provide technical advice on AI control and surveillance for both domestic and wild birds. It is worth highlighting that such programs include the LPNAIVs, which has increased the use of stamping-out programs in dealing with these two AI subtypes as a means to prevent emergence of HPAIV [237,251].

In Spain, during a 3-year period (2006-2009) monitoring, different AIV subtypes were detected in **wild birds** in the West Mediterranean area (Catalonia, Northeastern Spain), being the most common combinations H4N6 (22.2%) and H1N1 (18.5%) [32]. To date, the only reported case of H5N1 HPAIV in wild birds in Spain is a great crested grebe (*Podiceps cristatus*) found dead in July 2006 [14]. Besides, surveillance programs in **poultry** have demonstrated that Spanish commercial farms are usually free of AIV infections [143]. An exception is the H7N7 HPAIV epizootic occurred in 2009 in a layer farm in Guadalajara (Castilla-la Mancha) [95].

1.2.4. VACCINATION

From 1952 to 1992, most developed countries eradicated HPAI epizootics by traditional stamping-out programs. However, since 1992 with H7N2 and H5N1 HPAIV, many developing countries have not been able to achieve eradication through stamping-out programs alone but in combination with vaccination [245,246,249]. Even so, HPAI is still being reported continuously; so far this year 2013, the disease has been notified in Nepal, Mexico, India, Hong Kong, Cambodia, Buthan, and Bangladesh [157].

The goal of AI vaccination is the production of an immune response that will protect against the disease and prevent of infection. At this time, there is no universal vaccine which will protect against all AIVs [244,245]. In practice, protection is provided against the individual HA subtype(s) included within each vaccine. If a heterologous NA subtype is selected for the vaccine strain (i.e., being different from the outbreak virus), differentiation between infected and vaccinated birds will be possible (DIVA principle) [246]. Protection conferred by the vaccine depends on: 1) challenge virus dose; 2) content of the HA (in the inactivated vaccines) or titer (in the live vaccines); 3) adjuvants, which produces robust and long lasting immunes responses; 4) genetic similarity between the HA of vaccine and field viruses; 5) length of protection, which can be achieved either with high doses of antigen, high titers, or multiple vaccinations; 6) route of administration (parenteral, topical, or *in ovo*); 7) species of bird and number of vaccinations; 8) age of vaccination (the optimal age in most birds is after 2 weeks of age); and 9) field versus laboratory protection [246]. Based on experimental studies and field usage, AI vaccines can be categorized into the following groups [246]:

- Inactivated whole AIV vaccines

  These are the vaccines most widely used in the field, for both chickens [249] and exotic or endangered species [110]. Such vaccines have primarily utilized seed stock of LPAIV from the field outbreaks, such as in Mexico [266]. Occasionally they have used HPAIV, like in the case of Pakistan [151]. With reverse genetics, vaccine strains have been incorporated the HA and NA of recent field AIVs and remaining six gene segments from a high growth influenza A vaccine virus [124,132,258,273]. This type of technology allows to convert HPAIV into LPAIV vaccines by mutating the HA cleavage site. Therefore, the main advantage of inactivated vaccines is their safety, although they may require high antigen quantities and the inclusion of adjuvants to induce protective immunity [246,262,268]. In addition, parenteral administration is required for these vaccines, which can be a limitation from the pragmatic viewpoint.

- Live LPAIV vaccines

Live LPAIVs are used to provide rapid protection against homosubtypic HPAIV. However, these vaccines are not recommended in field conditions because: 1) they may produce important economically losses associated with signs of AI; 2) they can easily spread among birds and farms; 3) they can potentially revert to a HPAIV; and 4) they can reassort with field AIVs [246].

- Live vectored vaccines

These vaccines can provide some of the immunological advantages of a live virus vaccine but without the reassortment risk of using a live AIV. They are generated by recombinant technologies that incorporate genetic material from the AI genome into a viral backbone for gene expression in vivo. Many vector supports have been studied, but the most frequently reported systems have been the recombinant fowl poxvirus (rFPV) and the recombinant Newcastle disease virus (rNDV) with H5 or H7 AI HA gene inserts [243,244]. These vaccines replicate in the host providing similar immune protection than a live vaccine. However, their use may be limited by other factors, like the immune status of the host (they can induce immunity against the vector itself and interfere with other vaccines) and the host range. In China, both rFPV and rNDV vaccines have been extensively implemented [249].

- DNA vaccines

These vaccines are based on the inoculation of a plasmid DNA, which enhances the production of antigen in the host, resulting in both humoral and cellular immune responses [125]. They are currently economically prohibitive since they require a large amount of nucleic acid per dose to produce a protective immune response, and they entail at least three vaccinations.

- HA subunit vaccines

These vaccines are based on portions of influenza proteins (HA) that are chemically synthesized and formulated into a vaccine to stimulate a protective immune response in the host. The main disadvantage is that the peptide alone stimulates a very weak immune response. However, this can be improved using adjuvants or another method of delivering the peptide to the immune cells.

In Spain, similarly to other developed countries [249], preventive or routine vaccination of **domestic poultry** against AI is prohibited, although emergency vaccination programs previously approved by the European Commission (EC) could be used [143]. Preventive vaccination programs with vaccines licensed for chickens (mainly inactivated) might be useful when targeted to high-value or high-risk **non-poultry** populations, such as zoo birds, hunting or endangered species [249]. In this light, the EC approved preventive vaccination programs against H5N1 HPAIV for birds kept in zoos in 17 Member States to avoid stamping-out measures for captive wild bird species [181]. Additionally to the EU program, vaccination of zoo or captive-held non-poultry birds has been conducted in several other countries [249].

1.2.5. PRACTICAL CONSIDERATIONS

Passive/diagnosis surveillance

Passive surveillance involves the sampling of dead or moribund birds, particularly migratory waterfowl and other species of high exposure risk, and intense efforts are conducted in high risk spots like coastal areas, wetlands, or when domestic poultry is nearby [191]. Wild birds admitted in wildlife rehabilitation centers are also considered. Besides, clinical diagnostic surveillance in farms is also performed [143,145].

LPAIV infection is commonly subclinical; thereby the diagnosis in a **live wild bird** can be performed by virus isolation or RT-PCR [39,149]. Samples of choice might be CS and, to a lesser extent, oropharyngeal swabs (OS). The diagnosis of LPAIV infection in a

dead wild bird can be made by virus isolation, RT-PCR or IHC [39,149]. Additionally to CS and OS, tissue samples from intestine, bursa of Fabricius, and lung are preferred for virus isolation and RT-PCR (fresh tissues), and for IHC (formalin-fixed tissues) [145,185].

Necropsy provides invaluable information for a presumptive diagnosis of **HPAIV** H5N1 infection, especially when gross and microscopic lesions characteristic of AI (previously described in this chapter) are observed. Similarly to LPAIV, virus isolation or RT-PCR can be useful for the diagnosis of HPAIV H5N1 infection in a **live wild bird**, although OS are favored to CS. As for LPAIV, the diagnosis of HPAIV H5N1 in a **dead wild bird** can be made by virus isolation, RT-PCR or IHC [185]. However, OS are preferred to CS, and ideal tissue sampling should include brain, lung, air sac, pancreas, liver, and kidney [145,185].

Active/scanning surveillance

Active surveillance consists in the sampling of a minimum number of specimens per year based on the density of migratory birds [143]. Scanning of **wild bird** populations for both **LPAIV and HPAIV** is most commonly done by RRT-PCR of OS and CS, using a probe for the *M1* gene. Positive samples are subsequently cultured by virus isolation to obtain the virus, identify HA and NA subtype, and determine pathogenicity [145,185].

Active/scanning surveillance activities in **domestic poultry** are based on a representative sampling of the farms census, including all avian species in both intensive and extensive farming. A serological-based diagnosis is performed, and confirmatory virological assays for positive samples are required, being similar to those of wild birds [143,145].

Protocols for AIV diagnosis and surveillance are summarized in Table 7.

Table 7. Testing and sampling protocols for AIV diagnosis and surveillance.

Passive surveillance (Diagnosis)		
	LPAIV	**HPAIV**
Wild birds (live)	VI: CS>OS RT-PCR: CS>OS	VI: OS>CS RT-PCR: OS>CS
Wild birds (dead)	VI: CS>OS, fresh tissues RT-PCR: CS>OS, fresh tissues IHC: formalin-fixed tissues	VI: OS>CS, fresh tissues RT-PCR: OS>CS, fresh tissues IHC: formalin-fixed tissues
Domestic birds	VI: OS, CS, tissues RT-PCR: OS, CS, tissues Serological: serum	
Active surveillance (Scanning)		
Wild birds (live)	RT-PCR: OS, CS VI: OS, CS	
Domestic birds	Serological: serum VI: OS, CS RT-PCR: OS, CS	

Chapter 2

Hypothesis and Objectives

To date, several experimental studies and natural infections have assessed the susceptibility of numerous major and minor avian species to both LPAIVs and HPAIVs. However, scarce information concerning the infection dynamics of AIV in the red-legged partridge (*Alectoris rufa*), European quail (*Coturnix c. coturnix*), and gyr-saker hybrid falcon (*Falco rusticolus* x *F. cherrug*) exist so far. Such non-conventional avian species may be of great interest in some specific geographical regions, including the Iberian Peninsula. These species not only belong to the autochthonous wildlife, but they are also raised for various human benefits, normally within extensive farming systems, which leads to close contact with both humans and local wildlife.

Consequently, the general objective of the present dissertation is to determine the epidemiologic role that the red-legged partridge, the European quail, and the gyr-saker hybrid falcon may play in the AIV infection. In order to address this issue, the susceptibility, pathogenesis, viral dynamics, and the ability of effective transmission among birds have been studied by means of experimental infections with LPAIVs and HPAIVs.

Ethics statement

These studies were carried out in strict accordance with the recommendations of the *Comissió d'Experimentació Animal de la Generalitat de Catalunya*. The protocols were approved by the *Comissió d'Ètica en l'Experimentació Animal i Humana* of the *Universitat Autònoma de Barcelona*. Every effort was made to minimize suffering.

Part II

Studies

CHAPTER 3

STUDY I: PATHOGENESIS AND TRANSMISSIBILITY OF HIGHLY (H7N1) AND LOW (H7N9) PATHOGENIC AVIAN INFLUENZA VIRUS INFECTION IN RED-LEGGED PARTRIDGE (*ALECTORIS RUFA*)

3.1. INTRODUCTION

In recent years, AI has become one of the most important challenges that have emerged from animal reservoirs [34,35]. The current outbreaks detected in poultry and wild birds in many Asian, European, and African countries are of concern not only to the poultry industry, in which they produce an economically devastating disease, but also to public health [7]. The potential of these viruses to cause a pandemic represents a constant threat to poultry, wild birds, and humans worldwide, underlining the importance of avian reservoirs for any subtype of AIV. Wild birds, particularly those belonging to the orders Anseriformes and Charadriiformes, have long been recognized as the natural reservoir for influenza A viruses [3]. However, the epidemiology of AIV is complex, and there are still many unknown aspects especially in relation to the reservoir.

Some studies suggest that turkeys, pheasants, and Japanese quail are more susceptible than chickens to infection by AIV transmitted from free-living aquatic birds [94,173]. Experimental infections have shown that HPAIV can cause specific clinical signs and mortality in the above mentioned species [176], and that pheasants are efficient shedders of LPAIV [94]. Furthermore, open range raising of birds has been identified as one of the factors contributing to the increase of AIV outbreaks and their impact [7]. Nevertheless, to date, most experimental studies on AI are based on either chickens, turkeys or waterfowl species, while investigation into the ability of influenza A viruses to replicate in minor poultry species is scarce [94,98,176]. Therefore numerous aspects of the epidemiology of both LPAIV and HPAIV in free-range raised poultry and game birds still remain unclear.

Surprisingly, there are no studies about the susceptibility to infection and the pathogenicity of AIV in red-legged partridge (*Alectoris rufa*). Such important game bird species is a non-migrant gallinaceous species widely distributed in Southwestern Europe (France, Northwestern Italy, and Iberian Peninsula), where it naturally breeds, and in the South of England, where it was introduced as game species and has become naturalized

[53]. During the last decades, natural populations of this game bird have declined in most of its distribution range [2]. The strategy of many hunting estate managers to overcome the lack of wild partridges has been the release of farm-reared birds. Red-legged partridges are raised in outdoor operations that are abundant in Spain, currently comprising 7.6% of the global avian production system [144]. Moreover, the number of partridges' extensive farms has increased almost 83% during the period 2007-2011, evidencing the importance of this type of production [144]. Although some authors have stated that every year between 3 and 4.5 million of farm reared red-legged partridges are released into the wild [141], considering recent information from hunters, farms, hunting estates, and numbers of captures, the real number of partridges released in Spain could be quite close to 10 million per hunting season [265]. The lack of adequate biosecurity measures in part of the red-legged partridge farms, together with limited sanitary control measures before and after release into the wild, could favor the introduction, adaptation, maintenance, and spread of pathogens including AIV.

In the present study, an experimental infection with both LPAIV and HPAIV was carried out in red-legged partridges in order to determine clinical signs, and gross and microscopic lesions. Viral distribution in tissues and the extent and duration of viral shedding were also evaluated by means of quantitative RRT-PCR (qRRT-PCR) and IHC. In addition, the ability of effective transmission among animals was also assessed.

3.2. MATERIALS AND METHODS

Viruses

For the present study, two strains of AIV were used. An H7N1 HPAIV [(A/Chicken/Italy/5093/1999) (H7N1/HP)] was kindly provided by Dr. Ana Moreno from the *Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna* (IZSLER). An H7N9 LPAIV [(A/*Anas crecca*/Spain/1460/2008) (H7N9/LP)] was obtained from the ongoing surveillance program carried out in Catalonia, Northeast Spain. The amino acid

sequences at the HA cleavage site were PEIPKGSRVRR*GLF for the isolate H7N1/HP and PEIPKGR/GLF for the isolate H7N9/LP, being typical of HPAIV and LPAIV, respectively [245].

Virus stocks were produced in 9-day-old embryonating specific pathogen free (SPF) chicken eggs, by a sixth passage for the H7N1/HP and by a first passage for the H7N9/LP. In both cases, the allantoic fluid was harvested at 48 hpi, aliquoted, and stored at -80ºC until use. Viruses were diluted tenfold in phosphate buffer saline (PBS) for titration in 9-day-old embryonating SPF chicken eggs. The mean egg lethal dose (ELD_{50}) and the EID_{50} for H7N1/HP and H7N9/LP, respectively, were determined using the Reed and Muench method [184]. The H7N1/HP subtype demonstrated an IVPI of 3.0 [37] and showed an amino acid sequence in the cleavage site characteristic of HPAIV [33].

Animals

Red-legged partridges of two months of age were used in this study. Male and female birds were included in approximately equal numbers. The animals were raised in the experimental farm of *Instituto de Investigación en Recursos Cinegéticos* (IREC), where serum samples were collected and tested prior to inoculation to ensure that were seronegative for AIV by a C-ELISA (ID-VET, Montpellier, France) and a specific HI test for the H7 subtype. Upon arrival at the *Centre de Recerca en Sanitat Animal* (CReSA), the birds were housed in the animal biosafety level 3 (ABSL-3) facilities. The partridges were kept one week for acclimation, and then they were randomly assigned to experimental groups and housed separately in negative-pressured isolators with HEPA-filtered air. Food and water were provided *ad libitum* throughout the experiment.

Experimental Design

Fifty-six birds were separated into five groups (Table 8). For each virus, the partridges were subdivided into two experimental groups composed of 12 partridges. Groups A (1A and 2A) were used to evaluate the mortality and transmissibility of the viruses, as well as the virus shedding pattern. Groups B (1B and 2B) were used for

pathological studies. Both groups infected with H7N1/HP (groups 1A and 1B) were inoculated intranasally with 10^6 ELD_{50} of the virus. In group 1A, 4 out of 12 partridges were not infected but placed into the isolator with the inoculated birds one hour after inoculation; these uninfected birds were referred to as contacts. Both groups infected with H7N9/LP (groups 2A and 2B) were inoculated intranasally with 10^6 ELD_{50} of the virus. As in the case of group 1A, in group 2A four contacts were included that were not infected but placed into the isolator with the inoculated birds one hour after inoculation. A fifth group (group 3) of 8 partridges was used as control; these birds were inoculated intranasally with PBS solution.

Table 8. Experimental design of Study I.

Group	Inoculum	Titer	No. animals
1A	H7N1/HP	10^6 ELD_{50}	12 (8+4)*
1B	H7N1/HP	10^6 ELD_{50}	12
2A	H7N9/LP	10^6 EID_{50}	12 (8+4)*
2B	H7N9/LP	10^6 EID_{50}	12
3	PBS	-	8

*In A groups, eight quail were inoculated and four quail were left as contact birds.
H7N1/HP, A/Chicken/Italy/5093/1999; H7N9/LP, A/*Anas crecca*/Spain/1460/2008; ELD_{50}, mean embryo lethal dose; EID_{50}, mean embryo infectious dose; PBS, phosphate buffer saline.

Sampling

All birds were monitored daily for clinical signs and scored following the OIE system [156]: healthy (0), sick (1), severely sick (2), moribund or dead (3). Since this is a subjective clinical assessment, “sick” birds would be the ones showing one of the following signs, and “severely sick” more than one of the following signs: respiratory involvement, depression, diarrhea, cyanosis of the exposed skin or wattles, edema of the face and/or head, nervous signs. During the first 10 dpi, at 12 dpi, and 15 dpi, OS, CS, and feather pulp (FP) samples were obtained from partridges of groups 1A and 2A in order to measure viral shedding. The same samples were collected at 3, 6, 10, and 15 dpi from the control group. Mortality and MDT were calculated from these three groups. At

3, 6, 10, and 15 dpi, three animals of groups 1B and 2B, and two animals of the control group, were euthanized. All euthanized and naturally dead partridges were necropsied to evaluate gross lesions and obtain samples for pathological studies. Blood samples were collected in tubes without anticoagulant at 0, 6, 8, 10, and 15 dpi from those animals ethically euthanized. Samples collected for detection of viral shedding and serum samples were stored at -80ºC until use.

Pathologic Examination and Immunohistochemical Testing

Necropsies and tissue sampling were performed according to a standard protocol [135]. After fixation in 10% neutral buffered formalin and embedding in paraffin, tissue sections were processed routinely for hematoxylin/eosin (HE) staining. The following tissues were examined: esophagus, crop, proventriculus, gizzard, duodenum, jejunum-ileum, cecum/cecal tonsil, colon, rectum, pancreas, liver, kidney, adrenal gland, gonad, nasal turbinates, trachea, lung, heart, breast muscle, skin, bone marrow, spleen, bursa of Fabricius, thymus, brain, spinal cord, and sciatic nerve. In addition, an IHC technique was performed as previously described [76,187]. The primary antibody was a mouse-derived monoclonal commercial antibody against NP of influenza A virus (IgG2a, Hb65, ATCC). As a secondary antibody, a biotinylated goat anti-mouse IgG antibody (GaMb, Dako E0433, Glostrup, Denmark) was used. As positive control, tissues previously demonstrated to be positive against NP of influenza A virus by IHC were used. Negative controls were tissues from sham-inoculated animals and tissues incubated without the primary antibody. The following score was used in order to measure the staining in tissues: no positive cells (–), single positive cells (+), scattered groups of positive cells (++), widespread positivity (+++).

Virus Quantitation by qRRT-PCR

Virus quantitation using one step qRRT-PCR was carried out in OS, CS, and FP samples, which were previously placed in 0.5 ml of Dulbecco's Modified Eagle's Medium (DMEM) with antibiotics. Viral RNA was extracted with QIAamp viral mini kit (Qiagen,

Valencia, CA, USA) and amplified as previously described [213] in Fast7500 equipment (Applied Biosystems, Foster City, CA, USA). A one step qRRT-PCR assay for the detection of a highly conserved region of the *M1* gene was applied to determine the viral RNA titer, detecting genomic vRNA, cRNA, and mRNA. The limit of detection of the technique was 1.46 $\log_{10}$ viral RNA copies/sample.

Serology

A C-ELISA test was carried out to detect antibodies against the NP of influenza A virus using the commercially available kit ID Screen® Influenza A Antibody Competition (IDVET, Montpellier, France), according to the manufacturer's instructions.

3.3. RESULTS

Morbidity and Mortality

Clinical signs were only observed in H7N1/HP-infected partridges, which showed signs from scores 1 to 3. There were no relevant differences in clinical signs between inoculated and contact animals. All H7N1/HP-infected birds showed clinical signs that started at 3 dpi and consisted in depression, apathy, and ruffled feathers. Impaired respiration and diarrhea were observed in some of the animals. At 8 dpi, 3 of the 4 surviving partridges presented severe neurological signs consisting in torticollis, circling, incoordination, leg/wing paralysis, opisthotonus, and head tremors while two birds were recumbent and unresponsive. Mortality started at 4 dpi and lasted until 8 dpi. Intranasal inoculation of H7N1/HP resulted in 100% mortality rate, and MDT was 6.42 dpi. Birds with neurological signs, together with the two other animals that presented prostration, were euthanized for ethical reasons. No mortality or clinical signs were observed in H7N9/LP-infected partridges, and in the controls.

Gross Findings

Lesions associated with AI were observed only in H7N1/HP-infected partridges from 3 dpi onwards. Both H7N1/HP-inoculated and -contact partridges were generally in bad body condition. Thymus atrophy was detected until the end of the experiment (Figure 5a). At 3 dpi, petechial hemorrhages on the fasciae sheaths of the muscles of rear legs were seen in some birds (Figure 5b). Some partridges showed brain congestion from 6 dpi onwards (Figure 5c), and in most cases, hyperemic vessels were detected in almost all organs. Kidney lesions were present from 3 dpi onwards and were characterized by parenchymal pallor and accentuated lobular surface architecture, often accompanied by urate deposits in the urethers (Figure 5d). No lesions were observed in H7N9/LP-infected birds, and birds from the control group.

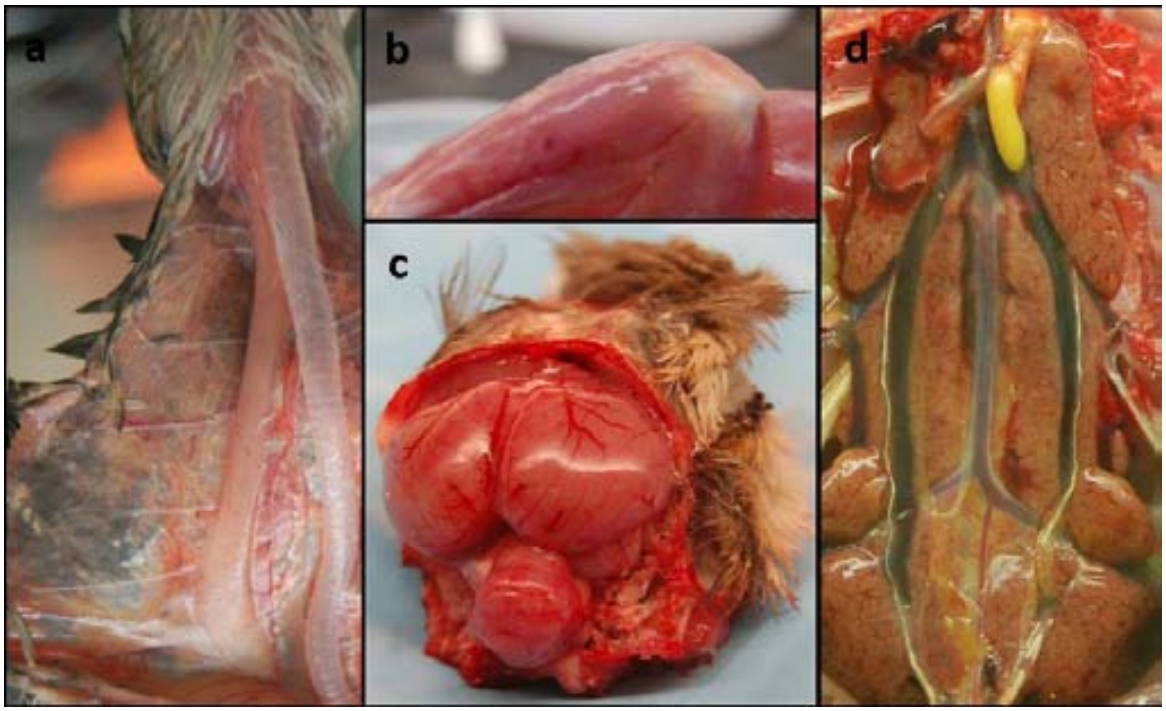


Figure 5. Gross lesions in H7N1/HP-infected red-legged partridges. a. Thymus atrophy, 7 dpi. **b.** Petechial hemorrhages on the fasciae sheaths of the muscles of rear legs, 8 dpi. **c.** Brain congestion, 8 dpi. **d.** Kidney lesions consisting in parenchymal pallor, lobular surface architecture and urate deposits in the urethers, 7 dpi.

Histopathological Findings

Histological lesions were only observed in H7N1/HP-infected partridges. The onset of microscopic findings was at 3 dpi, being the most intense between 6 dpi and 8 dpi. The most severely affected organs were the kidney, adrenal gland, feather follicles, brain, and spinal cord. Only the gonads, spleen, bone marrow, and sciatic nerve did not show significant histopathological changes. No significant lesions were observed in H7N9/LP-inoculated animals, and in the control birds.

Lesions in the digestive tract, liver, pancreas, kidney, adrenal gland, myocardium, breast muscle, bursa of Fabricius, and respiratory tract were mostly characterized by necrosis and mild to moderate heterophilic infiltrates (Figure 6). Necrosis of the epidermal collar epithelial cells, in some cases in association with heterophilic infiltrate, was observed in feather follicles from 6 dpi onwards. In the brain, the most striking finding consisted in multifocal areas of malacia (Figure 6g). Evident necrosis of ependymal cells of the ventricles and epithelial cells of the choroid plexus was present. The cerebellum frequently showed multifocal areas of moderate chromatolysis of Purkinje neurons. Similar lesions were seen in the spinal cord from 6 dpi onwards; multifocal areas of mild spongiosis of the neuropil and neuronal chromatolysis, especially surrounding the medullary canal, were observed. In addition, some animals at 8 dpi showed focal heterophilic inflammatory infiltrate in the leptomeninges. Influenza A viral antigen was only detected in tissues of H7N1/HP-infected partridges. In some organs the virus was more frequently and intensely detected, such as the gizzard, pancreas, kidney, adrenal gland, feather follicles, brain, and spinal cord (Figure 6). Antigenic staining was observed in parenchymal and endothelial cells; it was nuclear and also often cytoplasmic in distribution. In general, positive staining correlated well with histopathological findings (Table 9).

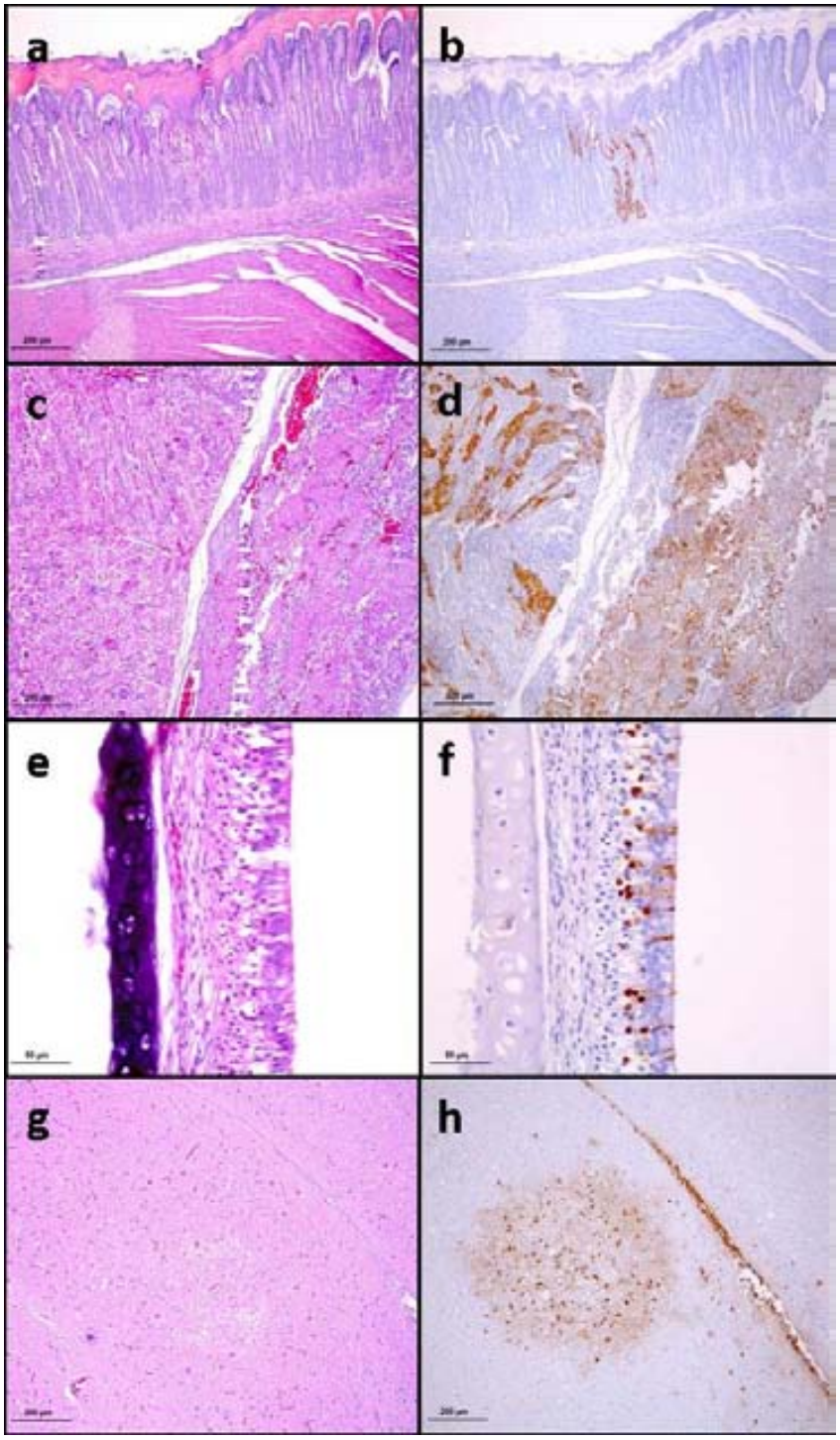


Figure 6. Microscopical lesions and positive NP-viral antigen cells in H7N1/HP-infected red-legged partridges. a. Gizzard, 3 dpi. Focal areas of degeneration and necrosis of the gastric glands (HE). **b.** Gizzard, 3 dpi. Epithelial cells of the gastric glands (IHC). **c.** Kidney and adrenal gland, 5 dpi. Necrosis of the tubular epithelial cells of the renal cortex, multifocal to coalescent areas of necrosis of corticotrophic and corticotropic cells (HE). **d.** Kidney and adrenal gland, 5 dpi. Tubular epithelial cells of the kidney, corticotrophic and corticotropic adrenal cells (IHC). **e.** Nasal turbinates, 6 dpi. Necrosis of single cells of the olfactory epithelium (HE). **f.** Nasal turbinates, 6 dpi. Olfactory epithelial cells (IHC). **g.** Brain, 5 dpi. Focal areas of malacia (HE). **h.** Brain, 5 dpi. Neurons, ependymal cells, and glyal cells (IHC).

Table 9. Average distribution of AIV-NP antigen in positive tissues from red-legged partridges intranasally challenged with H7N1/HP.

Tissue	3 dpi	6 dpi	8 dpi	Predominant cell types
Crop	–	+	–	Squamous polistratified epithelial cells
Proventriculus	–	+	–	Epithelial cells of the gastric glands
Gizzard	+	++	+	Epithelial cells of the gastric glands, cells of the muscularis externa
Cecum/cecal tonsil	–	+	–	Epithelial cells of the glands, cells of the muscularis externa
Pancreas	+	+	+	Acinar cells, endothelial cells
Liver	+	+	+	Kupffer cells, endothelial cells
Kidney	++	+++	++	Tubular epithelial cells, endothelial cells
Adrenal gland	+	+++	+++	Corticotropic and corticotrophic cells
Gonad	–	–	+	Epithelial cells of the oviduct
Nasal turbinates	–	+	+	Olfactory and respiratory epithelial cells, epithelial cells of the infraorbital sinuses, salivary and nasal glands
Trachea	+	–	+	Pseudostratified epithelial cells
Lung	+	+	+	Air capillaries cells, macrophages, endothelial cells
Heart	–	+	+	Myocardyocytes, endothelial cells
Pectoral muscle	–	+	–	Myocytes, endothelial cells
Skin	–	++	+	Epithelial cells of epidermal collar of feather follicles, endothelial cells of the pulp
Spleen	+	+	+	Macrophages, endothelial cells
Bursa of Fabricius	–	+	–	Macrophages, endothelial cells
Brain	–	++	+++	Neurons, ependymal cells, gliall cells
Spinal cord	–	+	++	Neurons, ependymal cells, cells of the leptomeninges

*Tissues not present appeared overtly normal on histopathological analysis and did not show positive IHC staining.
– = no positive cells; + = single positive cells; ++ = scattered groups of positive cells; +++ = widespread positivity.
dpi, days post-inoculation; NSL, no significant lesions.

Viral RNA Quantitation by qRRT-PCR

Quantitative RRT-PCR was performed on OS, CS, and FP samples. In H7N1/HP-inoculated birds, viral RNA was detected in OS from 1 dpi to the end of the experiment, and in CS and FP from 2 dpi to 8 dpi (Figure 7a). Concerning H7N1/HP-contact birds, detection was similar to that observed in inoculated partridges, although it started one day later (Figure 7b). Detection levels for these H7N1/HP-inoculated animals ranged

between 4 and 10 log_{10} viral RNA copies/sample, and FP shedding was higher than in both OS and CS, particularly between 2 and 8 dpi. Six out of 8 H7N9/LP-inoculated birds showed viral shedding mainly by the oropharyngeal route from 1 to 3 dpi (Figure 7c). One H7N9/LP-inoculated animal excreted virus by this route until 8 dpi. Only one animal shed minimal amounts of virus (2.37 log_{10} viral RNA copies/sample) by cloacal route at 1 dpi, and no viral shedding was detected in FP. H7N9/LP-contact animals did not shed virus by any of the studied routes during the whole experiment.

Serology

Serum samples from H7N1/HP-challenged birds tested positive from 6 dpi onwards. Interestingly, 2 out of 4 seropositive partridges at 8 dpi were contact birds. On the contrary, serum samples from 3 out of 8 H7N9/LP-infected partridges were seropositive at 15 dpi. These three animals were the ones that excreted virus in a more consistent manner. None of the four H7N9/LP-contact animals seroconverted, suggesting that these birds did not get infected by contact.

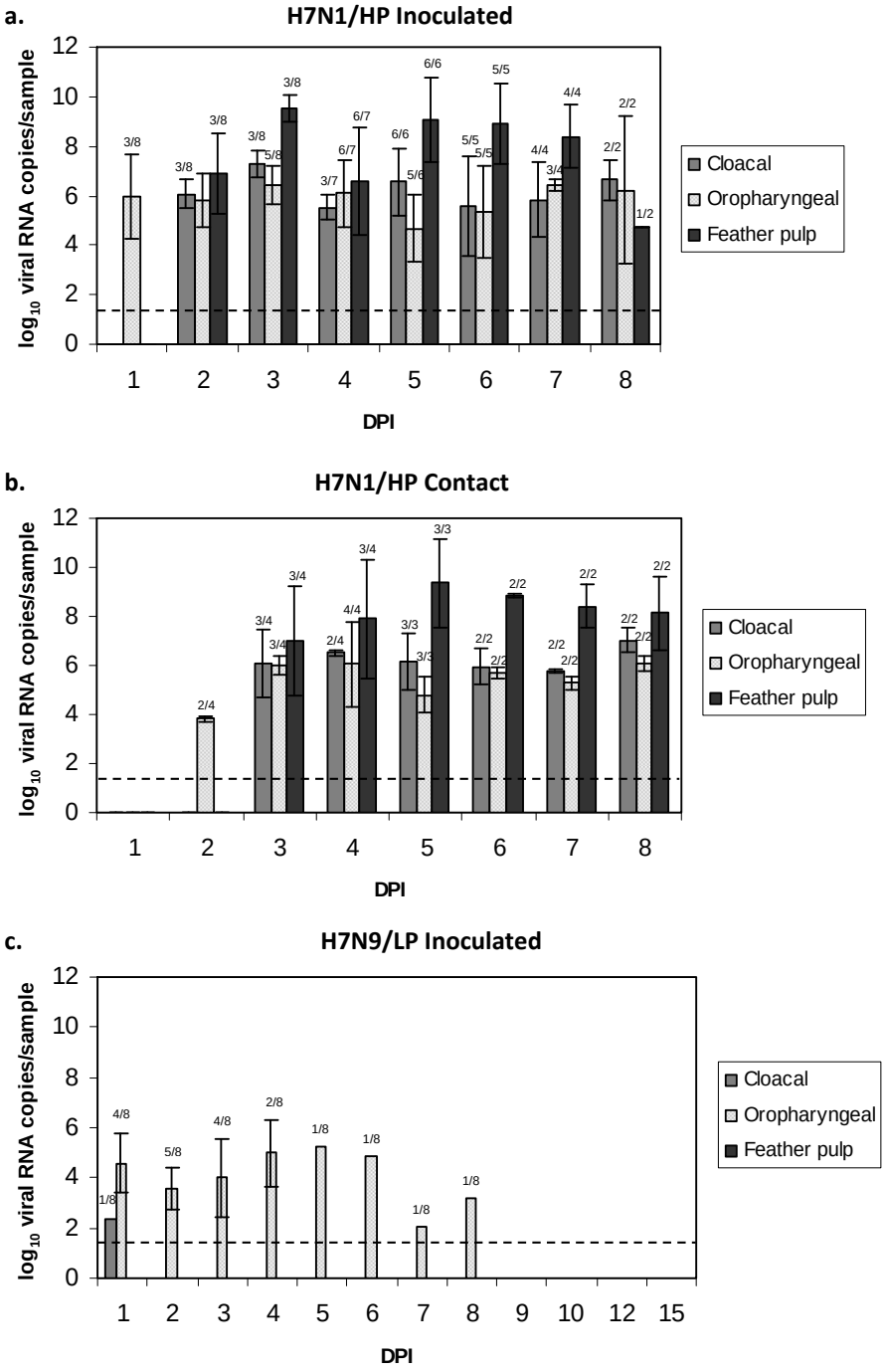


Figure 7. Viral shedding detected by qRRT-PCR in AIV-infected red-legged partridges. Results are expressed as $\log_{10}$ viral RNA copies/sample and shown as means of positive individuals ± SD. Rates above the bars indicate the ratio between positive birds and total birds examined. Limit of detection is indicated by the dashed line (1.46 log10 viral RNA copies/sample). DPI, days post-inoculation; OS, oropharyngeal swabs; CS, cloacal swabs; FP, feather pulps. **a.** H7N1/HP-inoculated partridges. **b.** H7N1/HP-contact partridges. **c.** H7N9/LP-inoculated partridges.

3.4. DISCUSSION

Although the red-legged partridge is one of the game bird species most frequently raised in outdoor operations, no studies had previously investigated AI infection dynamics in this species. In order to elucidate their putative role in the ecology of influenza A viruses, we evaluated the susceptibility of red-legged partridges to an infection with an H7N1 HPAIV (A/chicken/Italy/5093/1999) and an H7N9 LPAIV (A/*Anas crecca*/Spain/1460/2008) by studying pathogenesis, transmission, and viral shedding.

The high pathogenicity of this H7N1/HP strain, evidenced by 100% mortality in this study, is in accordance with standardized IVPI tests for influenza viruses [37], and in agreement with those obtained in natural H7N1 HPAIV infections in chickens [150]. The only experimental infection published so far with HPAIV in partridges used an H5N1 HPAIV strain as inoculum [176]. In this experiment, 75% of mortality rate was observed in chukar partridges and MDT was shorter than in our experiment (4.5 dpi). Therefore, mortality due to infection with H7N1 HPAIV in red-legged partridges seems to appear slightly later than in the H5N1 HPAIV-infected chicken and chukars [176]. Such delay in the onset of mortality could be due to the unique virulence of the H5N1 HPAIV [215]. Clinically, progressive neurologic dysfunction, the most pronounced sign in surviving birds, correlated with the observations of Perkins and Swayne [176] in chukar partridges. Gross lesions were observed in tissues that are known to be target organs for influenza A viruses in other gallinaceous species [98,176] such as the kidney or fasciae sheaths of the muscles. The general predilection of the virus for epithelia of the upper digestive, respiratory and urinary tract, pancreas, liver, feather follicles, brain, and spinal cord has been extensively described in chickens infected with other HPAIV subtypes [25,176,241]. Localization of H7N1/HP antigen in the parenchyma of other organs such as the lower digestive tract, bursa of Fabricius, and skeletal muscle was less consistent and more focalized, supporting the opinion that virus distribution in the host organism is dependent on particular host factors [176].

The onset of clinical signs in H7N1/HP-infected birds, both intranasally inoculated and contact animals, proved effective transmission of the virus from infected partridges to naïve contact birds. Moreover, not only H7N1/HP-inoculated birds seroconverted but also H7N1/HP-contact birds. Surprisingly, at 1 dpi all H7N1/HP-inoculated animals only showed oropharyngeal shedding, suggesting that contact birds had been infected by virus shed from the oral cavity of the inoculated animals. Such finding could indicate a shift from the classical fecal-oral route to the oral-oral route (possibly through shared drinking water) in H7N1 HPAIV infection, as some authors have already pointed out [27,34,98,109]. Our results suggest that feather follicles could be a potential source for HPAIV transmission in red-legged partridge, especially in recently dead individuals that are susceptible of feather picking. Interestingly, to date, few studies have evidenced the relevance of feathers as an important location for viral replication and potential origin of dissemination in HPAIV infection [31,278-280], and none of them have demonstrated the significance of this location in partridges.

The high susceptibility of red-legged partridges to H7N1/HP infection would make them a good sentinel species for detection of HPAIV. Since the partridges shed virus at high concentrations before death, this species could contribute to viral transmission during a local outbreak in free living birds, in countries where partridges are found in the wild or are reared in outdoor operations. The delay between the onset of virus shedding and the appearance of clinical signs (around three days in the present experiment) could have important consequences in relation to the risk of spreading disease into the wild by releasing apparently healthy farm-reared partridges for hunting purposes. The implementation of sanitary surveillance measures prior to and after release is of importance to avoid introduction of AIV, as well as other pathogens, in the natural ecosystem.

Our findings in H7N9/LP-infected birds correlate well with those obtained by Humberd *et al*. [94], where no clinical disease was observed in ring-necked pheasants and chukar partridges infected with different subtypes of LPAIV. By these authors, chukar partridges were considered as short-term shedders of LPAIV, with the respiratory

tract being the main viral excretion route. Likewise, in our study only limited viral shedding was detected in few H7N9/LP-inoculated birds most of which also seroconverted. Thus, our results suggest that partridges do not play a significant role as reservoir species for LPAIV, because only little, likely local, replication and short term shedding of low amounts of virus occurs in this species.

Based on our studies, firstly feathers (for HPAIV), but also OS and CS can be successfully used for AIV detection in surveillance programs. In addition, the brain, pancreas, and heart specimens are useful for both virus detection and histopathological diagnosis. In conclusion, although further studies with HPAIV and LPAIV strains should be performed, our observations suggest that the red-legged partridge may play a key role in the maintenance and spread of HPAIVs due to its high susceptibility and prolonged viral shedding when infected with H7N1 HPAIV. However, such partridge is not likely to be a reservoir species for LPAIV. Overall, this species should be included in passive surveillance programs in order to prevent economic losses from HPAIV outbreaks.

Chapter 4

Study II: Pathobiology and Transmission of Highly and Low Pathogenic Avian Influenza Viruses in European Quail (*Coturnix c. coturnix*)

4.1. INTRODUCTION

Since the first reported case of AI in Japanese quail in Italy (1966-1968) [152], influenza viruses of several subtypes have been isolated from quail in North America, Europe, and Asia through periodic surveillance and sporadic outbreaks [74,230,285]. Several experimental infections in Japanese quail have reported either higher, similar or lower susceptibilities than chickens to H5 HPAIV [98,176,192,252,271]. Moreover, inoculation of LPAIV representing subtypes H1 to H15 proved that Japanese quail may support the replication (predominantly in the respiratory tract) of almost all of them [136]. More recently, several studies suggested that multiple *in vivo* passages in Japanese quail facilitate the adaptation of duck AIV to chicken [47,67,91,210]. These cumulative observations along the years have been recently explained by molecular adaptation of quail AI strains, especially in HA and NA genes, which amino acids pattern might be intermediate between those of duck and chicken viruses [38,172,211]. In addition, quail carry sialic acid receptors functional for binding of avian and human influenza viruses [50,267]. Therefore, Japanese quail may provide an optimal environment for the adaptation of wild bird AIVs, generating novel variants that can cross the species barrier to domestic poultry and human beings. Surprisingly, the epidemiological significance observed for the Japanese quail has not yet been demonstrated for the European quail.

European quail (*Coturnix c. coturnix*), also called common or wild quail, is a partial migrant whose breeding range extends from the Atlantic to Lake Baikal and from the Arctic Circle to the tropics [53]. A decline in the number of European quail in the Western Palearctic over the past few decades has stimulated the release of Japanese quail as game birds in several European countries, leading to hybridization between both species in the field [12]. Even though European and Japanese quail show a high overall similarity in morphological, behavioral and ecological features that made some authors conclude that they belong to the same species [75], they are distinguishable by characteristic morphological traits and calls [12,54]. The Japanese quail, also called

domestic quail, is found in the wild in Asia [53], but is best known in its domestic form in Europe, Asia, North America, and India, where it is generally ranged in outdoor operations for meat and egg production [142]. Spain and France are the main European quail-meat producers; particularly in Spain, Japanese and European quail currently comprise 4.7% of the global avian meat production system, with an ever-increasing population size greater than 107% in number of farms along the past years [144]. Considering that open range rearing of birds has been identified as one of the factors contributing to the increase of AI outbreaks and their effect [7], specific attention should be paid to the quail, which may have a role in the spread and exacerbation of the disease.

To date, several studies have assessed the susceptibility of Japanese quail to H5 HPAIV and LPAIV [98,136,176,192,252,271]. However, the present study is the first experimental infection investigating the susceptibility of European quail to AIV. On the one hand, it is the first attempt to assess the potential viral shedding of HPAIV and LPAIV in this species, and the likelihood of effective transmission among quail. On the other hand, it represents a comparative study of the pathogenesis and viral distribution in tissues of two different HPAIV subtypes (H7 and H5). The overall results depict the role that European quail may play in the epidemiology of AI, and its putative responsibility in an interspecies outbreak.

4.2. MATERIALS AND METHODS

Viruses

Three strains of AIV were used: H7 HPAIV, H5 HPAIV, and H7 LPAIV. The H7 HPAIV [A/Chicken/Italy/5093/1999 (H7N1) (H7N1/HP)] was isolated during the 1999-2000 Italian epidemic [37]. The H5 HPAIV [A/Great crested grebe/Basque Country/06.03249/2006 (H5N1) (H5N1/HP)] was obtained from the only reported case of H5N1 HPAIV in wild birds in Spain so far [14]. The H7 LPAIV [A/*Anas*

platyrhynchos/Spain/1877/2009 (H7N2) (H7N2/LP)] was obtained from the ongoing surveillance program carried out in Catalonia (Northeast Spain). The deduced amino acid sequence of the region coding for the cleavage site of the precursor of the HA molecule were PEIPKGSRVRR*GLF for the H7N1/HP and PEIPKGR*GLF for the H7N2/LP, being typical of HPAIV and LPAIV, respectively [245].

Virus stocks were produced in SPF chicken eggs. The allantoic fluids were harvested at 48 hpi (H7N1/HP and H5N1/HP) and 72 hpi (H7N2/LP). Viruses were tenfold diluted in PBS for titration in 9-day-old embryonating SPF chicken eggs. The ELD_{50} and EID_{50} for the HPAIV and LPAIV isolates, respectively, were determined [184].

Animals

European quail (Urgasa S.A., Lleida, Spain) of approximately two months of age were used in this study. Male and female birds were included in almost equal numbers. Before the infection, serum samples of all individuals were confirmed to be seronegative for AIV by a C-ELISA test (IDVET, Montpellier, France). Furthermore, OS and CS were ensured to be negative for AIV by RRT-PCR. Each experimental group was housed in a different negative pressured isolator with HEPA-filtered air in the ABSL-3 facilities of CReSA. Quail were kept one week for acclimation, and feed and water were provided *ad libitum* throughout the experiment.

Experimental Design

Eighty birds were randomly separated into seven groups: six challenged groups with 12 birds/group and one control group with 8 birds (Table 10). For each virus, quail were subdivided into two experimental groups, A and B (n = 12/group). Groups 1A, 2A, and 3A were used to evaluate morbidity, mortality, transmissibility, and viral shedding pattern. Groups 1B, 2B, and 3B were used for the pathological studies. All animals were inoculated intranasally with 10^6 EID_{50} (for the LPAIV) or 10^6 ELD_{50} (for the HPAIV) of the corresponding challenge virus in a volume of 0.5 ml, except four birds of each A group which were used as contact animals. Contact birds were placed into the isolators four

hours after inoculating the other birds and after changing drinking water. A seventh group (group C) (*n* = 8) was used as negative controls; these quail were inoculated intranasally with PBS solution. Amounts of virus were verified by performing a RRT-PCR of both the original non-diluted viruses and the inocula.

Table 10. Experimental design of Study II.

Group	Inoculum	Titer	No. animals
1A	H7N2/LP	10^6 EID_{50}	12 (8+4)*
1B	H7N2/LP	10^6 EID_{50}	12
2A	H7N1/HP	10^6 ELD_{50}	12 (8+4)*
2B	H7N1/HP	10^6 ELD_{50}	12
3A	H5N1/HP	10^6 ELD_{50}	12 (8+4)*
3B	H5N1/HP	10^6 ELD_{50}	12
C	PBS	-	8

*In A groups, eight quail were inoculated and four quail were left as contact birds.
H7N2/LP, A/*Anas platyrhynchos*/Spain/1877/2009; H7N1/HP, A/Chicken/Italy/5093/1999; H5N1/HP, A/Great crested grebe/Basque Country/06.03249/2006; ELD_{50}, mean embryo lethal dose; EID_{50}, mean embryo infectious dose; PBS, phosphate buffer saline.

Sampling

All birds were monitored daily for clinical signs. During the first 10 dpi, at 12 dpi, and 15 dpi, OS, CS and FP samples were obtained from quail from the A groups to measure viral shedding by RRT-PCR. Drinking water was collected with a 1 ml syringe at the same time points, and it was changed on a daily basis. The same samples were collected from group C. Mortality and MDT were calculated from the A groups. At 3, 5, 8, and 15 dpi, three animals from groups B and two animals from group C were euthanized using intravenous sodium pentobarbital (100 mg/kg, Dolethal®, Vétoquinol, Cedex, France). Surviving birds were euthanized at the end of the experiment (15 dpi). Blood samples were collected before euthanasia to detect AI antibodies by C-ELISA testing. As it was terminal, bleeding was done from the heart after previous anesthesia with intramuscular injection of ketamine/xylazine (10 g/kg body weight, Imalgene® 1000 and 1 g/kg body weight, Xilagesic® 2%). All euthanized and naturally dead quail from the B

groups were necropsied to evaluate gross lesions and obtain samples for histopathological studies. Swabs and FP samples were placed in 0.5 ml of DMEM (BioWhittaker®, Lonza, Verviers, Belgium) with 600 µg/ml penicillin and streptomycin. These samples, together with drinking water samples and serum samples, were stored at -80ºC until further use.

Pathologic Examination and Immunohistochemical Testing

Necropsies and tissue sampling were performed according to standard protocols [135]. After fixation in 10% neutral buffered formalin and embedding in paraffin, tissue sections were processed routinely for HE staining. The following tissues were examined: esophagus, crop, proventriculus, gizzard, duodenum, jejunum-ileum, cecum/cecal tonsil, colon, rectum, pancreas, liver, kidney, adrenal gland, gonad, nasal turbinates, trachea, lung, heart, breast muscle, skin, bone marrow, spleen, bursa of Fabricius, thymus, brain, spinal cord, and sciatic nerve. In addition, an IHC technique was performed as previously described [76,187]. The primary antibody was a mouse-derived monoclonal commercial antibody against NP of influenza A virus (IgG2a, Hb65, ATCC). As a secondary antibody, a biotinylated goat anti-mouse IgG antibody (GaMb, Dako E0433, Glostrup, Denmark) was used. Tissues previously demonstrated to be positive against NP of influenza A virus by IHC were used as a positive control. Duplicated samples of all animals incubated without the primary antibody, as well as tissues from sham-inoculated animals processed as usual by IHC, served as negative controls. The following score was used to grade the staining in the tissues: no positive cells (–), single positive cells (+), scattered groups of positive cells (++), widespread positivity (+++).

Viral RNA Detection by RRT-PCR

Viral RNA from OS, CS, FP, and drinking water samples was extracted with NucleoSpin® RNA virus kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. The resulting viral RNA extracts were tested by one-step RRT-PCR for the detection of a highly conserved region of *M1* gene in Fast7500

equipment (Applied Biosystems, Foster City, CA, USA) using the primers and probe previously described [213] and the amplification conditions described by Busquets *et al*. [32]. Samples with a cycle threshold (Ct) value ≤ 40 were considered positive for influenza A viral RNA. Viral shedding was analyzed by ANOVA test for significant differences ($p < 0.05$) using the Statistical Package for the Social Sciences (SPSS) for Windows Version 20.0.

Serology

A C-ELISA test was carried out to detect antibodies against the NP of AIV using the commercially available kit ID Screen® Influenza A Antibody Competition (IDVET, Montpellier, France), according to the manufacturer's instructions. In addition, a HI test was performed to titrate antibodies against specific H5- (in H5N1/HP serum samples) and H7- (in H7N2/LP and H7N1/HP serum samples) subtypes. The HI assays were performed according to standard procedures [156] with chicken red blood cells and commercial inactivated H5- and H7-antigens (GD-Deventer, The Netherlands). To avoid nonspecific positive reactions, sera were pre-treated by adsorption with 10% chicken red blood cells. Titers were expressed as geometric mean titers (GMT-$\log_2$); GMT of 3 $\log_2$ or greater were considered positive. Previously known positive and negative sera were used as controls.

4.3. RESULTS

Morbidity and Mortality

Clinical signs and mortality were only observed in HPAIV-infected groups (groups 2 and 3) and were similar between inoculated and contact birds. Some of the quail (17% H7N1/HP-challenged and 58% H5N1/HP-challenged animals) displayed nonspecific clinical signs, consisting of lethargy, anorexia, and ruffled feathers, that progressed to death or severe neurological signs (e.g., incoordination, torticollis, circling, head tremors, head tilt, and opisthotonus) within 24 h. The onset times of these nonspecific

signs were 6 dpi for H7N1/HP-group and 4 dpi for H5N1/HP-group. Two H7N1/HP-challenged quail (17%) and three H5N1/HP-challenged quail (25%) presented an acute fatal progression of the infection, displaying neurological signs without previous nonspecific signs at 7 dpi and 5 dpi, respectively. However, in other cases (33% in H7N1/HP-group and 8% in H5N1/HP-group) quail were found dead without previous clinical signs. Only one bird, belonging to the H5N1/HP-group, recovered after showing nonspecific clinical signs at 6-7 dpi. All animals with neurological signs, recumbent or both were euthanized for ethical reasons. The survival rates and the MDTs of the HPAIV-infected groups (groups 2 and 3) throughout the experiment are summarized in Figure 8 and Table 11.

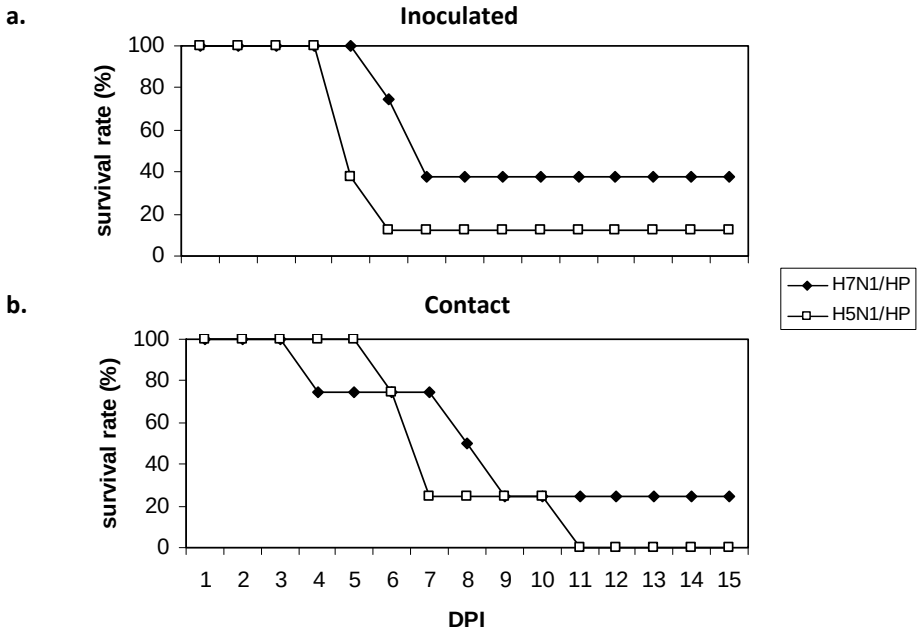


Figure 8. Survival rates of quail intranasally challenged with H7N1/HP and H5N1/HP. a. Intranasally inoculated quail. **b.** Contact quail.

Table 11. Survival rates and MDT of quail intranasally challenged with either H7N1/HP or H5N1/HP.

Virus	Mortality*		
	Inoculated (MDT)	Contact (MDT)	Total
H7N1/HP	63% (6.6)	75% (7)	67%
H5N1/HP	88% (5.3)	100 (6)	92%

*#dead/total X 100.
MDT, mean death time (dpi); H7N1/HP, A/Chicken/Italy/5093/1999; H5N1/HP, A/Great crested grebe/Basque Country/06.03249/2006.

Gross Findings

Consistent gross lesions were only observed in HPAIV-infected groups (groups 2 and 3) and were similar between inoculated and contact birds. At 3 dpi, one H7N1/HP-quail (group 2) presented multifocal petechia on the proventriculus-gizzard junction mucosa. However, the foremost lesions in the H7N1/HP-group were observed at 5 dpi, which consisted of moderate splenomegaly with pallor or parenchymal mottling and pancreatic lesions characterized by multifocal necrotic areas of 1mm-diameter. Lesions in H5N1/HP-quail (group 3) were most pronounced and were detected throughout the experiment in all necropsied birds. At 3 dpi, liver pallor in one bird was observed. The quail found dead at 4 dpi presented spleen pallor and multifocal areas in the pancreas. Such pancreatic lesion, as well as thymus atrophy, was observed until the end of the experiment in all necropsied birds. At 5 dpi, spleen pallor was observed in one bird. No gross lesions were observed in H7N2/LP-infected birds (group 1) or in birds from the control group (group C).

Histopathological Findings

Histological lesions and influenza A viral NP were only observed in HPAIV-infected quail (groups 2 and 3) (Tables 12 and 13). In H7N1/HP-quail, prevailing histological lesions were observed at 5 and 8 dpi mainly in the pancreas, heart, and brain, but also in the gizzard, cecal tonsil, and spinal cord (Table 12). H5N1/HP-challenged birds consistently showed marked lesions in the tissues mentioned for the H7N1/HP-infected quail and also, to a lesser extent, in the rectum, kidney, and skeletal muscle from the breast (Table 13). Accordingly, presence of H5N1/HP in tissues, as determined by IHC, was more intense than H7N1/HP. The most consistent finding, prevalent throughout almost all the experiment within both HPAIV-challenged groups, was moderate to severe multifocal to coalescent lytic necrosis of the acinar epithelium of the pancreas and endothelial activation indicative of acute inflammation. The main findings in the brain consisted of moderate to severe multifocal areas of malacia in the cerebral hemispheres, associated with spongiosis of the neuropil, neuronal chromatolysis, and gliosis (Figures

9a, 9b). Overt severe necrosis of ependymal cells of the ventricles was present in all affected quail. The cerebellum frequently showed multifocal areas of moderate to severe chromatolysis of Purkinje neurons at 3, 5, and 7 dpi of H5N1/HP-infected quail, sometimes associated with non-suppurative perivascular inflammatory infiltrate. The heart was also consistently affected, with multifocal to diffuse myocardial degeneration and necrosis consisting of hyalinization and fragmentation of cardiac myocytes, often associated with mild lymphoplasmacytic infiltrate (Figures 9c, 9d). In general, IHC staining was mainly nuclear and sometimes also cytoplasmic in distribution and correlated well with histopathological findings.

Table 12. Average distribution of AIV-NP antigen in positive tissues from quail intranasally challenged with H7N1/HP.

Tissue*	3 dpi	5 dpi	8 dpi	15 dpi	Predominant cell types	Associated lesion
Gizzard	–	++	–	–	Epithelial cells of the ventricular glands	Severe multifocal necrosis, mild lymphoplasmacytic infiltrate
Cecal tonsil	–	+	–	–	Epithelial cells of the glands, cells of the muscularis externa	Focal necrosis, mild lymphoplasmacytic infiltrate
Pancreas	–	++	+++	–	Acinar cells, endothelial cells	Severe multifocal to coalescent lytic necrosis, lymphoplasmacytic infiltrate, edema
Nasal turbinates	+	–	–	–	Respiratory epithelial cells	Lymphoplasmacytic infiltrate in lamina propria
Trachea	–	+	–	–	Goblet cells	NSL
Heart	–	++	–	–	Myocardiocytes, endothelial cells	Severe multifocal necrosis, mild lymphoplasmacytic infiltrate
Spleen	–	+	+	–	Endothelial cells, macrophages	NSL
Brain	–	+++	++	–	Neurons, Purkinje cells, ependymal cells, glial cells, endothelial cells	Malacia in cortex, necrosis of ependymal cells of ventricles and epithelial cells of choroid plexus, chromatolysis of Purkinje cells, lymphoplasmacytic infiltrate
Spinal cord	–	+++	–	–	-	Malacia in grey matter, necrosis of the ependyma and neuropil

*Tissues not present appeared overtly normal on histopathological analysis and did not show positive IHC staining.
– = no positive cells; + = single positive cells; ++ = scattered groups of positive cells; +++ = widespread positivity.
dpi, days post-inoculation; NSL, no significant lesions.

Table 13. Average distribution of AIV-NP antigen in positive tissues from quail intranasally challenged with H5N1/HP.

Tissue*	3 dpi	4 dpi	5 dpi	6 dpi	7 dpi	Predominant cell types	Associated lesion
Proventriculus	–	–	–	+	–	Epithelial cells of the proventricular glands, cells of the muscularis externa	Severe multifocal necrosis, mild lymphoplasmacytic infiltrate
Gizzard	–	–	+	++	++	Epithelial cells of the ventricular glands, cells of the muscularis externa	Severe multifocal necrosis, mild lymphoplasmacytic infiltrate
Cecal tonsil	–	–	+	+++	+	Cells of the lamina propria	Mild lymphoplasmacytic infiltrate
Rectum	–	–	–	+	+	Cells of the muscularis externa of the lamina propria	Vacuolation, degeneration, mild lymphoplasmacytic infiltrate
Pancreas	+	–	++	++	+	Acinar cells, endothelial cells	Severe multifocal to coalescent lytic necrosis, lymphoplasmacytic infiltrate, edema
Kidney	+	+	+	+	+++	Collecting tubular epithelial cells, endothelial cells	Moderate to severe necrosis, mild lymphoplasmacytic infiltrate
Adrenal gland	+	–	–	–	–	Corticotrophic and corticotropic cells	NSL
Nasal turbinates	–	++	–	–	–	Respiratory epithelial cells	Lymphoplasmacytic infiltrate in lamina propria
Heart	+	++	+++	++	+++	Myocardiocytes, endothelial cells	Severe multifocal necrosis, mild lymphoplasmacytic infiltrate
Skeletal muscle	–	–	+	++	++	Myocytes, endothelial cells	Moderate multifocal necrosis, mild lymphoplasmacytic infiltrate
Spleen	–	–	–	–	+	Endothelial cells, macrophages	NSL
Brain	++	+++	+++	+++	+++	Neurons, Purkinje cells, ependymal cells, glial cells, endothelial cells	Malacia in cortex, necrosis of ependymal cells of ventricles and epithelial cells of choroid plexus, chromatolysis of Purkinje cells, lymphoplasmacytic infiltrate

*Tissues not present appeared overtly normal on histopathological analysis and did not show positive IHC staining.
– = no positive cells; + = single positive cells; ++ = scattered groups of positive cells; +++ = widespread positivity.
dpi, days post-inoculation; NSL, no significant lesions.

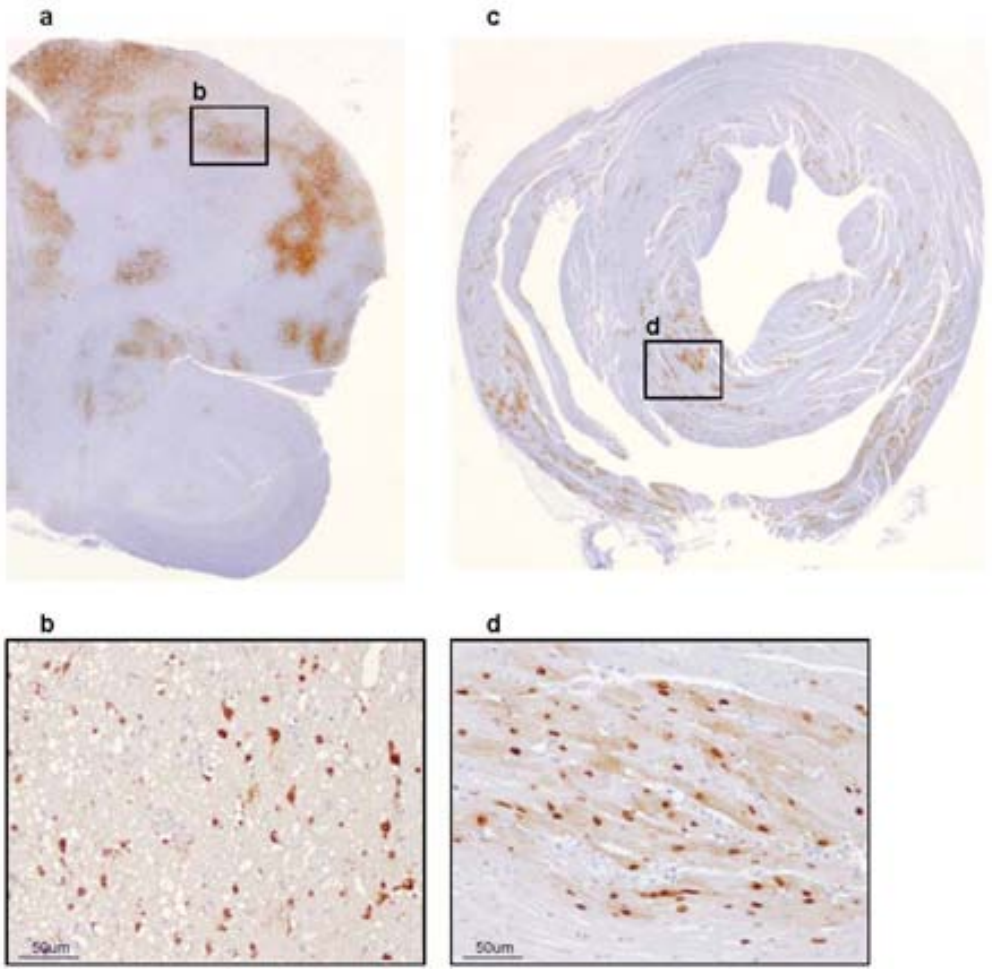


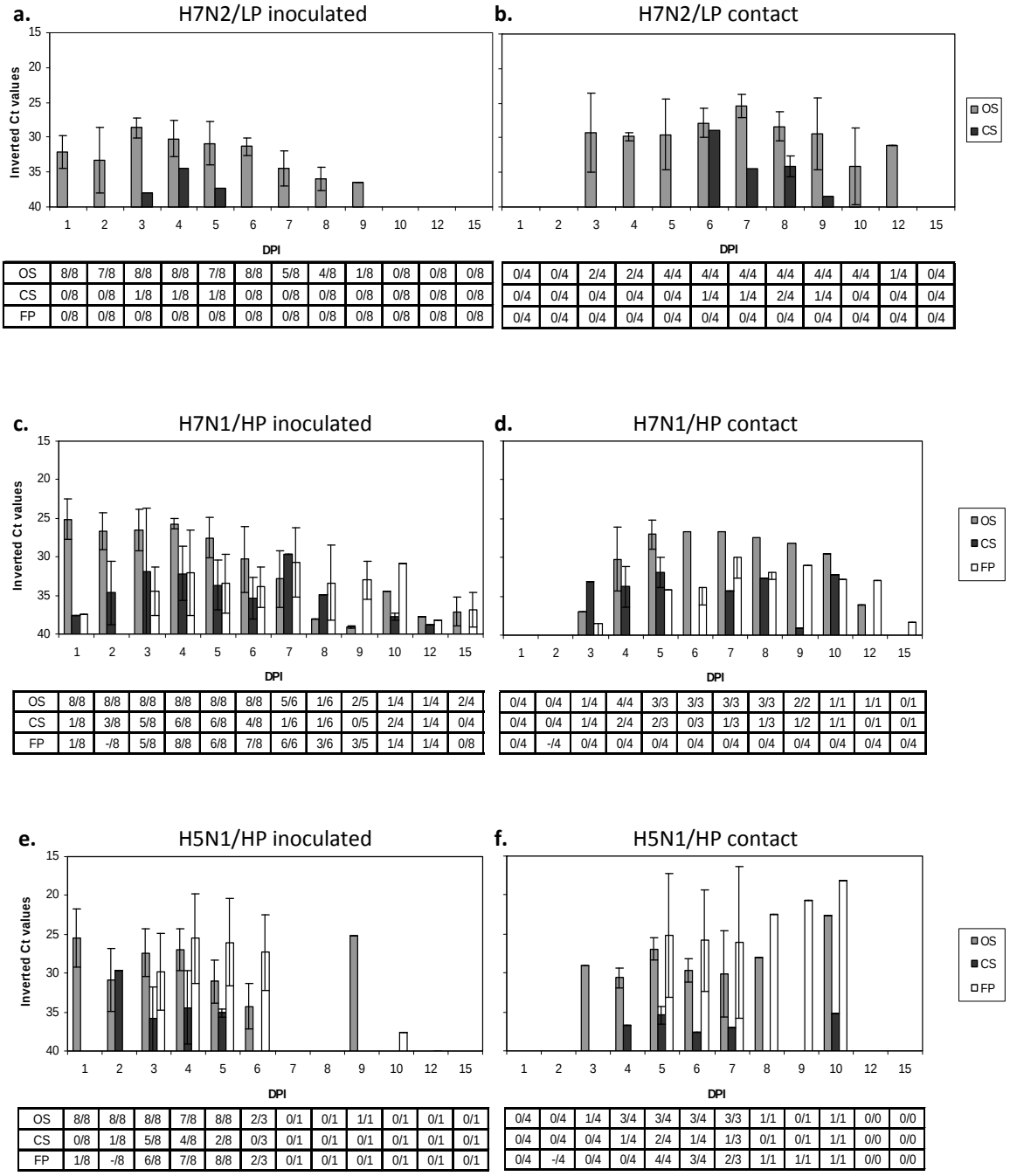
Figure 9. Distribution of NP antigen in positive tissues of a quail intranasally challenged with H5N1/HP. **a.** Brain, 7 dpi. **b.** Positive staining in nucleus and cytoplasm of neurons and glial cells. **c.** Heart, 5 dpi. **d**. Positive staining in nucleus and cytoplasm of myocardiocytes.

Viral RNA Detection by RRT-PCR

Real time RT-PCR was performed on OS, CS, FP, and drinking water samples of the A groups. Oropharyngeal swabs of H7N2/LP-challenged birds (group 1) tested positive until 9 dpi for inoculated birds peaking at 3 dpi, and until 12 dpi for contact birds peaking at 7 dpi (Figures 10a, 10b). Viral RNA from CS was detected in one animal during 3 days (3-5 dpi) and in two contact animals for 4 days (6-9 dpi). Feather pulp samples tested negative in this H7N2/LP-group. In H7N1/HP-inoculated quail (group 2), viral RNA was detected in all the studied samples (OS, CS, FP) from 1 dpi until before death, although oral shedding was predominant (Figure 10c). Viral RNA detection from contact

H7N1/HP-birds was similar to that observed in inoculated quail, although with two days of delay (Figure 10d). For H5N1/HP-inoculated quail (group 3), oral shedding was also higher than cloacal shedding, although FP samples had high amounts of viral RNA as well (Figure 3E). H5N1/HP viral RNA amounts were less homogenous than for H7N1/HP among dpi and types of sample. Contact H5N1/HP-quail had a similar shedding profile to the inoculated ones, although starting two days later (Figure 10f). HPAIV-challenged quail orally shed significantly higher amounts of viral RNA than the LPAIV-challenged quail ($p < 0.05$), especially on 1, 2, and 4 dpi. Moreover, FP from H5N1/HP-challenged quail contained significantly more viral RNA than FP from H7N1/HP-infected quail ($p < 0.05$).

Presence of H7N2/LP viral RNA in drinking water samples coincided with the days where quail's samples reached maximum viral RNA values (4-6 dpi). H7N1/HP viral RNA was present in water during almost all the experiment (3-15 dpi), being quite stable in time. Existence of H5N1/HP viral RNA in drinking water was manifested at 2 dpi and at 6 dpi, at levels akin to those of H7N1/HP.



OS	8/8	7/8	8/8	8/8	7/8	8/8	5/8	4/8	1/8	0/8	0/8	0/8
CS	0/8	0/8	1/8	1/8	1/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8
FP	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8	0/8

0/4	0/4	2/4	2/4	4/4	4/4	4/4	4/4	4/4	4/4	1/4	0/4
0/4	0/4	0/4	0/4	0/4	1/4	1/4	2/4	1/4	0/4	0/4	0/4
0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4

OS	8/8	8/8	8/8	8/8	8/8	8/8	5/6	1/6	2/5	1/4	1/4	2/4
CS	1/8	3/8	5/8	6/8	6/8	4/8	1/6	1/6	0/5	2/4	1/4	0/4
FP	1/8	-/8	5/8	8/8	6/8	7/8	6/6	3/6	3/5	1/4	1/4	0/8

0/4	0/4	1/4	4/4	3/3	3/3	3/3	3/3	2/2	1/1	1/1	0/1
0/4	0/4	1/4	2/4	2/3	0/3	1/3	1/3	1/2	1/1	0/1	0/1
0/4	-/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4	0/4

OS	8/8	8/8	8/8	7/8	8/8	2/3	0/1	0/1	1/1	0/1	0/1	0/1
CS	0/8	1/8	5/8	4/8	2/8	0/3	0/1	0/1	0/1	0/1	0/1	0/1
FP	1/8	-/8	6/8	7/8	8/8	2/3	0/1	0/1	0/1	0/1	0/1	0/1

0/4	0/4	1/4	3/4	3/4	3/4	3/3	1/1	0/1	1/1	0/0	0/0
0/4	0/4	0/4	1/4	2/4	1/4	1/3	0/1	0/1	1/1	0/0	0/0
0/4	-/4	0/4	0/4	4/4	3/4	2/3	1/1	1/1	1/1	0/0	0/0

Figure 10. Viral RNA shedding detected by RRT-PCR in quail experimentally challenged with AIV. Results are expressed as inverted Ct-values and shown as means of positive individuals ± SD. Tables indicate the ratio between positive quail and total quail examined per day and sample. Ct, cycle of threshold; DPI, days post-inoculation; OS, oropharyngeal swabs; CS, cloacal swabs; FP, feather pulps. **a**. Quail intranasally inoculated with H7N2/LP. **b**. Contact quail of H7N2/LP. **c**. Quail intranasally inoculated with H7N1/HP. **d**. Contact quail of H7N1/HP. **e**. Quail intranasally inoculated with H5N1/HP. **f**. Contact quail of H5N1/HP.

Serology

Before infection, all serum samples tested negative for antibodies against influenza A NP. Almost all the H7N2/LP-inoculated quail (7/8) and all the H7N2/LP-contact quail (4/4) were antibody-positive at 15 dpi, with GMT of 7.9 and 7.3, respectively (Table 14). Besides, all HPAIV-inoculated birds (both H7N1/HP and H5N1/HP) seroconverted from 5 dpi onwards, with GMT steadily increasing until 15 dpi from 4.7 to 7.0 in the case of H7N1/HP-inoculated quail (Table 14).

Table 14. Serological data of quail intranasally challenged with either H7N2/LP, H7N1/HP or H5N1/HP.

Group	3 dpi		5 dpi		9 dpi		15 dpi	
	ELISA	HI*	ELISA	HI*	ELISA	HI*	ELISA	HI*
H7N2/LP Inoculated	nd	nd	nd	nd	nd	nd	7/8	7/8 (7.9)
H7N2/LP Contact	nd	nd	nd	nd	nd	nd	4/4	4/4 (7.3)
H7N1/HP Inoculated†	0/3	0/3	3/3	3/3 (4.7)	3/3	3/3 (6.3)	3/3	3/3 (7.0)
H5N1/HP Inoculated†	0/3	0/3	3/3	3/3 (6.0)	nd	nd	nd	nd

dpi, days post-inoculation; ELISA, C-ELISA; HI, hemagglutination inhibition; nd, no data; H7N2/LP, A/*Anas platyrhynchos*/Spain/1877/2009; H7N1/HP, A/Chicken/Italy/5093/1999; H5N1/HP, A/Great crested grebe/Basque Country/06.03249/2006.
*GMT (log_2) are indicated in parenthesis. GMT include only positive birds.
†No data is available for contact quail in these groups.

4.4. DISCUSSION

This is the first experiment which demonstrates that European quail can be infected with both HPAIV and LPAIV. This quail subspecies can be found not only in the wild all over the Palearctic region, but also in many parts of Europe as a game bird species raised in outdoor operations. Despite the relevance of this game bird species, no studies had previously investigated its AIV infection dynamics. The aim of the present study was to elucidate the putative role of European quail in the ecology of influenza A viruses by assessing the pathogenesis, transmissibility, and viral shedding of quail experimentally infected with two different HPAIV subtypes (H7N1 and H5N1) and one LPAIV (H7N2).

Quail exhibited a high susceptibility to both HPAIV used in this study, as demonstrated by severe clinical signs and high mortality rates. With the earliest onset, most rapid progression of disease, and shortest MDT in H5N1/HP-infected quail, it is apparent that this virus is more virulent for this species than the H7N1/HP [215]. The high pathogenicity observed for both HPAIV is in accordance with natural and experimental H5N1 HPAIV infections in chickens and other gallinaceous species, among which are Japanese quail [19,98,176]. Although previous studies with Japanese quail reported minimal clinical signs or even sudden deaths without apparent symptoms [98,176,192], clinically neurological dysfunction was an evident sign in most of the HPAIV-infected quail of the present study. Certain gross findings indicative of AI were not as extensive and obvious as for chickens (e.g., presence of edematous, hemorrhagic, and necrotic cutaneous lesions), but affected tissues were known target organs for influenza A viruses in other gallinaceous species, including Japanese quail [8,19,98,176]. Interestingly, H5N1/HP showed a broader tissue distribution compared with H7N1/HP, suggesting that virus replication in a particular target organ other than respiratory or intestinal organs may contribute to the virulence of the HPAIV in quail, as previously stated [192]. Particularly, neurotropism is considered one of the main factors for the fatal course of AI in birds [23,42], evidenced in our study by the higher virulence of the H5N1/HP compared with H7N1/HP. Our findings in H7N2/LP correlate well with those of Makarova *et al*. [136], in which a wide range of LPAIV subtypes could replicate efficiently in Japanese quail, predominantly in the respiratory tract. In our study, European quail could also maintain the infection without clinical involvement, and shed the virus mainly orally during a substantial period.

Effective viral transmission from inoculated quail to naïve contact birds was confirmed for the three studied viruses, even though their origin avian hosts were as diverse as chicken, mallard, and great crested grebe. This finding suggests that adaptation may not be needed to allow AIV to replicate and transmit in European quail, confirming the substantial role that this species may play in AI epidemiology. As in a previous work with H5N1 HPAIV in Japanese quail [98], both HPAIV used in our study

confirmed to be able to transmit among European quail. Moreover, transmission of H7 isolates (both H7N1/HP and H7N2/LP) is of great importance because: 1) this is the first transmission evidence of an H7 HPAIV in quail so far; and 2) previous experiments with LPAIV failed to confirm this capability in Japanese quail [136]. Not only had the onset of clinical signs proved infection in contact birds, but also their antibody responses (in the case of H7N2/LP challenge) and their efficient viral shedding. Given that quail shed virus mainly orally, contact birds might have been infected by the oral-oral route. In fact, such viral shedding predominance, also stated in previous studies with Japanese quail [98,136,192], is already known to differ from that observed in LPAIV waterfowl reservoirs [274].

Ingestion of contaminated water has already been suggested as a possible transmission route [270]. Interestingly, the earlier detection of viral H5N1/HP RNA in water followed by H7N1/HP and finally by H7N2/LP could mirror the initial ability of the virus to replicate in host cells, be shed, and thus, be more likely transmissible to naïve birds. Drinking water should be particularly taken into account for quail and other game birds raised in outdoor operations, where AI viruses from wild birds could be introduced to the poultry flock. Furthermore, contamination of the environment by respiratory secretions and infected carcasses likely would result in indirect oral transmission of the virus. Although minor, cloacal shedding was consistently detected in HPAIV-infected quail, confirming that European quail might have functional binding receptors in both trachea and intestine, as already confirmed for both quail subspecies [50,73,267,286]. Besides, feathers could likely act as potential source for virus transmission in European quail, especially in recently dead birds susceptible to feather picking. To date, the relevance of feathers as a location for viral replication and potential origin of dissemination in HPAIV infection has been evidenced in certain bird species [19,31,280], but had not yet been demonstrated in quail.

The high degree of correlation between C-ELISA and HI results suggests that such tests seem to be equally sensitive and specific when assessing quail serological responses, as previously stated for Japanese quail [98]. Antibody response in HPAIV-

inoculated quail started as early as 5 dpi, further confirming infection of the birds and an early humoral immune response. Seroconversion in H7N2/LP-infected quail at the end of the experiment proved effective infection not only among inoculated birds but also among contacts. In general, antibody titers in the present study were akin to those previously observed in AIV-infected Japanese quail [6,58,284] and gradually increased throughout the experiment, as already observed in H9N2 LPAIV-infected Japanese quail [58].

The high susceptibility of European quail to H7N1/HP and H5N1/HP would make this species a good sentinel of the presence of HPAIV in the environment, both in the wild or in semi-extensive farms. On the other hand, infected quail can shed a considerable amount of AIV before the appearance of overt clinical signs, death or both (around four days in the present experiment). Therefore, spreading disease into the wild by releasing apparently healthy farm-reared quail for hunting purposes could represent a substantial threat, even higher if assuming that this species could act as a mixing vessel like already stated for the Japanese quail. Furthermore, European quail may be considered sentinels (for both HPAIV and LPAIV) and reservoirs (for LPAIV), which is of special interest as most wild individuals are migratory [53]. The application of surveillance measures on quail flocks before and after release is of importance to avoid introduction of HPAIV, as well as other pathogens, in the natural ecosystem.

Current active AI surveillance activities include sampling of both OS and CS, as well as blood [145,191]. Passive surveillance of dead or moribund birds involves the same samples as for active surveillance (when possible) along with tissue collection through necropsy [145,191]. On the basis of our findings, OS could be used as a unique tool for successful virus detection in active AI surveillance programs in quail, as it has been assessed for other minor species in which pathogenesis is still poorly understood [19]. In addition, brain, pancreas, and heart specimens would be suitable in passive surveillance when HPAIV is suspected. Our results suggest that European quail, like Japanese quail, could play a key role in AI epidemiology because of the high susceptibility to HPAIV and the noteworthy spread of both HPAIV and LPAIV. Taking into account the similarities in

viral dynamics between Japanese and European quail, the latter would also presumably have the capability to act as an intermediate host for avian/mammalian reassortant viruses, although further experiments are needed to address this issue. In addition, future studies comparing AI infection dynamics between Japanese and European quail by experimental infections with the same AIV strains would strengthen the present data. Altogether, our results underline the complexity of managing AI outbreaks when different susceptible species are involved.

Chapter 5

Study III: Highly (H5N1) and Low (H7N2) Pathogenic Avian Influenza Virus Infection in Falcons Via Nasochoanal Route and Ingestion of Experimentally Infected Prey

5.1. INTRODUCTION

Although AI typically courses as an asymptomatic infection in wild birds, recent HPAI epidemics resulted in unprecedented high mortality rates for certain wild bird species. In the past, HPAIVs were rarely found in birds of prey and were restricted to only a few isolated cases [134,137]. However, during recent H5N1 HPAIV outbreaks, increasing number of birds of prey have been reported to be infected, probably as a result of improvements in sampling and diagnostic tools. It is worth highlighting that Hong Kong had a series of cases of natural infection of peregrine falcons (*Falco peregrinus*) with H5N1 HPAIV in 2004, 2006 and 2008 [46,88,183], although other countries subsequently reported HPAI cases in different prey species, such as Hodgson's hawk eagles (*Spizaetus nipalensis*) in Belgium [261], saker falcons (*Falco cherrug*) in Saudi Arabia [193], and more recently in Saudi Arabia, houbara bustards (*Chlamydotis undulate macqueenii*), which interestingly infected falcons that came into contact with them [105].

Even though the number of AI natural cases in raptor species has gradually increased, data prevalence is still scarce. However, some countries have performed active AI surveillance of these species. In Sweden, neither HPAI nor LPAI infections were found in white-tailed sea eagle (*Haliaeetus albicilla*) or peregrine falcons by standard screening using RRT-PCR and serology [72]. Besides this, 7.7% of the Falconiformes tested between August 2005 and February 2006 in the United Arab Emirates were seropositive [155]. It should be noted that in the Middle East, in addition to the circulation of the H5N1 viruses, there is also evidence of extensive circulation of LPAIV, mainly H9N2 viruses [1,146]. The cocirculation of H9N2 and H5N1 subtypes of AI in these species may increase the risk of generating reassortant viruses with pandemic potential [147].

Falconry is an ancient tradition in the Arabian Peninsula that has spread worldwide, resulting in a strong trade of all species of falcons around the world. Nowadays, falconry is most popular in European countries such as the United Kingdom,

Germany, and Spain, in that order [10]. It is worth highlighting that Spain is the main falcon producer worldwide, with some 1300 falcons being reared and exported to the Arabian countries. Of such amount, more than a half of the falcons (approximately 800) are reared in a breeder located in Catalonia (Northeast Spain) (personal communication). Different prey species are utilized widely in falconry; among them, gyr-saker hybrid falcons are considered precious hunting birds and are commonly used. The gyrfalcon (*Falco rusticolus*) breeds on Arctic coasts and the islands of North America, Europe, and Asia, although after the breeding season or in winter it is dispersed throughout much of the Northern Hemisphere, with populations in Northern America, Greenland, and Northern Europe [53]. The saker falcon breeds from Eastern Europe eastwards across Asia to Manchuria. It is mainly migratory except in the southernmost parts of its range, wintering in Ethiopia, the Arabian Peninsula, Northern Pakistan, and Western China [53]. BirdLife International categorizes the saker falcon as endangered species, due to a rapid population decline particularly on the central Asian breeding grounds [20].

It is well known that migration of infected wild birds is one of the mechanisms in the spreading of AI viruses [103], thus many falcon species may contribute to the movement of both HPAIVs and LPAIVs within or between countries. Wild birds of prey are at an increased risk of acquiring AIVs because they regularly feed on avian carcasses and diseased avian prey [29,114,255]. In falconry, birds of prey are kept in captivity and come into close contact with humans. Although there is still no direct evidence of virus transmission from falcons to humans, birds of prey could represent a bridging species for AIVs and, consequently, the practice of falconry may pose an enhanced risk of transmission to humans and poultry. However, a recent study by Kohls *et al*. [111] indicates that the AIV prevalence of prey birds from falconry is generally low, and that falconry birds which come into contact with AIVs through their prey do not necessarily become infected, as in most cases the falconer does not allow them to eat the whole prey. However, concerning ornithophagous free ranging raptors, the risk of infection

would be higher, since these usually feed on the whole prey. There is no evidence to confirm this so far.

To date, scarce experimental infections have been performed in birds of prey. Lierz *et al*. [130] studied the effects of H5N1 HPAIV infection by performing an experimental vaccination trial on captive gyr-saker falcon hybrids via the oculo-oronasal route. Recently, an experimental infection in American kestrels (*Falco sparverius*) with various doses of H5N1 HPAI virus inoculated via the intranasal and intrachoanal route was performed [77]. Both studies showed that these birds are extremely susceptible to the H5N1 HPAIV.

Although it is evident that birds of prey can be infected with HPAIVs, their susceptibility to LPAIVs still remains unclear, and the pathogenicity of both HPAIVs and LPAIVs to these species has not been described extensively. Moreover, raptor's putative role as reservoirs in AI ecology and their potential to shed viruses need to be investigated. In the present study, the pathogenesis of HPAIV and LPAIV in ¾ gyr-saker hybrid falcons was determined. The birds were experimentally inoculated via the nasochoanal route and by ingestion of virus-infected preys. Viral load distribution in several tissues and the extent and duration of viral shedding were also evaluated. In addition, localization of influenza virus receptors in different tissues was also assessed in order to identify the target cells of AIVs in this species.

5.2. MATERIALS AND METHODS

Viruses

Two strains of AIV were used: the H5N1 HPAIV A/Great crested grebe/Basque Country/06.03249/2006 (H5N1/HP) and the H7N2 LPAIV A/*Anas platyrhynchos*/Spain/1877/2009) (H7N2/LP). The deduced amino acid sequences of the region coding for the cleavage site of the HA molecule were PEIPKGSRVRR*GLF for the

isolate H5N1/HP and PEIPKGR*GLF for the isolate H7N2/LP, being typical of HPAIVs and LPAIVs, respectively. In addition, the H5N1/HP subtype demonstrated an IVPI of 3.0 [37].

Virus stocks were produced in 9-day-old embryonating SPF chicken eggs. In both cases, the allantoic fluid was harvested at 48 hpi, aliquoted and stored at -80ºC until use. Viruses were diluted tenfold in PBS for titration in 9-day-old embryonating SPF chicken eggs. The ELD_{50} for H5N1/HP and the EID_{50} for H7N2/LP were determined using the Reed and Muench method [184].

Animals

Juvenile (5–10 weeks old) male captive-reared ¾ gyr-saker hybrid falcons were obtained from a breeder (Roc Falcon S.L.). From 3 to 10 weeks of age, falcons were imprinted by a person from the research group in order to minimize further stress. In order to assess that optimal health conditions existed, a complete cell blood count (CBC) was performed and a peripheral blood smear evaluated to rule out the presence of hemoparasites in all birds. Results of the CBC and blood smears were unremarkable. Also, an anti-parasite treatment (Baycox®) was carried out. In addition, non-diluted serum samples were collected to ensure that birds were serologically negative for AI antibodies by a C-ELISA test (ID-VET, Montpellier, France). Also, OS and CS were collected in order to make certain that falcons were negative for AI virus by RRT-PCR. Birds were weighed and tagged with numbered aluminum leg bands, and brought to ABSL-3 facilities in CReSA, where they were randomly distributed within the experimental groups and separately housed in negative-pressured isolators with HEPA-filtered air. It is worth highlighting that isolators were environmentally adapted for falcons' welfare. Whole chick-preys supplemented with vitamins were provided twice a day. Birds were adapted to their new environment for 5 days before infection.

Experimental Design

The experimental design is summarized in Table 15. Seventeen falcons were distributed into five experimental groups (A to E). Groups A (n = 4) and B (n = 4) were

challenged with 10^6 EID_{50} of the H7N2/LP, whereas groups C (*n* = 3) and D (*n* = 4) were challenged with 10^6 ELD_{50} of the H5N1/HP. Two falcons from group E were inoculated nasochoanally with PBS solution and served as negative controls. Groups A and C were inoculated nasochoanally; in group A, one of the 4 falcons was not-inoculated and was referred to as the contact animal. Groups B and D were challenged via the natural feeding route with previously infected SPF chicks. Briefly, one-day-old SPF chicks, confirmed negative for AIV by RRT-PCR, were inoculated via oculonasal route with either 10^6 EID_{50} of the H7N2/LP or 10^6 ELD_{50} of the H5N1/HP. At 3 dpi, chicks were confirmed positive for AIV by performing a RRT-PCR on OS and CS. Two whole H7N2/LP-infected chicks and five whole H5N1/HP-infected chicks were used to infect each falcon from groups B and D, respectively. In order to estimate the viral load ingested by the falcons, viral titration in MDCK cells was performed on tissue homogenates from H7N2/LP- and H5N1/HP-infected chicks. Two out of three homogenates of trachea, lung, kidney, and small intestine from H7N2/LP-infected chicks contained $10^{3.2}$ to $10^{7.2}$ $TCID_{50}$/g of tissue, whereas the viral load from homogenates of liver, lung, kidney, and brain of all H5N1/HP-infected chicks reached titers from $10^{6.5}$ to $10^{7.4}$ $TCID_{50}$/g of tissue. Inocula titers of all the experimental groups were verified by performing a RRT-PCR of both the original non-diluted viruses and the diluted inocula.

Table 15. Experimental design of Study III.

Group	Inoculum	Titer	Infection route	No. animals
A	H7N2/LP	10^6 EID_{50}	Nasochoanal	4 (3+1)*
B	H7N2/LP	2 chicks/falcon	Feeding	4
C	H5N1/HP	10^6 ELD_{50}	Nasochoanal	3
D	H5N1/HP	5 chicks/falcon	Feeding	4
E	PBS	-	Nasochoanal	2

*In group A, three falcons were inoculated and one falcon was left as contact bird.
H7N2/LP, A/*Anas platyrhynchos*/Spain/1877/2009; H5N1/HP, A/Great crested grebe/Basque Country/06.03249/2006; EID_{50}, mean embryo infectious dose; ELD_{50}, mean embryo lethal dose; PBS, phosphate buffer saline.

Sampling

All falcons were monitored daily for the development of clinical signs, and OS and CS were obtained to measure viral shedding by RRT-PCR. Besides, blood samples were collected before euthanasia to detect AI antibodies by C-ELISA testing. As it was terminal, bleeding was done from the heart after previous anesthesia with intramuscular injection of ketamine/xylazine (10 g/kg body weight, Imalgene® 1000 and 1 g/kg body weight, Xilagesic® 2%). Mortality and MDT were calculated. Ethically euthanized and naturally dead falcons were necropsied to evaluate gross lesions and obtain samples for pathological and molecular studies. Negative control falcons and surviving infected falcons were euthanized at the end of the experiment (11 dpi for LPAIV-infected groups and 10 dpi for HPAIV-infected groups). Falcons were euthanized using intravenous sodium pentobarbital (100 mg/kg, Dolethal®, Vétoquinol, Cedex, France). Swabs, blood samples, and tissue samples for molecular studies were stored at -80ºC until further use.

Pathologic Examination and Immunohistochemical Testing

Necropsies and tissue sampling were performed according to a standard protocol [135]. After fixation in 10% neutral buffered formalin and paraffin embedding, tissue sections were processed routinely for HE staining. The following tissues were examined: esophagus, crop, proventriculus-ventriculus, duodenum, jejunum-ileum, cecum/cecal tonsil, rectum, pancreas, liver, kidney, adrenal gland, gonad, nasal turbinates, trachea, lung, heart, breast muscle, skin, bone marrow, spleen, bursa of Fabricius, thymus, brain, spinal cord, and sciatic nerve. An IHC technique was performed as previously described [76,187]. The primary antibody was a mouse-derived monoclonal commercial antibody against NP of influenza A virus (IgG2a, Hb65, ATCC). As a secondary antibody, a biotinylated goat anti-mouse IgG antibody (GaMb, Dako E0433, Glostrup, Denmark) was used. Tissues previously demonstrated to be positive against NP of influenza A virus by IHC were used as a positive control. Tissues from sham-inoculated animals were incubated without the primary antibody and served as a negative control. The following

score was used in order to grade the staining in tissues: no positive cells (–), single positive cells (+), scattered groups of positive cells (++), widespread positivity (+++).

Viral RNA Detection by RRT-PCR

Swabs were placed in 0.5 ml of DMEM with antibiotics. Additionally, tissue samples from trachea, lung, kidney, and small intestine of H7N2/LP-infected falcons, and from lung, kidney, duodenum/pancreas, liver, and brain of H5N1/HP-infected falcons were placed in 0.5 ml of PBS. Viral RNA was extracted with NucleoSpin® RNA Virus kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. A RRT-PCR assay was used to detect the viral *M1* gene in Fast7500 equipment (Applied Biosystems, Foster City, CA, USA) using the primers and probe previously described [213] and the amplification conditions described by Busquets *et al*. [32]. Samples with a Ct value ≤ 40 were considered positive for influenza A viral RNA.

Oral viral shedding results obtained from RRT-PCR assay were analyzed by Kruskal-Wallis test for significant differences (p <0.05) between H7N2/LP- and H5N1/HP-infected groups and between routes of infection. The statistical tests were performed using the SPSS for Windows Version 17.0.

Serology

A C-ELISA test was carried out to detect antibodies against the NP of influenza A virus using the commercially available kit ID Screen® Influenza A Antibody Competition (IDVET, Montpellier, France), according to the manufacturer's instructions.

Lectin Histochemistry Detection of Influenza Virus Receptors

Lectin histochemistry was carried out in respiratory (nasal turbinates, trachea, and lung) and digestive (proventriculus, duodenum, ileum, cecum, and rectum) tracts of control falcons using the lectins *Maackia amurensis* agglutinin II (MAAII) and *Sambucus*

nigrans agglutinin (SNA), which show affinity for α-2,3 (avian type) and α-2,6 (human type) receptors, respectively.

Lectin histochemistry was performed using previously described procedures [283] with minor modifications. Briefly, 3 mm-thick sections were dewaxed and treated with 3% H_2O_2 in methanol to eliminate endogenous peroxidase activity, washed with TNT (0.1 M Tris HCl, 0.15 M NaCl, pH 7.5) and blocked with TNB (TNT plus blocking reagent) (Perkin Elmer, US) for 30 min at room temperature (RT). Tissue sections were then incubated with biotynilated SNA (10 mg/ml) and MAAII (15 mg/ml) (Vector Laboratories Inc, CA, US) in TNB at 4ºC, overnight. After washing with TNT, sections were incubated with streptavidinhorse radish peroxidase 1:100 for 1 h, and again incubated with SA-HRP for 30 min at RT. The reaction was developed with diaminobenzidine (Sigma-Aldrich, MO, US) at RT for 30 sec followed by counterstaining with Mayer's hematoxylin. To rule out the non-specific binding of lectins, two sequential slides were used as negative controls. One slide was pretreated with NA, which cleaves both α-2,3 and α-2,6 residues, as previously described [283], and the other was incubated with PBS instead of the lectins. Negative controls consisted of the substitution of the lectin with a TNB buffer. Human, pig, and mice tissue samples were used as positive controls because of previous publications reporting the lectin pattern of staining of these species [96,153,154,283]. For each slide, and in order to compare receptor expression patterns among the tissues included in this study, the relative intensity of receptor expression was scored based on the percentage of cells in a section showing positivity, and was graded as: no positive cells (–), single positive cells (+), scattered groups of positive cells (++), widespread positivity (+++).

5.3. RESULTS

Morbidity and Mortality

There were no relevant differences between nasochoanally inoculated animals and animals challenged via the natural feeding route in both H5N1/HP- and H7N2/LP-infection groups. Falcons from both H5N1/HP-challenged groups (groups C and D) showed first clinical signs at 5 dpi which consisted of depression, apathy, impaired respiration and, above all, slight neurological signs that in a matter of hours turned into moderate or severe, and included torticollis, head tilt, ataxia, circling, incoordination, leg/wing paralysis, opisthotonus, and tremors. Some birds were also found recumbent and unresponsive. Following the endpoint criteria established in the ethical protocol, one falcon per group was ethically euthanized at 5 dpi. At 6 dpi, one falcon infected by feeding and showing severe neurological signs was also ethically euthanized, while at 7 dpi one falcon per group was found dead. Remaining falcons (one per group) survived until the end of the experiment (10 dpi) and did not develop clinical signs. To sum up mortality data, two out of three nasochoanally inoculated falcons, and three out of four falcons challenged by feeding died between 5 and 7 dpi, and the MDT was 6 dpi. Neither morbidity nor mortality was observed in the negative control group (group E) or in the H7N2/LP-infected groups (groups A and B).

Gross Findings

Findings associated with AIV infection were only observed in some of the H5N1/HP-infected falcons (groups C and D). Lesions were very similar regardless of the infection route and day of necropsy, being the pancreas, proventriculus, ventriculus, and brain the most affected organs. Regarding the nasochoanal group (group C), multifocal hemorrhagic necrosis in the pancreas was found in the falcon necropsied at 5 dpi (Figure 11), while the falcon found dead at 7 dpi showed multifocal petechia on the proventriculus-ventriculus junction mucosa, as well as brain and eyelid congestion. The surviving falcon from this group also showed multifocal petechia in the proventriculus-

ventriculus junction mucosa at time of necropsy (10 dpi). Concerning the prey ingestion group (group D), the falcon necropsied at 5 dpi did not show significant lesions, whereas multifocal petechia on the proventriculus-ventriculus junction mucosa and brain and eyelid congestion were found in the falcon euthanized at 6 dpi. In this group, the falcon found dead at 7 dpi also showed multifocal petechia on the proventriculus-ventriculus junction mucosa and multifocal hemorrhagic necrosis in the pancreas. No gross lesions were observed in the H5N1/HP-infected falcon of the prey ingestion group euthanized at 10 dpi.

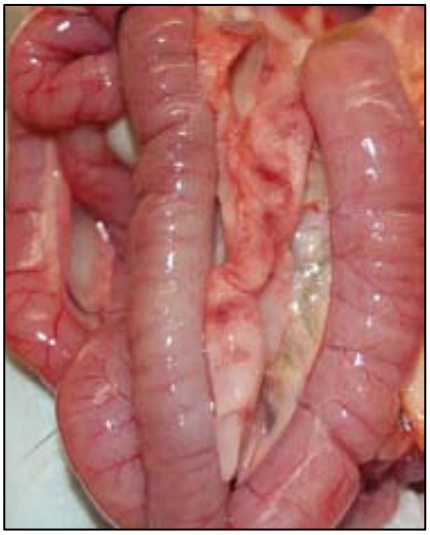


Figure 11. Gross lesions in a falcon infected via the nasochoanal route with H5N1/HP. Multifocal hemorrhagic necrosis in the pancreas, 5 dpi.

Histopathological Findings

Histological lesions and influenza A viral NP antigen were only observed in H5N1/HP-challenged falcons (groups C and D) (Tables 16 and 17). The most severely affected organ was the brain. The main findings consisted in moderate (5–6 dpi) to severe (7 dpi) multifocal areas of malacia in the cortex, present in all hemispheres of the brain, associated with spongiosis of the neuropil, chromatolysis, gliosis, and caryolysis (Figure 12a). Vascular endothelial swelling was also observed, especially at 5–6 dpi. Evident severe necrosis of ependymal cells of the ventricles and epithelial cells of the choroid plexus was present in almost all falcons. The cerebellum frequently showed multifocal areas of moderate to severe chromatolysis of Purkinje neurons, sometimes

associated with necrosis of the Purkinje cell layer and non-suppurative inflammatory infiltrate. Severe non-suppurative choroiditis was observed in two falcons of the prey ingestion group, particularly in the falcon found dead at 7 dpi and in the falcon euthanized at 10 dpi, as well as in the falcon found dead at 5 dpi of the nasochoanal group. Moderate multifocal areas of perivascular cuffing were present, being severe in the falcon from the prey ingestion group necropsied at 10 dpi. This falcon had the same histopathological findings as the neurologically affected falcons, whereas the falcon from the nasochoanal group necropsied at 10 dpi did not show any significant lesions in the brain. Significant microscopic lesions were seen in the spinal cord of the falcon from the nasochoanal group necropsied at 5 dpi, where severe malacia in the grey matter, and severe gliosis and necrosis of the ependym and neuropil surrounding the medullary canal were observed. Interestingly, almost all lung tissues showed positive IHC staining in bronchi, in particular bronchial epithelial cells and goblet cells. In general, antigenic staining was mainly nuclear and also often cytoplasmic in distribution and correlated well with histopathological findings (Figure 12b).

Table 16. Average distribution of AIV-NP antigen in positive tissues from falcons infected via the feeding route with H5N1/HP.

Tissue*	2VN6	4AN6	3ES4	6TN14	Predominant cell types	Associated lesion
	5 dpi	6 dpi	7 dpi	10 dpi		
Pancreas	–	–	–	–	-	NSL
Kidney	+	–	–	–	Collecting tubular epithelial cells	NSL
Nasal turbinates	nd	+	+	–	Lateral nasal gland epithelial cells	Vacuolation, degeneration, mild lymphoplasmacytic infiltrate
Trachea	–	+	–	–	Pseudostratified epithelial cells	Necrosis of pseudostratified epithelium, mild focal tracheitis
Lung	+++	–	nd	nd	Bronchial epithelial cells, goblet cells	NSL
Brain	+++	+++	++	+	Neurons, ependymal cells, glial cells, endothelial cells	Malacia in cortex, necrosis of ependymal cells of ventricles and epithelial cells of choroid plexus, perivascular cuffing,, endothelial hypertrophy, chromatolysis of Purkinje cells
Spinal cord	–	–	–	–	-	NSL

– = no positive cells; + = single positive cells; ++ = scattered groups of positive cells; +++ =widespread positivity.
NSL, no significant lesions; nd, not determined.

Table 17. Average distribution of AIV-NP antigen in positive tissues from falcons infected via the nasochoanal route with H5N1/HP.

Tissue*	1VN6	4CS1	1US18	Predominant cell types	Associated lesion
	5 dpi	7 dpi	10 dpi		
Pancreas	–	–	–	Acinar cells	Lytic necrosis, heterophilic infiltrate
Kidney	+	–	–	Collecting tubular epithelial cells	NSL
Nasal turbinates	–	nd	–	-	NSL
Trachea	–	–	–	-	NSL
Lung	+	+	+	Bronchial epithelial cells, goblet cells	NSL
Brain	+++	+++	–	Neurons, ependymal cells, glial cells, endothelial cells	Malacia in cortex, necrosis of ependymal cells of ventricles and epithelial cells of choroid plexus, perivascular cuffing,, chromatolysis of Purkinje cells, endothelial hypertrophy
Spinal cord	–	–	–	Neurons, ependymal cells, glial cells	Malacia in grey matter, necrosis of the ependyma and neuropil

– = no positive cells; + = single positive cells; ++ = scattered groups of positive cells; +++ =widespread positivity.
NSL, no significant lesions; nd, not determined.

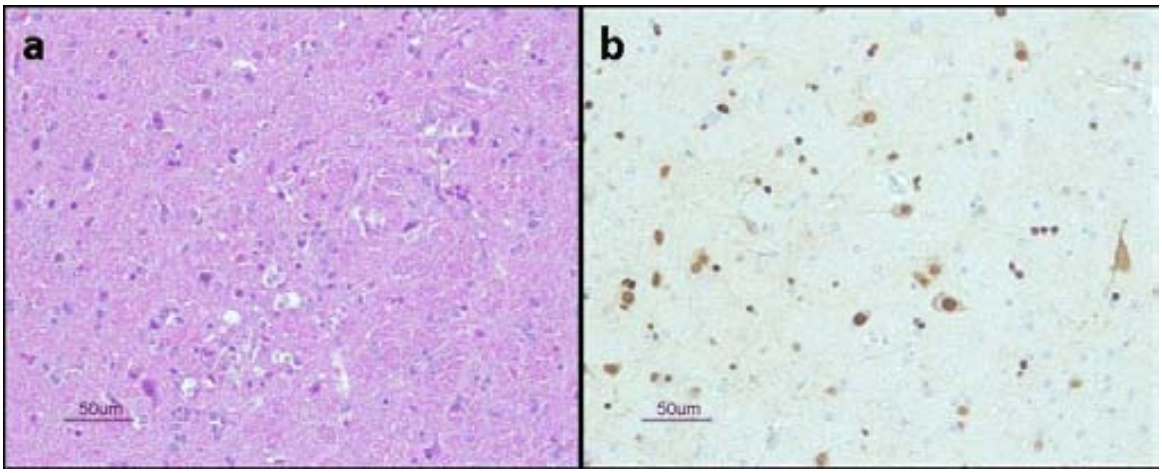


Figure 12. Microscopical lesions and positive NP-viral antigen cells in brain of a falcon infected via the nasochoanal route with H5N1/HP, 5 dpi. a. Focal area of malacia in the cortex (HE). **b.** Neurons and glyal cells (IHC).

Viral RNA Detection by RRT-PCR

Real time RT-PCR was performed on OS and CS and on various tissues obtained at necropsy. In both H7N2/LP-infected groups (groups A and B) (Figure 13a), viral RNA was only detected on OS. Regarding H7N2/LP prey-infected falcons (group B) the amount of viral RNA detected orally was consistent and reached minimum Ct values of 22.29 at 2 dpi. Besides, viral RNA was already detected at 1 dpi in one animal, with a Ct value of 37.31. H7N2/LP viral RNA was detected in all falcons until the end of the experiment (11 dpi), although amounts of viral RNA were evidently declining. Regarding falcons inoculated via the nasochoanal route (group A), detection was similar to that observed in the feeding group, although quantities of viral RNA declined more rapidly. No viral RNA was detected in swabs from the contact animal during the whole experiment.

For H5N1/HP-infected falcons (groups C and D) (Figure 13b), viral RNA detection had a different profile than for H7N2/LP-infected animals. Both nasochoanally and feeding-infected falcons showed viral RNA orally from 1 dpi until prior to death, peaking at 2 dpi (Ct 24.08). Falcons that survived until the end of the experiment (10 dpi) stopped shedding at 7 dpi. In contrast with H7N2/LP-infected falcons, H5N1/HP-infected animals showed some viral RNA detection in CS. In particular, the falcon from the feeding group that was euthanized at 5 dpi showed low amounts of virus (Ct 33.15) at the time of necropsy, as well as two falcons from the nasochoanal group: the one that was euthanized at 5 dpi showed virus from 3 to 5 dpi (Ct 33.77, Ct 37.96, and Ct 29.21), and the one found dead at 7 dpi had a Ct value of 31.51 at 5 dpi.

The statistical analysis performed on the results of the OS until 7 dpi (both individual and mean Ct) showed no significant differences between routes of infection for both H5N1/HP- and H7N2/LP-infected groups (p >0.05), whereas amounts of viral RNA of H7N2/LP-infected groups were significantly higher than in H5N1/HP-infected groups (p <0.05).

Results of viral RNA detection by RRT-PCR in tissues are shown in Table 18. In general, all falcons that showed clinical signs and died, or were ethically euthanized had positive results for almost all the selected tissues. The highest amounts of viral RNA were detected in the brain. The surviving falcon's brain from the feeding group was positive for RRT-PCR.

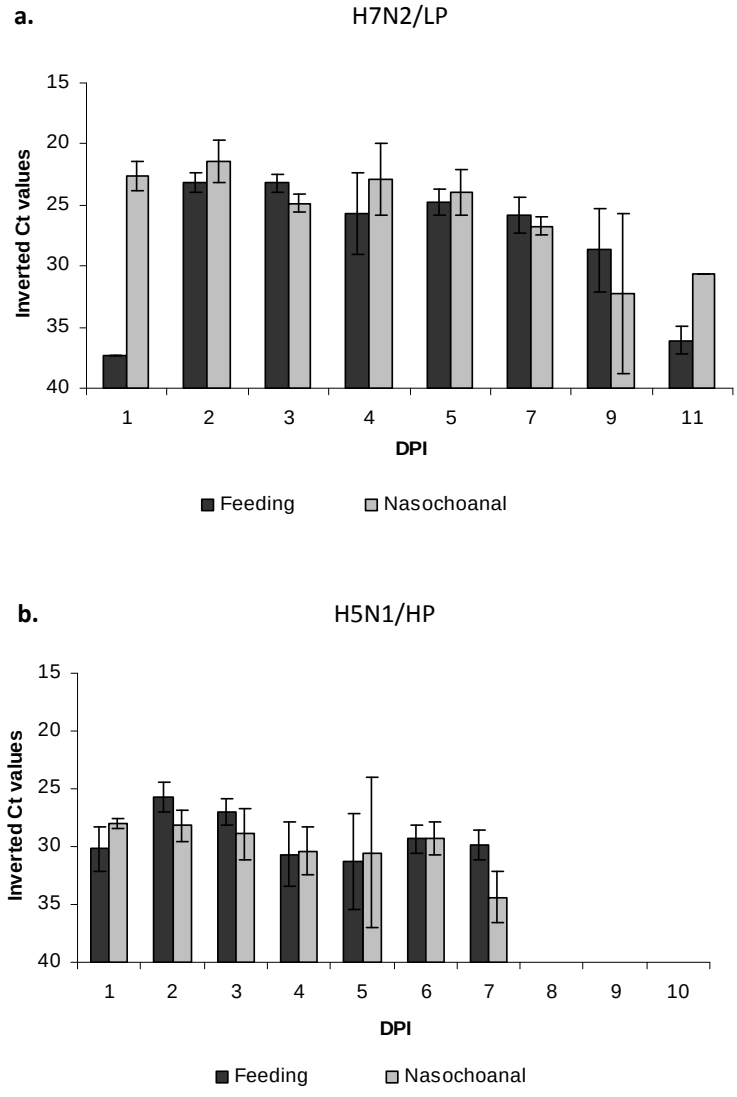


Figure 13. Oral RNA viral shedding from falcons experimentally infected with AIV via the feeding route or via the nasochoanal route. Results are expressed as inverted Ct-values and shown as means of positive individuals ± SD. Ct, cycle of threshold; DPI, days post-inoculation. **a.** Falcons infected with H7N2/LP and euthanized at 11 dpi. **b.** Falcons infected with H5N1/HP.

Table 18. Viral RNA in tissues of falcons infected with H5N1/HP.

Infection route, animal	Day of death	RNA in tissue, Ct value				
		Lung	**Kidney-adrenal gland**	Duodenum-pancreas	Brain	Liver
Feeding						
2VN6	5	24.23	25.42	25.58	15.82	undet
4AN6	6	28.77	undet	34.92	14.45	undet
3ES4	7	29.23	32.67	36.31	14.73	undet
6TN14	10	undet	undet	undet	19.22	undet
Nasochoanal						
1VN6	5	23.90	24.56	26.16	14.43	29.36
4CS1	7	26.48	29.38	28.45	17.84	undet
1US18	10	undet	undet	undet	undet	undet

Ct, cycle of threshold; undet, not detected by RRT-PCR.

Serology

With the exception of the contact bird, all H7N2/LP-infected falcons were seropositive at the end of the experiment (11 dpi). Concerning H5N1/HP-infected falcons, only serum samples from 10 dpi were positive. No seroconversion was observed in the negative control falcons (group E).

Lectin Histochemistry Detection of Influenza Virus Receptors

Lectin histochemistry was carried out in the respiratory and digestive tracts of control falcons in order to assess the distribution pattern of SNA (α-2,6) and MAAII (α-2,3). Regarding respiratory tract (Figure 14), moderate expression of α-2,6 was observed in ciliated epithelial cells and mucous gland cells of the respiratory tract of nasal turbinates and in salivary gland epithelium. Mild expression of α-2,6 receptors was observed in ciliated epithelial cells, mucous gland epithelium, and goblet cells of the trachea. However, expression of receptors was predominantly α-2,3 (avian type) on the respiratory tract, being as follows: strong in bronchial epithelial cells; moderate in

ciliated epithelial cells and goblet cells of the trachea; and mild in ciliated epithelial cells of the respiratory tract of nasal turbinates and in nasal gland epithelium, and in mucous gland epithelium of the trachea. Regarding digestive tract (Figure 15), strong α-2,3 expression was noted in enterocytes of the rectum, and mild α-2,3 expression was observed in goblet cells of the same region. Other cell types, such as endothelial cells, macrophages, and lymphocytes gave mild positive results for both α-2,3 and α-2,6 receptors in the cecum/cecal tonsil and rectum.

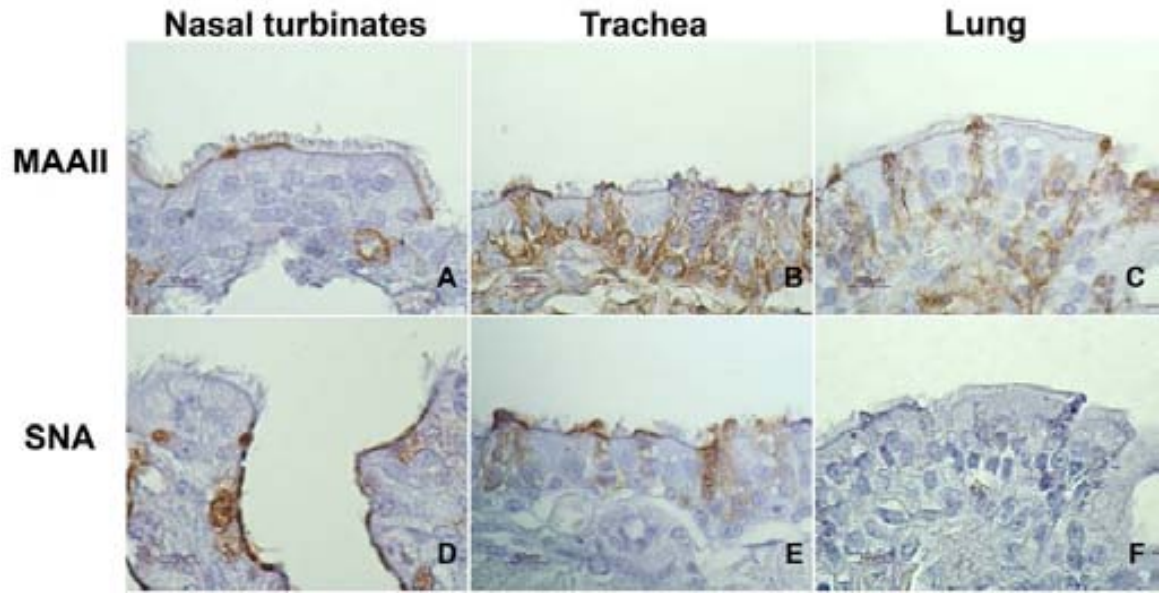


Figure 14. Distribution of α -2,3 and α -2,6 receptors in the respiratory tract of falcons demonstrated by means of MAAII and SNA lectin histochemistry. a. Nasal turbinates stained by MAAII lectin. **b.** Trachea stained by MAAII lectin. **c.** Lung stained by MAAII lectin. **d.** Nasal turbinates stained by SNA lectin. **e.** Trachea stained by SNA lectin. **f.** Lung stained by SNA lectin.

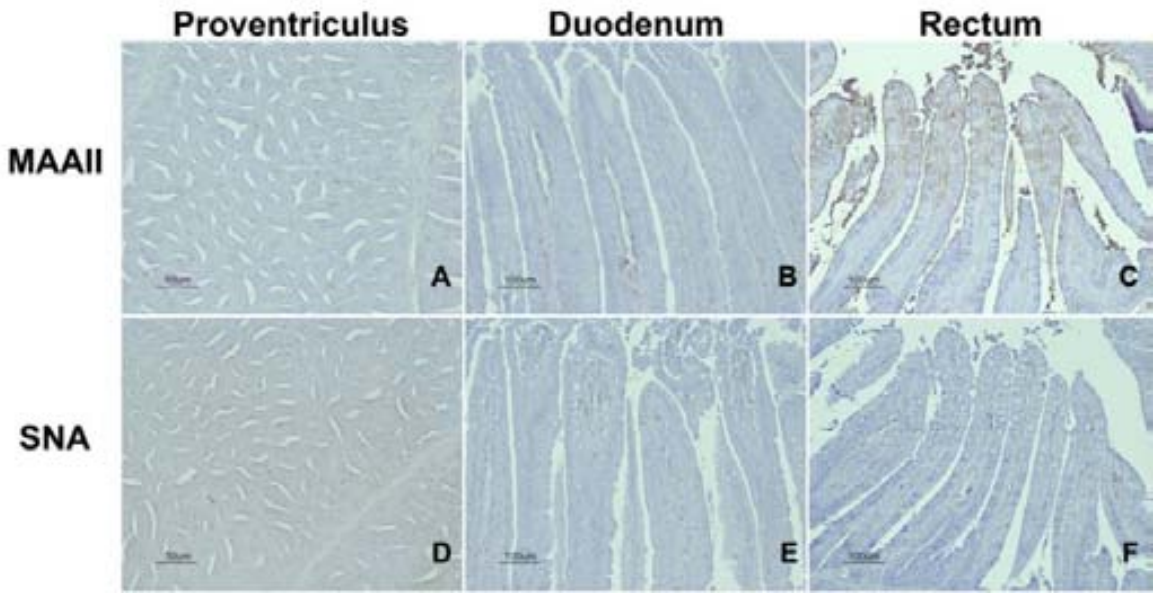


Figure 15. Distribution of α -2,3 and α -2,6 receptors in the digestive tract of falcons demonstrated by means of MAAII and SNA lectin histochemistry. a. Proventriculus stained by MAAII lectin. **b.** Duodenum stained by MAAII lectin. **c.** Rectum stained by MAAII lectin. **d.** Proventriculus stained by SNA lectin. **e.** Duodenum stained by SNA lectin. **f.** Rectum stained by SNA lectin.

5.4. DISCUSSION

This is the first experiment which demonstrates that falcons can be infected with both HPAIV and LPAIV, not only via the nasochoanal route but also by feeding on infected prey. For both viruses, infected falcons exhibited similar infection dynamics despite the different routes of exposure, demonstrating that ingestion of infected SPF chicks is as effective as direct nasochoanal route to produce infection. To the best of our knowledge, this is the first study demonstrating that the consumption of infected prey is a viable route of transmission for both HPAIV and LPAIV in falcons. Other studies have addressed the role of feeding on influenza-infected prey in other animal species [29,114,186]. Infectious dose to which the falcons were experimentally exposed via feeding on infected-SPF chicks could be comparable to the infectious dose to which wild falcons would be exposed during feeding on infected wild preys. This situation could also be feasible in falcons raised for falconry or in wild falcons clinically-admitted in wildlife

rehabilitation centers when fed on AI infected preys (either LPM or wild birds). Therefore, this practice should be closely monitored.

The high pathogenicity of the H5N1 HPAIV strain used in the present study is in agreement with that obtained in other H5N1 HPAI experimental infections in falcons [77,130]. In our study, 5 out of 7 falcons died between 5 and 7 dpi; whether the two surviving falcons would have died in a longer experiment (at some point after the 10 dpi) remains unclear. Nevertheless, clinical signs of H5N1/HP-infected animals were very acute and extremely severe, similar to those observed in Hall's study [77]. Such neurologic disorders would be feasible in natural infected falcons; these signs could be seen in free range birds under surveillance, and would certainly be noticed in animals under captivity. However, evident external lesions that are expected in AI-infected gallinaceous species, such as cutaneous hemorrhages on the legs [176], were not observed in falcons, and thus, may be overlooked during routine necropsy when AI is not suspected. Viral H5N1/HP RNA in tissues correlated well with IHC results, the brain being the most affected organ. Indeed, falcons only demonstrated severe neurological signs prior to death, and only after necropsy and histopathological studies did some of the other tissues exhibit significant lesions. The feeding H5N1/HP-infected falcon that survived until the end of the experiment could possibly have demonstrated clinical signs later on, since the virus was detected in the brain by both IHC and RRT-PCR, and in the lungs by IHC.

Viral shedding was considered mainly oral for H5N1/HP-infected falcons, being consistent and lasting for one week. In both experimental studies with H5N1 HPAIV in falcons performed up to date [77,130] oral shedding seemed to be predominant over cloacal shedding. In addition, the only viral shedding route observed in H7N2/LP-infected falcons was the oral one, which was significantly higher and lasted longer (up to 11 dpi) than in H5N1/HP-infected animals. Moreover, the distribution pattern of influenza virus receptors seems to be in agreement with the pattern of viral shedding observed. The presence of avian-type receptors (α-2,3) in the nasal turbinates, trachea, and bronchial epithelium, and their absence in other parts of the lung, support previous

findings regarding domestic birds that AIVs mainly localize in the upper respiratory tract [3,178], and thus, successful oral shedding is detected. The scarce cloacal shedding observed in some H5N1/HP-infected falcons is in accordance with the absence of avian-type receptors in the intestinal tract, which was only expressed in rectum.

Given that falcons can shed a considerable amount of AIV before the appearance of overt clinical signs or death (if so), this species may contribute to viral transmission within the geographical limits in free-living birds, or to a local outbreak when reared in outdoor operations. Therefore, infected falcons shedding AIV could represent a risk for humans and other valuable bird species when admitted in wildlife rehabilitation centers or during shipping for falconry trade.

Raptors are at the top of their food chain, representing a natural surveillance system that target those subjects more likely to have had HPAIV exposure. However, the possible introduction of HPAIV or LPAIV in raptor populations could have a negative impact on already threatened species. Therefore, surveillance could be an invaluable tool in studying the epidemiological situation of AIVs in raptor and other related wild bird populations. Data obtained in the present study indicates that OS can be successfully used for virus detection in falcon surveillance programs, as is also recommended for other species [19,103]. In addition, not only brain but also pancreas specimens are useful for AIV detection and histopathological diagnosis.

In conclusion, our observations suggest that gyr-saker hybrid falcons are highly susceptible to infection with the H5N1 HPAIV used in this study, and that they may play a major role in spreading AIVs, given that a prolonged and consistent viral shedding has been demonstrated, especially with the H7N2 LPAIV used in this study. Therefore, this species, whether wild or in captivity, should be included in passive surveillance programs in order to prevent risk to humans and other wild bird species, and to minimize the threat of spreading, particularly of HPAIVs within and among countries via animal trade or natural movements.

Part III

General Discussion and Conclusions

Chapter 6

General Discussion

To date, experimental studies and natural infections have assessed the susceptibility of numerous different avian species to LPAIV and HPAIV. Knowledge about AI infection dynamics has traditionally been focused in large-scale produced avian species such as chickens, turkeys, and domestic ducks [5,165,178,212,215,227,247,260]. Besides, some minor avian species like chukar partridges, pheasants, Japanese quail, and ostriches, as well as wild ducks have also been studied to a certain extent [6,94,98,136,176,192]. However, limited information concerning the infection dynamics of AIVs in the red-legged partridge (*Alectoris rufa*), European quail (*Coturnix c. coturnix*), and gyr-saker hybrid falcon (*Falco rusticolus* x *F. cherrug*) exist so far. These species are of considerable interest in some geographical regions, including the Iberian Peninsula, because not only they belong to the local fauna, but they are also raised in small-scale extensive farming systems, leading to close contact with poultry, humans, and neighboring wildlife. To effectively perform surveillance efforts, much more information on host range, transmission cycles, and potential wild avian reservoirs is needed globally.

In the present dissertation, three experimental infections have been presented with the aim to understand the dynamics of AIV infection in three non-conventional avian species, as well as their epidemiological role in an interspecies AI outbreak to better define surveillance strategies. The pathogenesis of the infection with both LPAIV and HPAIV was determined in the red-legged partridge (Study I), European quail (Study II), and gyr-saker hybrid falcon (Study III), with special emphasis on describing the clinical disease, gross and microscopic lesions, together with the presence of viral antigen in tissues. In addition, viral shedding pattern for each avian species and each AIV was defined, and the likelihood of effective viral transmission among birds was assessed. Study II also represents a comparative study of infection dynamics of two different HPAIV subtypes (H7 and H5). In Studies I and II, special attention was paid on feathers as a potential origin of AIV dissemination. Besides, in Study II drinking water was investigated as for being a possible transmission route. In Study III, the natural AIV infection route in falcons (i.e., by ingestion of infected prey) was efficiently reproduced,

and the influenza virus receptors' pattern in this species was elucidated for the first time. The main findings of the present dissertation are reviewed in this general discussion, along with possible improvements that would respond the encountered limitations. Specific aspects of each study were explained in the discussion section of the correspondent chapter and are not going to be considered in this general discussion.

Overall, successful infection was confirmed for the three species. It is worth mentioning that the origin avian hosts of the AIVs tested were as diverse as chicken (H7N1/HP), Eurasian teal (*Anas crecca*) (H7N9/LP), mallard (*Anas platyrhinchos*) (H7N2/LP), and great crested grebe (H5N1/HP). This finding suggests that adaptation may not be needed at this viral dose to allow AIV to replicate and transmit in these species. Therefore, introduction of AIVs could be easily accomplished from such wildlife to poultry populations, and vice versa.

The high pathogenicity of the HPAIVs used in the present experimental infections was confirmed for the three avian species under study, and it was in accordance with previous natural and experimental HPAIV infections in chickens [215,247], other gallinaceous species like chukar partridges [176] and Japanese quail [98,176,192], and birds of prey [77,130]. A peracute progression of the disease prevented the display of overt clinical involvement before death in some individuals. However, progressive neurologic dysfunction (following nonspecific signs) was the most pronounced sign in birds that survived this peracute syndrome, as already observed by diverse authors in other avian species [77,98,130,176,192]. Such neurologic disorders could be seen in free-range birds under surveillance, and would certainly be noticed in reared animals or under captivity. Nevertheless, neurological signs are not pathognomonic for HPAI and can also result from velogenic Newcastle disease, other infectious diseases or non-infectious causes [236]. Gross lesions were observed in tissues that are known to be target organs for influenza A viruses in gallinaceous species [98,164,176,215] and birds of prey [77,130]. However, some evident external gross findings associated with damaged vascular endothelial cells that are expected in AIV-infected land fowl species [164,215] were not exhibited by red-legged partridges dying after 3 dpi, Japanese quail

or gyr-saker hybrid falcons. Such lack of lesions may be partially due to the longer survival of Galliformes other than chickens or turkeys, in which HPAIV produce viremia with minimal vascular endothelial cell replication, but tend to extensively replicate in parenchymal cells of visceral organs [164]. Nevertheless, even if gross lesions in visceral organs were evident, they were not as extensive and severe as in chickens, probably due to differences among bird species or virus strain susceptibility [174,275]. Consequently, AI could be easily disregarded in these species during routine necropsy if the disease is not suspected.

It is believed that the clinical presentation and gross lesions are the mirror of the viral replication extension [248]. In this way, birds in the acute phase (from 5-6 dpi to 7-8 dpi) exhibited lesions in multiple organs, mainly necrosis often with inflammation, hemorrhage, and edema, together with presence of abundant viral antigen by IHC. Interestingly, the longer the birds survived, the less prominent were the necrosis and apoptosis, and the more prominent was the inflammation, as already stated by other authors [164,248]. On the contrary, HPAIV-infected birds in the peracute stage (within 3 dpi) showed mild and multifocal presence of viral antigen mainly in vascular endothelial cells and macrophages and almost without microscopic lesions in visceral organs.

Some considerations may be taken regarding the detection of viral NP antigen in tissues by IHC assay. Such technique allows the detection of virus actually present in the parenchymal cells of a tissue, rather than the overall viral load in a tissue which also includes virus present in the bloodstream (especially limiting in organs such as the heart, lung, and kidney). Moreover, IHC not only allows assessing a semiquantification of the virus-infected cells within a tissue, but also co-localizing histopathological lesions with the presence of AIV antigen. It is worth mentioning that NP antigen may be detected by IHC staining from an active replicating virus in host cells (both in the nucleus and cytoplasm) or from a non-specific uptake of the virus by phagocytic cells (in the cytoplasm). Indeed, target host cells may present NP antigen in the nucleus during polymerase transcription, and when being transported between the nucleus and the

cytoplasm to form RNPs [159,229]. On the contrary, particles of phagocytosed virus in macrophages and heterophils may be only present in their cytoplasm [248].

To date, AI viral pathogenesis in poultry has been extensively studied [176,231,239,245,248]. Based on our findings, HPAIV seems to behave similarly when infecting red-legged partridges, European quail or gyr-saker hybrid falcons. After an initial replication in nasal epithelium, the HPAIV replicates within endothelial cells and spreads via the vascular or lymphatic systems to infect and replicate in the parenchymal cells of a variety of organs. The general predilection of HPAIVs for epithelia of the upper digestive, respiratory, and urinary tracts, pancreas, brain, and myocardium has been widely described in chickens infected with other HPAIV subtypes [25,164,176,241]. In particular, neurons of the brain, kidney tubular cells, pancreatic acinar epithelium, cardiac myocytes, adrenal cortical cells, and pulmonary epithelial cells are the most frequently reported sites of necrosis in H5N1 HPAIV-infected chickens [231], other gallinaceous species [176], and Falconiformes [77,130]. In Study II, where the infection dynamics of two different HPAIV subtypes (H7 and H5) were compared, it is evident that H5N1/HP is more virulent for European quail than the H7N1/HP. Interestingly, H5N1/HP showed a distinct neurotropism, characterized by an earlier arrival into the brain, a more widespread presence of the virus, and more severe histopathological lesions. The central nervous system is the preferred site for HPAIV replication especially in those birds that survive the initial stages of viremia, and it is considered a strong determinant factor for the fatal course of AI in birds [23,42,174]. In addition to the lesions induced directly by viral replication, other mechanisms may contribute to cell, tissue, and organ damage in these avian species, such as the release of cytokines by phagocytic cells that cause vasodilation, increased vascular permeability, and hypercoagulability [18,81,87]. These effects significantly contribute to the development of vascular infection, multiorgan failure (myocardial insufficiency, neurologic dysfunction, adrenal insufficiency), and shock [176]. In addition to indirect endothelial effects, cytokines can cause marked lymphocytolysis via apoptosis [68,81,176], leading to lymphocellular depletion that may immunocompromise the resistance to other potential pathogens in birds surviving

HPAIV infection [176]. Based on the already existing pathobiology classification for the prototype H5N1 HPAIV A/chicken/Hong Kong/220/1997 [164,174], the game birds under study in the present work (red-legged partridges and European quail) would be categorized within Group One when challenged either with H7N1/HP or H5N1/HP (Table 3). Such pathobiology group is defined by a widespread dissemination with relatively rapid and high mortality, and contains other seven gallinaceous species [174]. Interestingly, gyr-saker hybrid falcons would also fit into the same pathobiology group, giving emphasis to the recognition of non-gallinaceous species in their possible epidemiological involvement with HPAIVs.

Regarding LPAIV infection, and in correlation with previous data [164,245,248], LPAIVs produced subclinical infections in the three experimentally infected bird species. Moreover, there was no evidence of lesions (gross or microscopic) associated with LPAIV infection, which fits with the general idea that LPAIV in wild birds does not cause clinical disease and indicates that selection of LPAIV in reservoir species is directed toward low virulence [51]. This concept has been recently investigated in the mallard, an already-recognized reservoir host, and it has been suggested that a strong selection by LPAIV to cause minimal virulence may be favored in maintenance host species, although morphologically unapparent functional changes cannot be ruled out [51]. Besides, such absence of clinical involvement could represent a threat of spreading AIVs. Still, under the natural conditions of a production system, a rehabilitation facility or into the wild, individuals may produce mild to moderate disease syndromes because of accompanying secondary pathogens. Differently to the H7N2/LP infections in quail and falcons, H7N9/LP only produced successful infection in approximately half of the challenged partridges. This could be due to the virus strain, the host species, or both. The HA receptor binding affinity has been identified as an important factor in species adaptation and virulence [139]. In this light, partridges have been evidenced to harbor moderate and low influenza receptors in respiratory and digestive tracts, respectively, compared to strong and moderate presence of receptors in respiratory and digestive tracts of quail

[50]. Nevertheless, further studies should be performed to elucidate the differences in LPAI susceptibility observed among host species.

With the exception of HPAIV-infected red-legged partridges that shed virus almost equally between oral and cloacal routes, oropharyngeal shedding was the principal route of excretion, concurring with previous studies in chickens [215], other game birds [98,136,192], and raptors [77,130]. This is in accordance with the viral antigen detection by IHC in the upper respiratory tract. In contrast, cloacal shedding was generally low and variable, as already observed [31,242]. Previous studies have suggested the intestinal content, urine, bile, and pancreatic excretions to be a source of the virus in feces [264], as all these tracts empty into the cloaca. Because viral antigen was found in intestinal epithelial cells, hepatocytes, kidney, adrenal gland, and pancreas, it is likely that all these tissues could have contributed to the presence of virus in feces, as previously described [264]. Based on the viral shedding and transmission dynamics, the natural infection route in Galliformes may be the oral-oral route, irrespective of being HPAIV (H5 or H7) or LPAIV. Such viral transmission predominance coincides with that observed in chickens [215] and other game fowl [94,98,136,192], although it differs from the classical fecal-oral route described for LPAIVs in waterfowl reservoirs [85,86,274]. What impact this oral shedding pattern may have on persistence in wild populations and habitats, environmental contamination, and transmission between birds (both wild and domestic) is unknown.

Regarding natural AIV infection routes, to the best of our knowledge Study III is the first experimental infection which demonstrates that falcons can be infected with HPAIVs and LPAIVs not only via the nasochoanal route but also by feeding on AIV-infected prey. Recently, Kwon and Swayne [118] suggested that alimentary tract exposure of H5N1 HPAIV in chickens and ducks initiates virus replication in upper alimentary sites with production of prominent AIV-associated lesions, followed by a systemic virus spread and lesions in multiple visceral organs and brain at 2 dpi. In our study, given that: 1) similar infection dynamics were observed regardless of viral exposure routes; and 2) there is a lack of information on AIV replication sites during the

first 5 dpi, we cannot specify if the primary replication site in feeding-infected falcons is the alimentary tract, the respiratory tract or both. However, because oropharyngeal shedding was predominant from 1 dpi onwards, feeding-infected falcons likely inhaled virus particles while consuming preys, a phenomenon that has already been described in foxes [186]. Therefore, it is expected that the natural AIV infection route in birds of prey could be a combination of ingestion and inhalation of virus when cannibalizing infected carcasses. Nonetheless, in order to assess susceptibility, the relative dose of virus required to produce infection via the respiratory versus alimentary tracts in this species should be compared. Besides, pathological studies during earlier dpi would clarify the initial replication sites of feeding-infected falcons. Overall, as it has been stated for other animal species [29,114,186], evidence of effective AIV infection in species of prey through the natural ingestion route is of crucial importance because it highlights the need of closely monitoring falcons raised for falconry purposes or wild type falcons clinically-admitted in wildlife rehabilitation centers when fed on AIV-infected preys (either LPM or wild birds).

Contaminated water has been previously suggested to play a key role in viral transmission in aquatic bird populations [86]; considering results from Study II, it may also be relevant in landfowl. A better ability of HPAIVs (especially H5N1/HP) to replicate in host cells, be shed, and thus, be more likely transmissible to naïve birds was observed. However, H7N2/LP seemed to have a higher persistence in water, which agrees with previous reports [30]. The results from our study support the careful selection of diagnostic approaches for detecting or evaluating AIV persistence and infectivity in either experimental or field settings. Indeed, although AI viral RNA has been previously detected from sediment samples using RRT-PCR [119], future experiments should consider to concentrate water samples through direct culture [78], filters [218] or with formalin-fixed chicken erythrocytes [97,104]. Moreover, in order to define water as an environmental reservoir not only for waterfowl [218] but also for gallinaceous species, long-term experimental studies should be performed. Finally, it is worth highlighting that RNA detection-based diagnostics can be effectively used, but transmission potential will

ultimately require demonstration of infectivity. Overall, our findings indicate that drinking water should be particularly taken into account for game birds raised in outdoor operations, where AIVs from waterfowl could be introduced to the poultry flock. Nevertheless, more sensitive detection methods to evaluate field samples and a standardized protocol for environmental studies or surveillance are needed.

Feathers could be an apt source for HPAIV transmission in both game fowl species investigated herein. In fact, HPAIV replicated in the feather epithelium, and viral RNA was present in FP samples in high loads – particularly in partridges, even higher than in swabs. Previously, Busquets *et al*. [31] already detected high viral loads in FP during active H7N1 HPAIV infection and in line with viremia levels. As FP is highly vascularized, the elevated amounts of HPAIV may not only be due to an active replication in feather epidermal cells [280], but it may also be favored by the blood vascularizing these tissues [31]. Hence, probably because LPAIV infection is not systemic, viral RNA was never detected in feathers from LPAIV-infected birds. Alarmingly, it is believed that high H7N1 HPAIV titers may be present in feathers not only during active infection, as also observed in the present studies, but also for several days post-mortem [31]. To date, the relevance of feathers as a location for viral replication and potential origin of dissemination in H5N1 HPAIV infection has been evidenced in call ducks (*Anas platyrhinchos* var. *domestica*) [278], Pekin ducks [133], domestic geese (*Anser cygnoides* var. *domestica*) [279], naturally infected whooper swans (*Cygnus Cygnus*) [281], and chickens [31]. Altogether, these results suggest that feathers could represent a source of environmental contamination, as well as a source of infection for birds (including birds of prey) or mammals that pick dead individuals, and thus, eat infected feathers or inhale their dusts. Furthermore, feathers may represent a risk of zoonotic infection when being plucked from dead infected birds without appropriate protective equipment, as already suggested for waterfowl [281]. Consequently, biosecurity measures consisting in fast elimination of bird carcasses should be implemented on farms and open-range facilities. As already done in ducks [278], further experimental studies in red-legged partridges and European quail confirming oral infection through feathers should be performed.

Moreover, assessment of the viral replication in feather follicles of naturally infected gallinaceous birds would clarify whether there is an epidemiological risk of viral transmission to predators in the wild.

The viral shedding pattern observed for the three species seems to be in agreement with the distribution pattern of influenza virus receptors. Red-legged partridges may have moderate presence of avian-type receptors (α-2,3) and human-type receptors (α-2,6) in the nasal cavity, and relatively low presence of receptors throughout the intestinal tract [50]. Besides, moderate to strong presence of functional binding of both types of receptors in the respiratory and intestinal tracts have been detected in European quail [50], as well as in the Japanese subspecies [73,267,286]. Based on the results in Study III, avian- and human-type receptors seem to co-localize mainly in the upper respiratory tract of falcons. Altogether, such results indicate that the three studied species harbor functional influenza virus receptors capable of producing effective infection and successful transmission to naïve birds. In addition, particularly European quail may have the potential of acting as an intermediate host for avian/mammalian reassortant viruses, as already stated for Japanese quail [136,173,257]. Nevertheless, further experimental infections with the mentioned species should be performed to address such reassortant ability.

It is noteworthy that the viral loads obtained from shedding samples (Studies I, II, and III), tissues (Study III), and drinking water (Study II) were detected using a RT-PCR technique. Such technique has been previously reported as a feasible alternative to the expensive and time-consuming AIV titration in embryonating chicken eggs [127]. However, RT-PCR assay is unable to discriminate RNA forming encapsidated and intact envelope viruses from defective particles which are not considered infectious [31,213]. Therefore, the use of other techniques such as virus titration in eggs or in MDCK cells [213,214] would be helpful to confirm the infectiveness of the viruses present in these samples. In spite of the limitations of RT-PCR assay, effective viral transmission from inoculated birds to naïve contact birds was confirmed in European quail for the three studied viruses (H5N1/HP, H7N1/HP, and H7N2/LP) and in red-legged partridges for the

H7N1/HP. Such evidence indicates that particularly European quail may be prone to act as an intermediate host species, similarly to what has been suggested for Japanese quail [38,47,67,91,136,172,210,257]. Moreover, because European quail have shown to be adapted to AIVs from diverse hosts, they could readily re-introduce these viruses to their original host population, playing a key role in a secondary dissemination or farm-to-farm spread.

Although further studies with HPAIV and LPAIV strains should be performed, our observations suggest that the red-legged partridge, European quail, and gyr-saker hybrid falcon could be considered avian species of special interest under the AI epidemiological viewpoint, and thus, should be included in surveillance programs. On the one hand, farm-reared red-legged partridges and European quail could contribute to a local outbreak and spread the disease into the wild when releasing apparently healthy individuals for hunting purposes. As a result, the implementation of sanitary surveillance measures before and after release would avoid introduction of AIVs in the natural ecosystem and would provide perspective to anticipate the course of emerging AIV problems related to domestic animal, wildlife, and public health. On the other hand, shedder falcons could represent a risk for humans and other valuable bird species when admitted in wildlife rehabilitation centers or during shipping for falconry trade. Besides, free-living birds of the three species may contribute to viral transmission within their geographical limits.

Currently, active AI surveillance activities include sampling of both OS and CS, as well as blood [145,185]. Passive surveillance of dead or moribund birds involves the same samples as for active surveillance (when possible) along with tissue collection through necropsy [145,185]. Our results strongly favor OS as the most suitable sample for AIV diagnosis in the three studied species, and FP for HPAIV diagnosis at least in the two gallinaceous species, in accordance with previous reports with chickens [31]. In addition, brain, pancreas, and heart specimens of the same species would be suitable in passive surveillance for virus detection and histopathological diagnosis when HPAIV is suspected.

In summary, the present dissertation highlights the importance of studying the susceptibility, infection dynamics, and transmission likelihood of AIVs in host avian species in the domestic-wild interface. A complete understanding of the host range of these viruses, maintenance cycles, and potential for dissemination is critical. Furthermore, putative impacts associated with introduction of AIVs within avian species that come into close contact with poultry, wild birds, and humans need to be defined. Such knowledge is crucial to improve surveillance efforts, develop appropriate preventive measures, and successfully manage AI outbreaks when different avian species are involved.

Chapter 7

Conclusions

1. Experimentally infected red-legged partridges (*Alectoris rufa*) and European quail (*Coturnix c. coturnix*) have been proved to be highly susceptible to and effective disseminators of the tested HPAIV. Therefore, both land-based birds could act as sentinel species and potential spreaders of AIV into the wild.

2. The H7N9 LPAIV does not produce an effective infection and transmission in red-legged partridges (*Alectoris rufa*).

3. Experimental infection of European quail (*Coturnix c. coturnix*) with H7N2 LPAIV resulted in long-lasting shedding and efficient transmission of the virus. Thus, such game bird could be considered as reservoir species for LPAIVs.

4. Gyr-saker hybrid falcons (*F. rusticolus x F. cherrug*) were successfully infected with H5N1 HPAIV and H7N2 LPAIV via the nasochoanal route and by feeding on infected prey. Both routes induced similar infection dynamics for each tested AIV, and viral shedding was in accordance with the expression of influenza virus receptors in this species.

5. Regardless of the challenge route, high susceptibility to and consistent shedding of H5N1 HPAIV were observed in gyr-saker hybrid falcons (*F. rusticolus x F. cherrug*), representing a potential risk for humans and other valuable bird species.

6. Red-legged partridges (*Alectoris rufa*), European quail (*Coturnix c. coturnix*), and gyr-saker hybrid falcons (*F. rusticolus x F. cherrug*) displayed a predominant oral shedding pattern for the studied AIVs. Therefore, oral swabs could be the most suitable sample for AIV diagnosis in the three investigated species.

7. Feathers have been confirmed as a location for viral replication and potential source of dissemination of H7N1 HPAIV in red-legged partridges (*Alectoris rufa*), and H5N1 HPAIV and H7N1 HPAIV in European quail (*Coturnix c. coturnix*). Hence, feathers can be used for HPAIV diagnosis in these two gallinaceous species.

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VETERINARY RESEARCH

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Pathogenesis and transmissibility of highly (H7N1) and low (H7N9) pathogenic avian influenza virus infection in red-legged partridge (*Alectoris rufa*)

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Abstract

An experimental infection with highly pathogenic avian influenza virus (HPAIV) and low pathogenic avian influenza virus (LPAIV) was carried out in red-legged partridges (*Alectoris rufa*) in order to study clinical signs, gross and microscopic lesions, and viral distribution in tissues and viral shedding. Birds were infected with a HPAIV subtype H7N1 (A/Chicken/Italy/5093/1999) and a LPAIV subtype H7N9 (A/*Anas crecca*/Spain/1460/2008). Uninoculated birds were included as contacts in both groups. In HPAIV infected birds, the first clinical signs were observed at 3 dpi, and mortality started at 4 dpi, reaching 100% at 8 dpi. The presence of viral antigen in tissues and viral shedding were confirmed by immunohistochemistry and quantitative real time RT-PCR (qRRT-PCR), respectively, in all birds infected with HPAIV. However, neither clinical signs nor histopathological findings were observed in LPAIV infected partridges. In addition, only short-term viral shedding together with seroconversion was detected in some LPAIV inoculated animals. The present study demonstrates that the red-legged partridge is highly susceptible to the H7N1 HPAIV strain, causing severe disease, mortality and abundant viral shedding and thus contributing to the spread of a potential local outbreak of this virus. In contrast, our results concerning H7N9 LPAIV suggest that the red-legged partridge is not a reservoir species for this virus.

Introduction

In recent years, avian influenza has become one of the most important challenges that have emerged from animal reservoirs [1,2]. The current outbreaks detected in poultry and wild birds in many Asian, European and African countries are of concern not only to the poultry industry, in which they produce an economically devastating disease, but also to public health [3]. The potential of these viruses to cause a pandemic represents a constant threat to poultry, wild birds and humans worldwide, underlining the importance of avian reservoirs for any subtype of avian influenza virus. The epidemiology of avian influenza viruses is complex, and there are still many unknown aspects, especially in relation to the reservoir. Wild birds, particularly those belonging to the orders *Anseriformes* and *Charadriiformes*, have long been recognised as the natural reservoir for influenza A viruses [4]. Since its first isolation from wild birds in 1961, influenza A viruses have been isolated from 105 wild bird species belonging to 26 families [3,5].

Some studies suggest that turkeys, pheasants, and Japanese quails are more susceptible than chickens to infection by avian influenza viruses transmitted from free-living aquatic birds [6,7]. Experimental infections have shown that highly pathogenic avian influenza virus (HPAIV) can cause specific clinical signs and mortality in the above mentioned species [8], and that pheasants are efficient shedders of low pathogenic avian influenza virus (LPAIV) [6]. Furthermore, open range raising of birds has been identified as one of the factors contributing to the increase of avian influenza virus outbreaks

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and their impact [3]. Nevertheless, to date, most experimental studies on avian influenza are based on either chickens, turkeys or waterfowl species, while investigation into the ability of influenza A viruses to replicate in minor poultry species is scarce [6,8,9], and numerous aspects of the epidemiology of both LPAIV and HPAIV in free-range raised poultry and game birds still remain unclear.

Surprisingly, there are no studies about the susceptibility to infection and the pathogenicity of avian influenza in red-legged partridge (*Alectoris rufa*). This important game bird species is widely distributed in south-western Europe and in the south of England. During the last decades, natural populations of this game bird have declined in most of its distribution range [10]. The strategy of many hunting estate managers to overcome the lack of wild partridges has been the release of farm-reared birds. Red-legged partridges are raised in outdoor operations that are abundant in Spain, comprising currently 7% of the global avian production system [11]. Although some authors have stated that every year, between 3 and 4.5 million of farm reared red legged partridges are released into the wild [12], considering recent information from hunters, farms, hunting estates and numbers of captures, the real number of partridges released in Spain could be quite close to 10 million per hunting season [13]. The lack of adequate biosecurity measures in part of the red-legged partridge farms, together with limited sanitary control measures prior to and after release into the wild, could favour the introduction, adaptation, maintenance, and spread of pathogens including avian influenza (AI) viruses.

In the present study, an experimental infection with both LPAIV and HPAIV was carried out in red-legged partridges in order to determine clinical signs, gross and microscopic lesions. Viral distribution in tissues and the extent and duration of viral shedding were also evaluated by means of qRRT-PCR and immunohistochemistry. In addition, the ability of effective transmission among animals was also assessed.

Materials and methods

Viruses

For the present study, two strains of avian influenza virus were used. An HPAIV H7N1 subtype isolate (A/Chicken/Italy/5093/1999) was kindly provided by Dr Ana Moreno from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna (IZSLER). A LPAIV H7N9 subtype isolate (A/*Anas crecca*/Spain/1460/2008) was obtained from the ongoing surveillance program carried out in Catalonia, north-east Spain. Designations hereafter will be H7N1 for the A/Chicken/Italy/5093/1999 virus and H7N9 for the A/*Anas crecca*/Spain/1460/2008 virus. The amino acid sequences at the HA0 cleavage site were PEIPKGSRVRR*GLF for the isolate H7N1 and PEIPKGR/GLF for the isolate H7N9.

Stocks of avian influenza viruses were produced in 9-day-old embryonated specific pathogen free (SPF) chicken eggs, by a sixth passage in the H7N1 strain and by a first passage in the H7N9 strain. In both cases, the allantoic fluid was harvested at 48 hours post inoculation, aliquoted and stored at -80°C until use. Virus was diluted tenfold in phosphate buffer saline (PBS) for titration in 9-day-old embryonated chicken eggs. The 50% egg lethal dose (ELD) for H7N1 subtype, and the 50% egg infective dose (EID) for H7N9 subtype, were determined using the Reed and Muench method [14]. The H7N1 subtype demonstrated an intravenous pathogenicity index (IVPI) of 3.0 [15] and showed an amino acid sequence in the cleavage site characteristic of HPAIV [16].

Animals

Fifty-six red-legged partridges of two months of age were used in this study. Male and female birds were included in approximately equal numbers. The animals were raised in the experimental farm of Instituto de Investigación en Recursos Cinegéticos (IREC), where serum samples were collected and tested, prior to inoculation, to ensure that birds were serologically negative for avian influenza virus by a competition ELISA test (ID-VET, Montpellier, France) and a specific hemagglutination inhibition (HI) test for the H7 subtype. Upon arrival at the Centre de Recerca en Sanitat Animal (CReSA), the animals were housed in biosafety level 3 (BSL-3) facilities. The partridges were kept one week for acclimation, and then they were randomly assigned to experimental groups and housed separately in negative-pressured isolators with HEPA-filtered air. Food and water were provided *ad libitum* throughout the experiment.

Experimental design

Fifty-six birds were separated into five groups. For each virus, the partridges were subdivided into two experimental groups composed of twelve partridges. Groups A (1A and 2A) were used to evaluate the mortality and transmissibility of the viruses, as well as the virus shedding pattern. Groups B (1B and 2B) were used for pathological studies. Both groups infected with the HPAIV subtype (groups 1A and 1B) were inoculated intranasally with 10^6 ELD_{50} of the H7N1 strain. In group 1A, 4 out of 12 partridges were not infected but placed into the isolator with the inoculated birds one hour after inoculation; these uninfected birds were referred to as contacts. Both groups infected with the LPAIV subtype (groups 2A and 2B) were inoculated intranasally with 10^5 ELD_{50} of the H7N9 strain. As in the case of group 1A, in group 2A four contacts were

included that were not infected but placed into the isolator with the inoculated birds one hour after inoculation. A fifth group (3) of eight partridges was used as control; these birds were inoculated intranasally with PBS solution. All procedures were performed according to the requirements of the Ethics Committee of Animal and Human Experimentation of the Universitat Autònoma de Barcelona.

Sampling

All birds were monitored daily for clinical signs and scored following the OIE system [17]: healthy (0), sick (1), severely sick (2), moribund or dead (3). Since this is a subjective clinical assessment, "sick" birds would be the ones showing one of the following signs, and "severely sick" more than one of the following signs: respiratory involvement, depression, diarrhoea, cyanosis of the exposed skin or wattles, oedema of the face and/or head, nervous signs. Every day during the first 10 days post infection (dpi), and also at 12 dpi and 15 dpi, oropharyngeal and cloacal swabs, and feather pulp samples were obtained from partridges of groups 1A and 2A in order to measure viral shedding. The same samples were collected at 3, 6, 10 and 15 dpi from the control group. Mortality and mean death time (MDT) were calculated from these three groups. At 3, 6, 10 and 15 dpi, three animals of groups 1B and 2B, and two animals of the control group, were euthanised. All euthanised and naturally dead partridges were necropsied to evaluate gross lesions and obtain samples for pathological studies. Blood samples were collected in tubes without anticoagulant at 0, 6, 8, 10 and 15 dpi from those animals ethically euthanised. Samples collected for detection of viral shedding and serum samples were stored at -80°C until use.

Histopathology

Necropsies and tissue sampling were performed according to a standard protocol. After fixation in 10% neutral buffered formalin and embedding in paraffin, tissue sections were processed routinely for haematoxylin/eosin (H/E) staining. The following tissues were examined: oesophagus, crop, proventriculus, gizzard, duodenum, jejunum-ileum, caecum/cecal tonsil, colon, rectum, pancreas, liver, kidney, adrenal gland, gonad, nasal turbinates, trachea, lung, heart, breast muscle, skin, bone marrow, spleen, bursa of Fabricius, thymus, brain, spinal cord and sciatic nerve.

Avian influenza virus detection by immunohistochemistry (IHC)

An immunohistochemical technique based on Avidin-biotin complex immunoperoxidase (ABC) system was performed as previously described [18,19]. The primary antibody was a mouse-derived monoclonal commercial antibody against nucleoprotein of influenza A virus (IgG2a, Hb65, ATCC). As a secondary antibody, a biotinylated goat anti-mouse IgG antibody (GaMb, Dako E0433, Glostrup, Denmark), was used. As positive control, tissues previously demonstrated to be positive against nucleoprotein of influenza A virus by IHC were used. Negative controls were tissues from sham-inoculated animals and tissues incubated without the primary antibody. The following score was used in order to measure the staining in tissues: no positive cells (-), single positive cells (+), scattered groups of positive cells (++), widespread positivity (+++).

Avian influenza virus quantitation by real time RT-PCR (qRRT-PCR)

Viral RNA quantitation using one step qRRT-PCR was carried out in oropharyngeal and cloacal swabs, and feather pulp samples. Viral RNA was extracted with QIAamp viral mini kit (Qiagen, Valencia, CA, USA) and amplified as previously described [20] in Fast7500 equipment (Applied Biosystems, Foster City, CA, USA). A one step qRRT-PCR assay for *M* gene was applied to determine the viral RNA titre, detecting viral RNA genome (vRNA), the copy of vRNA (cRNA) and mRNA. The limit of detection of the technique was 1.46 $\log_{10}$ viral RNA copies/sample.

Serology

A competitive enzyme-linked immunosorbent assay (C-ELISA) test was carried out in order to detect avian influenza antibodies using a commercially available C-ELISA kit (ID-VET, Montpellier, France) performed according to the manufacturer's instructions.

Results

Clinical signs

Clinical signs were only observed in H7N1 infected partridges, which showed signs from scores 1 to 3. There were no relevant differences in clinical signs between inoculated and contact animals.

All birds infected with H7N1 showed clinical signs that started at 3 dpi and consisted in depression, apathy and ruffled feathers. Impaired respiration and diarrhoea were observed in some of the animals. At 8 dpi, 3 of the 4 surviving partridges presented severe neurological signs consisting in torticollis, circling, incoordination, leg/wing paralysis, opisthotonus and head tremors while two birds were recumbent and unresponsive. Mortality started at 4 dpi and lasted until 8 dpi. Intranasal inoculation of the H7N1 virus resulted in 100% mortality, and mean death time (MDT) was 6.42 dpi. Birds with neurological signs, together with the two other animals that presented prostration, were euthanised for ethical

reasons. No mortality or clinical signs were observed in H7N9 infected partridges, and in the controls.

Gross findings

Lesions associated with influenza were observed only in H7N1 infected partridges from 3 dpi onwards. HPAIV infected partridges, both inoculated and contact, were generally in bad body condition. At 3 dpi, petechial haemorrhages on the *fasciae* sheaths of the muscles of rear legs were seen in some birds, and thymus atrophy was detected until the end of the experiment. Kidney lesions were present from 3 dpi onwards and were characterised by parenchymal pallor and accentuated lobular surface architecture, often accompanied by urate deposits in the urethers (Figure 1). Some partridges showed brain congestion from 6 dpi onwards, and, in most cases, hyperaemic vessels were detected in almost all organs. No lesions were observed in H7N9 infected birds, and birds from the control group.

Histopathological findings

Histological lesions were only observed in H7N1 infected partridges. The onset of microscopic findings was at 3 dpi, with the lesions being the most intense between 6 dpi and 8 dpi. The most severely affected organs were the kidney, adrenal gland, feather follicles and CNS (brain and spinal cord). Only the gonads, spleen, bone marrow and sciatic nerve did not show significant histopathological changes. No significant lesions were observed in H7N9 inoculated animals, and in the control birds.

Lesions in the digestive tract, liver, pancreas, kidney, adrenal gland, myocardium, breast muscle, Bursa of Fabricius and respiratory tract (Figure 2A) were mostly characterised by necrosis and light to moderate heterophilic infiltrates. Necrosis of the epidermal collar epithelial cells, in some cases in association with heterophilic infiltrate, was observed in feather follicles from 6 dpi onwards. In the brain, the most striking finding consisted in multifocal areas of malacia (Figure 3A). Evident necrosis of ependymal cells of the ventricles and epithelial cells of the choroid plexus was present. The cerebellum frequently showed multifocal areas of moderate chromatolysis of Purkinje neurons. Similar lesions were seen in the spinal cord from 6 dpi onwards; multifocal areas of mild spongiosis of the neuropil and neuronal chromatolysis, especially surrounding the medullary canal, were observed. In addition, some animals at 8 dpi showed focal heterophilic inflammatory infiltrate in the leptomeninges.

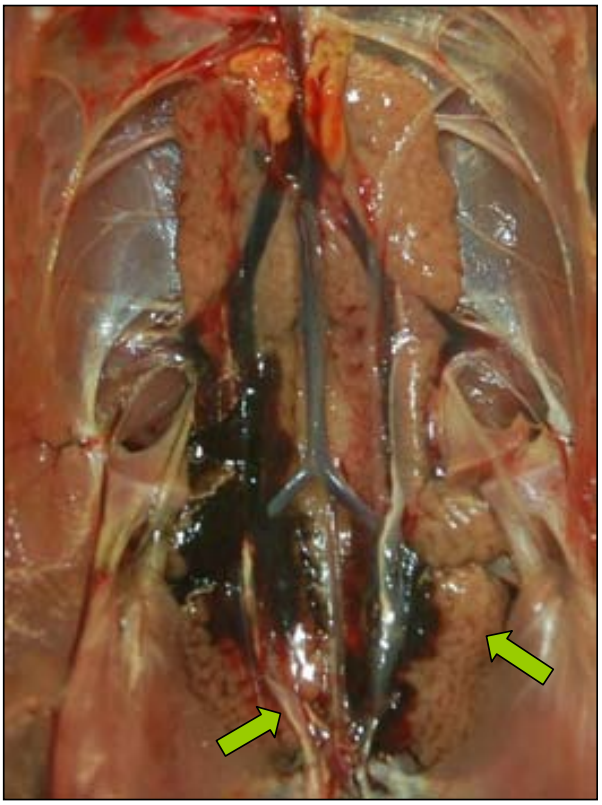

Figure 1 Kidney lesions of a H7N1 HPAIV (A/Chicken/Italy/5093/1999) infected partridge consisting in parenchymal pallor, lobular surface architecture and urate deposits in the urethers, 6 dpi.

Avian influenza virus detection by immunohistochemistry (IHC)

Influenza A viral antigen was only detected in tissues of H7N1 infected partridges. In some organs, virus was more frequently and intensely detected, such as the gizzard, pancreas, kidney, adrenal gland, feather follicles and CNS (brain and spinal cord) (Figures 2B, 3B). Antigenic staining was observed both in parenchymal and endothelial cells; it was nuclear and also often cytoplasmic in distribution. In general, positive staining correlated well with histopathological findings (Table 1).

Avian influenza virus quantitation by real time RT-PCR (qRRT-PCR)

qRRT-PCR was performed on oropharyngeal and cloacal swabs, and feather pulp samples. In H7N1 inoculated birds, virus was detected in oropharyngeal swabs from 1 dpi to the end of the experiment; in cloacal swabs and feather pulp, viral shedding was observed from 2 dpi to 8 dpi (Figure 4A). Concerning H7N1 contact birds, detection was similar to that observed in inoculated partridges, although it started one day later (Figure 4B). Detection levels for these H7N1 inoculated animals ranged between 4 and 10 $\log_{10}$ viral RNA copies/sample, and feather pulp shedding was higher than in both

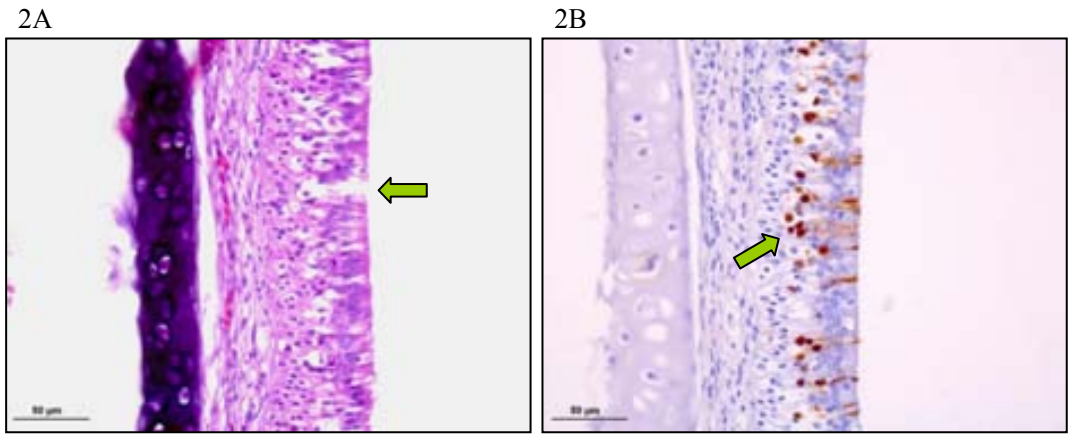

Figure 2 Nasal turbinates, 6 dpi; (A) Necrosis of single cells of the olfactory epithelium, H/E. (B) Positive staining in olfactory epithelial cells, IHC.

oropharyngeal and cloacal swabs, particularly between 2 and 8 dpi.

Among the H7N9 infected birds, 6 out of 8 inoculated birds showed viral shedding mainly by the oropharyngeal route from 1 to 3 dpi (Figure 4C). One infected animal excreted virus by this route until 8 dpi. Only one animal shed minimal amounts of virus (2.37 $\log_{10}$ viral RNA copies/sample) by cloacal route at 1 dpi, and no viral shedding was detected in the feather pulps. Contact animals in this group did not shed virus by any of the studied routes during the whole experiment.

Serology

H7N1 infected birds were ELISA positive from 6 dpi onwards; interestingly, 2 out of 4 seropositive partridges at 8 dpi were contact birds. On the contrary, 3 out of 8 H7N9 infected partridges showed ELISA positive results at 15 dpi. These three animals were the ones that excreted virus in a more consistent manner. None of the four contact animals in this group seroconverted, suggesting that these birds did not get infected by contact.

Discussion

Although the red-legged partridge is one of the game bird species most frequently raised in outdoor operations, no studies had previously investigated the infection dynamics of avian influenza viruses in this species. In order to elucidate their putative role in the ecology of influenza A viruses, we evaluated the susceptibility of red-legged partridges to an infection with a HPAIV H7N1 strain (A/Chicken/Italy/5093/1999) and a LPAIV H7N9 strain (A/*Anas crecca*/Spain/1460/2008) by studying pathogenesis, transmission and viral shedding.

The high pathogenicity of this H7N1 HPAIV strain, evidenced by 100% mortality in this study, is in accordance with standardised IVPI tests for influenza viruses [15], and in agreement with those obtained in natural H7N1 HPAIV infections in chickens [21]. The only

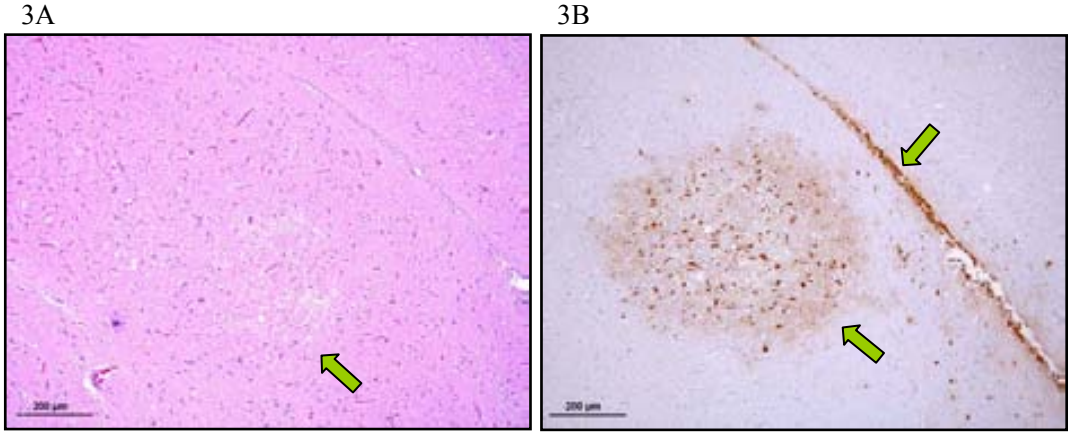

Figure 3 Brain, 5 dpi; (**A**) Focal areas of malacia, H/E. (**B**) Positive staining in neurons, ependymal cells and glial cells, IHC.

Table 1 Average distribution of nucleoprotein antigen, as determined by immunohistochemistry, in tissues sampled from red-legged partridges (*Alectoris rufa*) intranasally inoculated with A/Chicken/Italy/5093/1999 (H7N1) influenza virus

Tissue	3 dpi	6 dpi	8 dpi	Predominant cell types
Esophagus	-	-	-	-
Crop	-	+	-	Squamous polistratified epithelial cells
Proventriculus	-	+	-	Epithelial cells of the gastric glands
Gizzard	+	++	+	Epithelial cells of the gastric glands, cells of the muscularis externa
Duodenum	-	-	-	-
Jejunum-Ileum	-	-	-	-
Cecum/Cecal tonsil	-	+	-	Epithelial cells of the glands, cells of the muscularis externa
Colon	-	-	-	-
Rectum	-	-	-	-
Pancreas	+	+	+	Acinar cells, endothelial cells
Liver	+	+	+	Kupffer cells, endothelial cells
Kidney	++	+++	++	Tubular epithelial cells, endothelial cells
Adrenal gland	+	+++	+++	Corticotrophic, corticotropic cells
Gonad	-	-	+	Epithelial cells of the oviduct
Nasal turbinates	-	+	+	Olfactory epithelial cells, respiratory epithelial cells, epithelial cells of the infraorbital sinuses, salivary and nasal glands
Trachea	+	-	+	Pseudostratified epithelial cells
Lung	+	+	+	Air capillaries cells, macrophages, endothelial cells
Heart	-	+	+	Myocardyocytes, endothelial cells
Breast muscle	-	+	-	Myocytes, endothelial cells
Skin	-	++	+	Epithelial cells of epidermal collar of feather follicles, endothelial cells of pulp
Bone marrow	-	-	-	-
Spleen	+	+	+	Macrophages, endothelial cells
Bursa of Fabricius	-	+	-	Macrophages, endothelial cells
Thymus	-	-	-	-
Brain	-	++	+++	Neurons, ependymal cells, glial cells
Spinal cord	-	+	++	Neurons, ependymal cells, cells of the leptomeninges
Sciatic nerve	-	-	-	-

- = no positive cells; + = single positive cells; ++ = scattered groups of positive cells; +++ = widespread positivity.

experimental infection published so far with HPAIV in partridges used an H5N1 HPAIV strain as inoculum [8]. In this experiment, 75% of mortality was observed in Chukar partridges (*Alectoris Chukar*) and MDT was shorter than in our experiment (4.5 dpi). Therefore, mortality due to infection with H7N1 in red-legged partridges seems to appear slightly later than in the H5N1 infected chicken and Chukar partridges [8]. This delay in the onset of mortality could be due to the unique virulence of the H5N1 HPAIV [22]. Clinically, progressive neurologic dysfunction, the most pronounced sign in surviving birds, correlated with the observations of Perkins and Swayne [8] in Chukar partridges. Gross lesions were observed in tissues that are known to be target organs for influenza A viruses in other gallinaceous species [8,9], such as the kidney or *fasciae* sheaths of the muscles. The general predilection of the virus for epithelia of the upper digestive, respiratory and urinary tract, pancreas and liver, feather pulp and CNS, has been extensively described in chickens infected with other HPAIV subtypes [8,23,24]. Localisation of H7N1 antigen in the parenchyma of other organs, such as the lower digestive tract, bursa of Fabricius and skeletal muscle, was less consistent and more focalised, supporting the opinion that virus distribution in the host organism is dependent on particular host factors [8].

The onset of clinical signs in H7N1 birds, both intranasally inoculated and contact animals, proved effective transmission of the virus from infected partridges to naïve contact birds. Moreover, not only inoculated birds seroconverted but also contact birds. Surprisingly, at 1 dpi all inoculated animals only showed oropharyngeal shedding, suggesting that contact birds had been infected by virus shed from the oral cavity of the inoculated animals. This finding could indicate a shift from the classical faecal-oral route to the oral-oral route

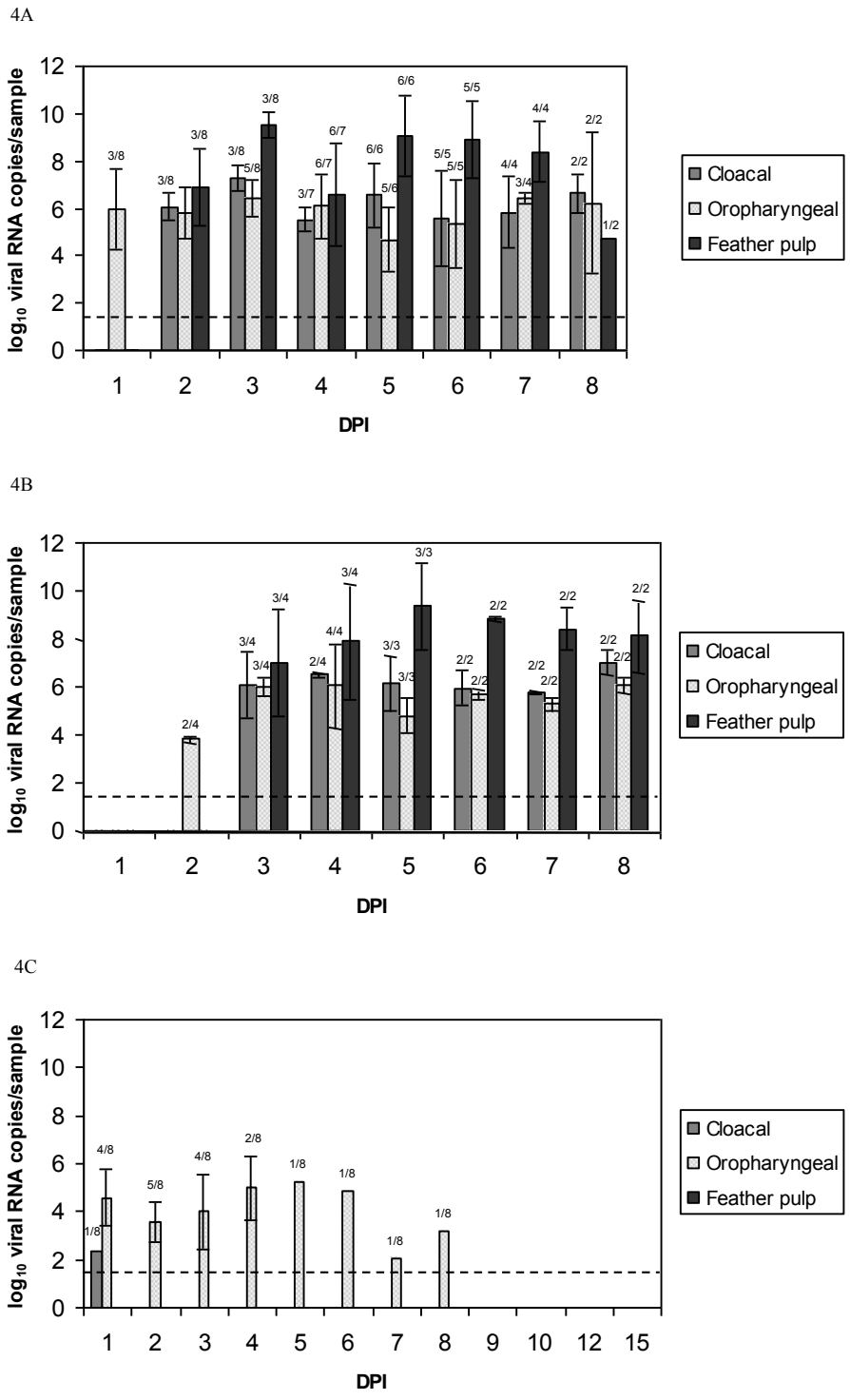

Figure 4 Viral shedding (expressed as log_{10} viral RNA copies/sample) detected by qRRT-PCR over 8 days in cloacal and oropharyngeal swabs and feather pulp samples of red-legged partridges (*Alectoris rufa*) infected with A/Chicken/Italy/5093/1999 H7N1 HPAIV and A/*Anas crecca*/Spain/1460/2008 H7N9 LPAIV. In **A** and **B**, rates above the bars indicate the relation between positive birds and the total number of animals examined. Limit of detection is indicated by the dashed line (1.46 log_{10} viral RNA copies/sample). (**A**) H7N1 HPAIV intranasally inoculated partridges, (**B**) H7N1 HPAIV contact partridges, (**C**) H7N9 LPAIV intranasally inoculated partridges.

(possibly through shared drinking water) in H7N1 infection, as some authors have already pointed out [2,9,25,26]. Our results suggest that in red-legged partridge, feather follicles could be a potential source for virus transmission, especially in recently dead individuals that are susceptible of feather picking. Interestingly, to date, few studies have evidenced the relevance of feathers as an important location for viral replication and potential origin of dissemination in HPAIV infection [27-29], and none of them have demonstrated the significance of this location in partridges.

The high susceptibility of partridges to H7N1 infection would make them a good sentinel species for detection of HPAIV. Since the partridges shed virus at high concentrations before death, this species could contribute to viral transmission during a local outbreak in free-living birds, in countries where partridges are found in the wild or are reared in outdoor operations. The delay between the onset of virus shedding and the appearance of clinical signs (around three days in the present experiment) could have important consequences in relation to the risk of spreading disease into the wild by releasing apparently healthy farm-reared partridges for hunting purposes. The implementation of sanitary surveillance measures prior to and after release is of importance to avoid introduction of avian influenza viruses, as well as other pathogens, in the natural ecosystem.

Our findings in H7N9 LPAIV infected birds correlate well with those obtained by Humberd et al. [6] in their experiment, in which no clinical disease was observed in ring-necked pheasants (*Phasianus colchicus*) and Chukar partridges infected with different subtypes of LPAIV. By this author, Chukar partridges were considered as short-term shedders of LPAIV, with the respiratory tract being the main viral excretion route. Likewise, in our study only limited viral shedding was detected in few inoculated birds most of which also seroconverted. Thus, our results suggest that partridges do not play a significant role as reservoir species for LPAIV, because only little, likely local, replication and short term shedding of low amounts of virus occurs in this species.

Based on our studies, firstly feather pulp, but also cloacal and oropharyngeal swabs, can be successfully used for virus detection in surveillance programs. In addition, the CNS and also pancreas and heart specimens are useful both for virus detection and histopathological diagnosis. In conclusion, although further studies with HPAIV and LPAIV strains should be performed, our observations suggest that the red-legged partridge is not likely to be a reservoir species for LPAI viruses but they are highly susceptible to H7N1 HPAIV and develop severe clinical disease and prolonged viral shedding. Thus, this species should be included in passive surveillance programs in order to prevent economical losses from HPAIV outbreaks.

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Authors' contributions

NB and FXA prepared the viruses used in this study. KB, EPR, RD, AC, JVA and NM participated in the daily monitoring of the clinical signs and the sampling of the animals during all the experimental period. KB, EPR, RD, AR, AC and NM performed the necropsies and the tissue sampling. RV performed the histopathology and immunohistochemistry techniques of the necropsy tissues. KB, RD and NM carried out the histopathological examinations. EPR and NB carried out the avian influenza virus quantitation by real time RT-PCR (qRRT-PCR) and the serology assays. RD, AD, FXA, MB, UH and NM conceived the study and participated in its design and coordination. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Pathobiology and transmission of highly and low pathogenic avian influenza viruses in European quail (*Coturnix c. coturnix*)

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Abstract

European quail (*Coturnix c. coturnix*) may share with Japanese quail (*Coturnix c. japonica*) its potential as an intermediate host and reservoir of avian influenza viruses (AIV). To elucidate this question, European quail were experimentally challenged with two highly pathogenic AIV (HPAIV) (H7N1/HP and H5N1/HP) and one low pathogenic AIV (LPAIV) (H7N2/LP). Contact animals were also used to assess the viral transmission among birds. Severe neurological signs and mortality rates of 67% (H7N1/HP) and 92% (H5N1/HP) were observed. Although histopathological findings were present in both HPAIV-infected groups, H5N1/HP-quail displayed a broader viral antigen distribution and extent of microscopic lesions. Neither clinical nor pathological involvement was observed in LPAIV-infected quail. Consistent long-term viral shedding and effective transmission to naive quail was demonstrated for the three studied AIV. Drinking water arose as a possible transmission route and feathers as a potential origin of HPAIV dissemination. The present study demonstrates that European quail may play a major role in AI epidemiology, highlighting the need to further understand its putative role as an intermediate host for avian/mammalian reassortant viruses.

Introduction

Avian influenza (AI) represents a major disease problem, not only for poultry but also for other avian species, mammals, and human beings [1]. The constant outbreaks detected around the world in poultry and wild birds are of concern to the economics of the poultry industry, to wildlife conservation, and to animal and public health [2]. Susceptibility to AI viruses (AIV) varies deeply among wild bird and poultry species, as well as their possible role as sentinels, intermediate hosts or reservoirs. Gallinaceous poultry are considered to be highly susceptible [3,4], whereas waterfowl have long been recognized as natural reservoirs, although they may show variable morbidity depending on the infective viral strain [5-7].

Since the first reported case of AI in Japanese quail (*Coturnix c. japonica*) in Italy (1966–1968) [8], influenza viruses of several subtypes have been isolated from quail in North America, Europe, and Asia through periodic surveillance and sporadic outbreaks [9-11]. Several experimental infections in Japanese quail have reported either higher, similar or lower susceptibilities than chickens to H5 highly pathogenic AIV (HPAIV) [3,12-15]. Moreover, inoculation of low pathogenic AIV (LPAIV) representing subtypes H1 to H15 proved that Japanese quail may support the replication (predominantly in the respiratory tract) of almost all of them [16]. More recently, several studies suggested that multiple in vivo passages in Japanese quail facilitate the adaptation of duck AIV to chicken [17-20]. These cumulative observations along the years have been recently explained by molecular adaptation of quail AI strains, especially in hemagglutinin (HA) and neuraminidase genes, which amino acids pattern might be intermediate between those of duck and chicken viruses [21-23]. In addition, quail carry sialic acid receptors functional for binding of avian and human influenza viruses [24,25]. Therefore, Japanese quail may provide an optimal environment for the adaptation of wild bird AIV, generating novel variants that can cross the species barrier to domestic poultry and human beings. Surprisingly, the epidemiological significance observed for the Japanese quail has not yet been demonstrated for the European quail (*Coturnix c. coturnix*).

The European quail, also called common or wild quail, is a partial migrant whose breeding range extends from the Atlantic to Lake Baikal and from the Arctic Circle to the tropics [26]. A decline in the number of European quail in the Western Palearctic over the past few decades has stimulated the release of Japanese quail as game birds in various European countries, leading to hybridization between both species in the field [27]. Even though European and Japanese quail show a high overall similarity in morphological, behavioral, and ecological features that made some authors conclude that they belong to the same species [28], they are distinguishable by characteristic morphological traits and calls [27,29]. The Japanese quail, also called domestic quail, is found in the wild in Asia [26], but is best known in its domestic form in Europe, Asia, North America, and India where it is generally ranged in outdoor game farms for restocking and hunting purposes [30] as well as for meat and egg production [31]. Particularly in Spain, Japanese and European quail currently comprise 4.7% of the global avian meat production system [32] with an ever-increasing population size along the years. Considering that open range rearing of birds has been identified as one of the factors contributing to the increase of AI outbreaks and their effect [2], specific attention should be paid to the quail, which may have a role in the spread and exacerbation of the disease.

To date, various studies have assessed the susceptibility of Japanese quail to H5 HPAIV and LPAIV [3,12-16]. However, the present study is the first experimental infection investigating the susceptibility of European quail to AIV. On the one hand, it is the first attempt to assess the potential viral shedding of HPAIV and LPAIV in this species, and the likelihood of effective transmission among quail. On the other hand, it represents a comparative study of the pathogenesis and viral distribution in tissues of two different HPAIV subtypes (H7 and H5). The overall results depict the role that European quail may play in the epidemiology of AI, and its putative responsibility in an interspecies outbreak.

Materials and methods

Viruses

Three strains of AIV were used: H7 HPAIV, H5 HPAIV, and H7 LPAIV. The H7 HPAIV [A/Chicken/Italy/5093/1999 (H7N1) (H7N1/HP)] was isolated during the 1999–2000 Italian epidemic [33]. The H5 HPAIV [A/Great crested grebe/Basque Country/06.03249/2006 (H5N1) (H5N1/HP)] was obtained from the only reported case of H5N1 HPAIV in wild birds in Spain so far [34]. The H7 LPAIV [A/*Anas platyrhynchos*/Spain/1877/2009 (H7N2) (H7N2/LP)] was obtained from the ongoing surveillance program carried out in Catalonia (Northeast Spain). The deduced amino acid sequence of the region coding for the cleavage site of the precursor of the HA molecule were PEIPKGSRVRR*GLF for the H7N1/HP and PEIPKGR*GLF for the H7N2/LP, being typical of HPAIV and LPAIV, respectively [35].

Virus stocks were produced in specific pathogen free (SPF) chicken eggs. The allantoic fluids were harvested at 48 hours post-inoculation (hpi) (H7N1/HP and H5N1/HP) and 72 hpi (H7N2/LP). Viruses were tenfold diluted in phosphate buffer saline (PBS) for titration in 9-day-old embryonating SPF chicken eggs. The mean embryo lethal dose (ELD_{50}) and the mean embryo infectious dose (EID_{50}) for the HPAIV and LPAIV isolates, respectively, were determined [36].

Animals

European quail (Urgasa S.A., Lleida, Spain) of approximately two months of age were used in this study. Male and female birds were included in almost equal numbers. Before the infection, serum samples of all individuals were confirmed to be seronegative for AIV by a competition ELISA test (C-ELISA) (IDVET, Montpellier, France). Furthermore, oropharyngeal (OS) and cloacal (CS) swabs were ensured to be negative for AIV by real time RT-PCR (RRT-PCR). Each experimental group was housed in a different negative pressured isolator with HEPA-filtered air in the animal biosafety level 3 (ABSL-3) facilities of *Centre de Recerca en Sanitat Animal* (CReSA). Quail were kept one week for acclimation, and feed and water were provided *ad libitum* throughout the experiment. All procedures were performed according to the requirements of the Ethical Commission of Animal Experimentation of the Autonomous Government of Catalonia.

Experimental design

Eighty birds were randomly separated into seven groups: six challenged groups with 12 birds/group and one control group with 8 birds (Table 1). For each virus, quail were subdivided into two experimental groups, A and B (n = 12/group). Groups 1A, 2A, and 3A were used to evaluate morbidity, mortality, transmissibility, and viral shedding pattern. Groups 1B, 2B, and 3B were used for the pathological studies. All animals were inoculated intranasally with 10^6 EID_{50} (for the LPAIV) or 10^6 ELD_{50} (for the HPAIV) of the corresponding challenge virus in a volume of 0.5 mL, except four birds of each A group which were used as contact animals. Contact birds were placed into the isolators four hours after inoculating the other birds and after changing drinking water. A seventh group (group C) (n = 8) was used as negative controls; these quail were inoculated intranasally with PBS solution. Amounts of virus were verified by performing a RRT-PCR of both the original non-diluted viruses and the inocula.

Sampling

All birds were monitored daily for clinical signs. During the first 10 days post-inoculation (dpi), at 12 dpi, and 15 dpi, OS, CS and feather pulp (FP) samples were obtained from quail from the A groups to measure viral shedding by RRT-PCR. Drinking water was collected with a 1 mL syringe at the same time points, and it was changed on a daily basis. The same samples were collected from group C. Mortality and mean death times (MDT) were calculated from the A groups. At 3, 5, 8, and 15 dpi, three animals from groups B and two animals from group C were euthanized using intravenous sodium pentobarbital (100 mg/kg, Dolethal®, Vétoquinol, Cedex, France). Surviving birds were euthanized at the end of the experiment (15 dpi). Blood samples were collected before euthanasia to detect AI antibodies by C-ELISA testing. As it was terminal, bleeding was done from the heart after previous anesthesia with intramuscular injection of ketamine/xylazine (10 g/kg body weight, Imalgene® 1000 and 1 g/kg body weight, Xilagesic® 2%). All euthanized and naturally dead quail from the B groups were necropsied to evaluate gross lesions and obtain samples for histopathological studies. Swabs and FP samples were placed in 0.5 mL of Dulbecco's Modified Eagle's Medium (DMEM) (BioWhittaker®, Lonza, Verviers, Belgium) with 600 µg/mL penicillin and streptomycin. These samples, together with drinking water samples and serum samples, were stored at −80 °C until further use.

Pathologic examination and immunohistochemical testing

Necropsies and tissue sampling were performed according to standard protocols [37]. After fixation in 10% neutral buffered formalin and embedding in paraffin, tissue sections were processed routinely for hematoxylin/eosin (HE) staining. The following tissues were examined: esophagus, crop, proventriculus, gizzard, duodenum, jejunum-ileum, cecum/cecal tonsil, colon, rectum, pancreas, liver, kidney, adrenal gland, gonad, nasal turbinates, trachea, lung, heart, breast muscle, skin, bone marrow, spleen, bursa of Fabricius, thymus, brain, spinal cord, and sciatic nerve. In addition, an immunohistochemical (IHC) technique was performed as previously described [38,39]. The primary antibody was a mouse-derived monoclonal commercial antibody against nucleoprotein (NP) of influenza A virus (IgG2a, Hb65, ATCC). As a secondary antibody, a biotinylated goat anti-mouse IgG antibody (GaMb, Dako E0433, Glostrup, Denmark) was used. Tissues previously demonstrated to be positive against NP of influenza A virus by IHC were used as a positive control. Duplicated samples of all animals incubated without the primary antibody, as well as tissues from sham-inoculated animals processed as usual by IHC, served as negative controls. The following score was used to grade the staining in the tissues: no positive cells (−), single positive cells (+), scattered groups of positive cells (++), widespread positivity (+++).

Viral RNA detection by RRT-PCR

Viral RNA from OS, CS, FP, and drinking water samples was extracted with NucleoSpin® RNA virus kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. The resulting viral RNA extracts were tested by one-step RRT-PCR for the detection of a highly conserved region of the matrix (*M*) gene in Fast7500 equipment (Applied Biosystems, Foster City, CA, USA) using the primers and probe previously described [40] and the amplification conditions described by Busquets et al. [41]. Samples with a threshold cycle (Ct) value ≤ 40 were considered positive for influenza A viral RNA. Viral shedding was analyzed by ANOVA test for significant differences ($p < 0.05$) using the Statistical Package for the Social Sciences for Windows Version 20.0.

Serology

A C-ELISA test was carried out to detect antibodies against the NP of AIV using the commercially available kit ID Screen® Influenza A Antibody Competition (IDVET, Montpellier, France), according to the manufacturer's instructions. In addition, a hemagglutination inhibition (HI) test was performed to titrate antibodies against specific H5- (in H5N1/HP serum samples) and H7- (in H7N2/LP and H7N1/HP serum samples) subtypes. The HI assays were performed according to standard procedures [42] with chicken red blood cells and commercial inactivated H5- and H7-antigens (GD-Deventer, The Netherlands). To avoid nonspecific positive reactions, sera were pre-treated by adsorption with 10% chicken red blood cells. Titers were expressed as geometric mean titers (GMT-$\log_2$); GMT of 3 $\log_2$ or greater were considered positive. Previously known positive and negative sera were used as controls.

Results

Morbidity and mortality

Clinical signs and mortality were only observed in HPAIV-infected groups (groups 2 and 3) and were similar between inoculated and contact birds. Some of the quail (17% H7N1/HP-challenged and 58% H5N1/HP-challenged animals) displayed nonspecific clinical signs, consisting of lethargy, anorexia, and ruffled feathers, that progressed to death or severe neurological signs (e.g., incoordination, torticollis, circling, head tremors, head tilt, and opisthotonus) within 24 h. The onset times of these nonspecific signs were 6 dpi for H7N1/HP-group and 4 dpi for H5N1/HP-group. Two H7N1/HP-challenged quail (17%) and three H5N1/HP-challenged quail (25%) presented an acute fatal progression of the infection, displaying neurological signs without previous nonspecific signs at 7 dpi and 5 dpi, respectively. However, in other cases (33% in H7N1/HP-group and 8% in H5N1/HP-group) quail were found dead without previous clinical signs. Only one bird, belonging to the H5N1/HP-group, recovered after showing nonspecific clinical signs at 6–7 dpi. All animals with neurological signs, recumbent or both were euthanized for ethical reasons. The survival rates and the MDT of the HPAIV-infected groups (groups 2 and 3) throughout the experiment are summarized in Figure 1 and Table 2.

Gross findings

Consistent gross lesions were only observed in HPAIV-infected groups (groups 2 and 3) and were similar between inoculated and contact birds. At 3 dpi, one H7N1/HP-quail (group 2) presented multifocal petechia on the proventriculus-gizzard junction mucosa. However, the foremost lesions in the H7N1/HP-group were observed at 5 dpi, which consisted of moderate splenomegaly with pallor or parenchymal mottling and pancreatic lesions characterized by multifocal necrotic areas of 1 mm-diameter. Lesions in H5N1/HP-quail (group 3) were most pronounced and were detected throughout the experiment in all necropsied birds. At 3 dpi, liver pallor in one bird was observed. The quail found dead at 4 dpi presented spleen pallor and multifocal areas in the pancreas. Such pancreatic lesion, as well as thymus atrophy, was observed until the end of the experiment in all necropsied birds. At 5 dpi, spleen pallor was observed in one bird. No gross lesions were observed in H7N2/LP-infected birds (group 1) or in birds from the control group (group C).

Histopathological findings

Histological lesions and influenza A viral NP were only observed in HPAIV-infected quail (groups 2 and 3) (Tables 3, 4). In H7N1/HP-quail, prevailing histological lesions were observed at 5 and 8 dpi mainly in the pancreas, heart, and brain, but also in the gizzard, cecal tonsil, and spinal cord (Table 3). H5N1/HP-challenged birds consistently showed marked lesions in the tissues mentioned for the H7N1/HP-infected quail and also, to a lesser extent, in the rectum, kidney, and skeletal muscle from the breast (Table 4). Accordingly, presence of H5N1/HP in tissues, as determined by IHC, was more intense than H7N1/HP. The most consistent finding, prevalent throughout almost all the experiment within both HPAIV-challenged groups, was moderate to severe multifocal to coalescent lytic necrosis of the acinar epithelium of the pancreas and endothelial activation indicative of acute inflammation. The main findings in the brain consisted of moderate to severe multifocal areas of malacia in the cerebral hemispheres, associated with spongiosis of the neuropil, neuronal chromatolysis,

and gliosis (Figures 2a, 2b). Overt severe necrosis of ependymal cells of the ventricles was present in all affected quail. The cerebellum frequently showed multifocal areas of moderate to severe chromatolysis of Purkinje neurons at 3, 5, and 7 dpi of H5N1/HP-infected quail, sometimes associated with non-suppurative perivascular inflammatory infiltrate. The heart was also consistently affected, with multifocal to diffuse myocardial degeneration and necrosis consisting of hyalinization and fragmentation of cardiac myocytes, often associated with mild lymphoplasmacytic infiltrate (Figures 2c, 2d). In general, IHC staining was mainly nuclear and sometimes also cytoplasmic in distribution and correlated well with histopathological findings.

Viral RNA detection by RRT-PCR

Real time RT-PCR was performed on OS, CS, FP, and drinking water samples of the A groups. Oropharyngeal swabs of H7N2/LP-challenged birds (group 1) tested positive until 9 dpi for inoculated birds peaking at 3 dpi, and until 12 dpi for contact birds peaking at 7 dpi (Figures 3a, 3b). Viral RNA from CS was detected in one animal during 3 days (3–5 dpi) and in two contact animals for 4 days (6–9 dpi). Feather pulp samples tested negative in this H7N2/LP-group. In H7N1/HP-inoculated quail (group 2), viral RNA was detected in all the studied samples (OS, CS, FP) from 1 dpi until before death, although oral shedding was predominant (Figure 3c). Viral RNA detection from contact H7N1/HP-birds was similar to that observed in inoculated quail, although with two days of delay (Figure 3d). For H5N1/HP-inoculated quail (group 3), oral shedding was also higher than cloacal shedding, although FP samples had high amounts of viral RNA as well (Figure 3e). H5N1/HP viral RNA amounts were less homogenous than for H7N1/HP among dpi and types of sample. Contact H5N1/HP-quail had a similar shedding profile to the inoculated ones, although starting two days later (Figure 3f). HPAIV-challenged quail orally shed significantly higher amounts of viral RNA than the LPAIV-challenged quail ($p < 0.05$), especially on 1, 2, and 4 dpi. Moreover, FP from H5N1/HP-challenged quail contained significantly more viral RNA than FP from H7N1/HP-infected quail ($p < 0.05$).

Presence of H7N2/LP viral RNA in drinking water samples coincided with the days where quail's samples reached maximum viral RNA values (4–6 dpi). H7N1/HP viral RNA was present in water during almost all the experiment (3–15 dpi), being quite stable in time. Existence of H5N1/HP viral RNA in drinking water was manifested at 2 dpi and at 6 dpi, at levels akin to those of H7N1/HP.

Serology

Before infection, all serum samples tested negative for antibodies against influenza A NP. Almost all the H7N2/LP-inoculated quail (7/8) and all the H7N2/LP-contact quail (4/4) were antibody-positive at 15 dpi, with GMT of 7.9 and 7.3, respectively (Table 5). Besides, all HPAIV-inoculated birds (both H7N1/HP and H5N1/HP) seroconverted from 5 dpi onwards, with GMT steadily increasing until 15 dpi from 4.7 to 7.0 in the case of H7N1/HP-inoculated quail (Table 5).

Discussion

This is the first experiment which demonstrates that European quail (*Coturnix c. coturnix*) can be infected with both HPAIV and LPAIV. This quail subspecies can be found not only in

the wild all over the Palearctic region, but also in many parts of Europe as a game bird species raised in outdoor operations. Despite the relevance of this game bird species, no studies had previously investigated its AIV infection dynamics. The aim of the present study was to elucidate the putative role of European quail in the ecology of influenza A viruses by assessing the pathogenesis, transmissibility, and viral shedding of quail experimentally infected with two different HPAIV subtypes (H7N1 and H5N1) and one LPAIV (H7N2).

Quail exhibited a high susceptibility to both HPAIV used in this study, as demonstrated by severe clinical signs and high mortality rates. With the earliest onset, most rapid progression of disease, and shortest MDT in H5N1/HP-infected quail, it is apparent that this virus is more virulent for this species than the H7N1/HP [43]. The high pathogenicity observed for both HPAIV is in accordance with natural and experimental H5N1 HPAIV infections in chickens and other gallinaceous species, among which are Japanese quail [3,4,14]. Although previous studies with Japanese quail reported minimal clinical signs or even sudden deaths without apparent symptoms [3,14,15], clinically neurological dysfunction was an evident sign in most of the HPAIV-infected quail of the present study. Certain gross findings indicative of AI were not as extensive and obvious as for chickens (e.g., presence of edematous, hemorrhagic, and necrotic cutaneous lesions), but affected tissues were known target organs for influenza A viruses in other gallinaceous species, including Japanese quail [3,4,14,44]. Interestingly, H5N1/HP showed a broader tissue distribution compared with H7N1/HP, suggesting that virus replication in a particular target organ other than respiratory or intestinal organs may contribute to the virulence of the HPAIV in quail, as previously stated [15]. Particularly, neurotropism is considered one of the main factors for the fatal course of AI in birds [45,46], evidenced in our study by the higher virulence of the H5N1/HP compared with H7N1/HP. Our findings in H7N2/LP correlate well with those of Makarova et al. [16], in which a wide range of LPAIV subtypes could replicate efficiently in Japanese quail, predominantly in the respiratory tract. In our study, European quail could also maintain the infection without clinical involvement, and shed the virus mainly orally during a substantial period.

Effective viral transmission from inoculated quail to naive contact birds was confirmed for the three studied viruses, even though their origin avian hosts were as diverse as chicken, mallard, and great crested grebe. This finding suggests that adaptation may not be needed to allow AIV to replicate and transmit in European quail, confirming the substantial role that this species may play in AI epidemiology. As in a previous work with H5N1 HPAIV in Japanese quail [14], both HPAIV used in our study confirmed to be able to transmit among European quail. Moreover, transmission of H7 isolates (both H7N1/HP and H7N2/LP) is of great importance because: I) this is the first transmission evidence of an H7 HPAIV in quail so far; and II) previous experiments with LPAIV failed to confirm this capability in Japanese quail [16]. Not only had the onset of clinical signs proved infection in contact birds, but also their antibody responses (in the case of H7N2/LP challenge) and their efficient viral shedding. Given that quail shed virus mainly orally, contact birds might have been infected by the oral-oral route. In fact, such viral shedding predominance, also stated in previous studies with Japanese quail [14-16], is already known to differ from that observed in LPAIV waterfowl reservoirs [47].

Ingestion of contaminated water has already been suggested as a possible transmission route [48]. Interestingly, the earlier detection of viral H5N1/HP RNA in water followed by H7N1/HP and finally by H7N2/LP could mirror the initial ability of the virus to replicate in host cells, be shed, and thus, be more likely transmissible to naive birds. Drinking water should be particularly taken into account for quail and other game birds raised in outdoor

operations, where AI viruses from wild birds could be introduced to the poultry flock. Furthermore, contamination of the environment by respiratory secretions and infected carcasses likely would result in indirect oral transmission of the virus. Although minor, cloacal shedding was consistently detected in HPAIV-infected quail, confirming that European quail might have functional binding receptors in both trachea and intestine, as already confirmed for both quail subspecies [24,25,49,50]. Besides, feathers could likely act as potential source for virus transmission in European quail, especially in recently dead birds susceptible to feather picking. To date, the relevance of feathers as a location for viral replication and potential origin of dissemination in HPAIV infection has been evidenced in certain bird species [4,51,52], but had not yet been demonstrated in quail.

The high degree of correlation between C-ELISA and HI results suggests that such tests seem to be equally sensitive and specific when assessing quail serological responses, as previously stated for Japanese quail [14]. Antibody response in HPAIV-inoculated quail started as early as 5 dpi, further confirming infection of the birds and an early humoral immune response. Seroconversion in H7N2/LP-infected quail at the end of the experiment proved effective infection not only among inoculated birds but also among contacts. In general, antibody titers in the present study were akin to those previously observed in AIV-infected Japanese quail [5,53,54] and gradually increased throughout the experiment, as already observed in H9N2 LPAIV-infected Japanese quail [54].

The high susceptibility of European quail to H7N1/HP and H5N1/HP would make this species a good sentinel of the presence of HPAIV in the environment, both in the wild or in semi-extensive farms. On the other hand, infected quail can shed a considerable amount of AIV before the appearance of overt clinical signs, death or both (around four days in the present experiment). Therefore, spreading disease into the wild by releasing apparently healthy farm-reared quail for hunting purposes could represent a substantial threat, even higher if assuming that this species could act as a mixing vessel like already stated for the Japanese quail. Furthermore, European quail may be considered sentinels (both for HPAIV and LPAIV) and reservoirs (for LPAIV), which is of special interest as most wild individuals are migratory [26]. The application of surveillance measures on quail flocks before and after release is of importance to avoid introduction of HPAIV, as well as other pathogens, in the natural ecosystem.

Current active AI surveillance activities include sampling of both OS and CS, as well as blood [55,56]. Passive surveillance of dead or moribund birds involves the same samples as for active surveillance (when possible) along with tissue collection through necropsy [55,56]. On the basis of our findings, OS could be used as a unique tool for successful virus detection in active AI surveillance programs in quail, as it has been assessed for other minor species in which pathogenesis is still poorly understood [4]. In addition, brain, pancreas, and heart specimens would be suitable in passive surveillance when HPAIV is suspected. Our results suggest that European quail, like Japanese quail, could play a key role in AI epidemiology because of the high susceptibility to HPAIV and the noteworthy spread of both HPAIV and LPAIV. Taking into account the similarities in viral dynamics between Japanese and European quail, the latter would also presumably have the capability to act as an intermediate host for avian/mammalian reassortant viruses, although further experiments are needed to address this issue. In addition, future studies comparing AI infection dynamics between Japanese and European quail by experimental infections with the same AIV strains would strengthen the present data. Altogether, our results underline the complexity of managing AI outbreaks when different susceptible species are involved.

Competing interests

The authors declare that they have no competing interests.

Author's contributions

KB, NB, and FXA prepared the viruses used in this study. KB, RD, VG, JVA, AJC, and NM participated in the daily monitoring of the clinical signs and the sampling of the animals. KB, RD, VG, JVA, AJC, AR, and NM performed the necropsies and the tissue sampling. KB and NM carried out the histopathological examinations. KB carried out the RRT-PCR and the serology assays, together with FXA in the case of H5N1/HP samples. RD, NB, FXA, UH, and NM conceived the study and participated in its design and coordination. All authors read and approved the final manuscript.

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Figure 1 Survival rates of quail intranasally challenged with H7N1/HP and H5N1/HP. a. Intranasally inoculated quail. **b**. Contact quail.

Figure 2 Distribution of NP antigen in positive tissues of a quail intranasally challenged with H5N1/HP. a. Brain, 7 dpi. **b**. Positive staining in nucleus and cytoplasm of neurons and glial cells. **c**. Heart, 5 dpi. **d**. Positive staining in nucleus and cytoplasm of myocardiocytes.

Figure 3 Viral RNA shedding detected by RRT-PCR in quail experimentally challenged with AIV. Results are expressed as inverted Ct-values and shown as means of positive individuals ± SD. Tables indicate the ratio between positive quail and total quail examined per day and sample. Ct, cycle of threshold; DPI, days post-inoculation; OS, oropharyngeal swabs; CS, cloacal swabs; FP, feather pulps. **a**. Quail intranasally inoculated with H7N2/LP. **b**. Contact quail of H7N2/LP. **c**. Quail intranasally inoculated with H7N1/HP. **d**. Contact quail of H7N1/HP. **e**. Quail intranasally inoculated with H5N1/HP. **f**. Contact quail of H5N1/HP.

Table 1 Experimental design of the study

Group	Inoculum	Titer	No. animals
1A	H7N2/LP	10^6 EID_{50}	12 (8+4)*
1B	H7N2/LP	10^6 EID_{50}	12
2A	H7N1/HP	10^6 ELD_{50}	12 (8+4)*
2B	H7N1/HP	10^6 ELD_{50}	12
3A	H5N1/HP	10^6 ELD_{50}	12 (8+4)*
3B	H5N1/HP	10^6 ELD_{50}	12
C	PBS	-	8

*In A groups, eight quail were inoculated and four quail were left as contact birds.
H7N2/LP, A/*Anas platyrhynchos*/Spain/1877/2009; H7N1/HP, A/Chicken/Italy/5093/1999; H5N1/HP, A/Great crested grebe/Basque Country/06.03249/2006; ELD_{50}, mean embryo lethal dose; EID_{50}, mean embryo infectious dose; PBS, phosphate buffer saline.

Table 2 Survival rates and MDT of quail intranasally challenged with either H7N1/HP or H5N1/HP

Virus	Mortality*		
	Inoculated (MDT)	Contact (MDT)	Total
H7N1/HP	63% (6.6)	75% (7)	67%
H5N1/HP	88% (5.3)	100% (6)	92%

*#dead/total X 100.
MDT, mean death time (dpi); H7N1/HP, A/Chicken/Italy/5093/1999; H5N1/HP, A/Great crested grebe/Basque Country/06.03249/2006.

Table 3 Average distribution of AIV-NP antigen in positive tissues from quail intranasally challenged with H7N1/HP

Tissue*	3 dpi	5 dpi	8 dpi	15 dpi	Predominant cell types	Associated lesion
Gizzard	–	++	–	–	Epithelial cells of the ventricular glands	Severe multifocal necrosis, mild lymphoplasmacytic infiltrate
Cecal tonsil	–	+	–	–	Epithelial cells of the glands, cells of the muscularis externa	Focal necrosis, mild lymphoplasmacytic infiltrate
Pancreas	–	++	+++	–	Acinar cells, endothelial cells	Severe multifocal to coalescent lytic necrosis, lymphoplasmacytic infiltrate, edema
Nasal turbinates	+	–	–	–	Respiratory epithelial cells	Lymphoplasmacytic infiltrate in lamina propria
Trachea	–	+	–	–	Goblet cells	NSL
Heart	–	++	–	–	Myocardiocytes, endothelial cells	Severe multifocal necrosis, mild lymphoplasmacytic infiltrate
Spleen	–	+	+	–	Endothelial cells, macrophages	NSL
Brain	–	+++	++	–	Neurons, Purkinje cells, ependymal cells, glial cells, endothelial cells	Malacia in cortex, necrosis of ependymal cells of ventricles and epithelial cells of choroid plexus, chromatolysis of Purkinje cells, lymphoplasmacytic infiltrate
Spinal cord	–	+++	–	–	-	Malacia in grey matter, necrosis of the ependyma and neuropil

*Tissues not present appeared overtly normal on histopathological analysis and did not show positive IHC staining.
– = no positive cells; + = single positive cells; ++ = scattered groups of positive cells; +++ = widespread positivity.
dpi, days post-inoculation; NSL, no significant lesions.

Table 4 Average distribution of AIV-NP antigen in positive tissues from quail intranasally challenged with H5N1/HP

Tissue*	3 dpi	4 dpi	5 dpi	6 dpi	7 dpi	Predominant cell types	Associated lesion
Proventriculus	–	–	–	+	–	Epithelial cells of the proventricular glands, cells of the muscularis externa	Severe multifocal necrosis, mild lymphoplasmacytic infiltrate
Gizzard	–	–	+	++	++	Epithelial cells of the ventricular glands, cells of the muscularis externa	Severe multifocal necrosis, mild lymphoplasmacytic infiltrate
Cecal tonsil	–	–	+	+++	+	Cells of the lamina propria	Mild lymphoplasmacytic infiltrate
Rectum	–	–	–	+	+	Cells of the muscularis externa of the lamina propria	Vacuolation, degeneration, mild lymphoplasmacytic infiltrate
Pancreas	+	–	++	++	+	Acinar cells, endothelial cells	Severe multifocal to coalescent lytic necrosis, lymphoplasmacytic infiltrate, edema
Kidney	+	+	+	+	+++	Collecting tubular epithelial cells, endothelial cells	Moderate to severe necrosis, mild lymphoplasmacytic infiltrate
Adrenal gland	+	–	–	–	–	Corticotrophic and corticotropic cells	NSL
Nasal turbinates	–	++	–	–	–	Respiratory epithelial cells	Lymphoplasmacytic infiltrate in lamina propria
Heart	+	++	+++	++	+++	Myocardiocytes, endothelial cells	Severe multifocal necrosis, mild lymphoplasmacytic infiltrate
Skeletal muscle	–	–	+	++	++	Myocytes, endothelial cells	Moderate multifocal necrosis, mild lymphoplasmacytic infiltrate
Spleen	–	–	–	–	+	Endothelial cells, macrophages	NSL
Brain	++	+++	+++	+++	+++	Neurons, Purkinje cells, ependymal cells, glial cells, endothelial cells	Malacia in cortex, necrosis of ependymal cells of ventricles and epithelial cells of choroid plexus, chromatolysis of Purkinje cells, lymphoplasmacytic infiltrate.

*Tissues not present appeared overtly normal on histopathological analysis and did not show positive IHC staining.
– = no positive cells; + = single positive cells; ++ = scattered groups of positive cells; +++ = widespread positivity.
dpi, days post-inoculation; NSL, no significant lesions.

Table 5 Serological data of quail intranasally challenged with either H7N2/LP, H7N1/HP or H5N1/HP

Group	3 dpi		5 dpi		9 dpi		15 dpi	
	ELISA	**HI***	**ELISA**	**HI***	**ELISA**	**HI***	**ELISA**	**HI***
H7N2/LP Inoculated	nd	nd	nd	nd	nd	nd	7/8	7/8 (7.9)
H7N2/LP Contact	nd	nd	nd	nd	nd	nd	4/4	4/4 (7.3)
H7N1/HP Inoculated†	0/3	0/3	3/3	3/3 (4.7)	3/3	3/3 (6.3)	3/3	3/3 (7.0)
H5N1/HP Inoculated†	0/3	0/3	3/3	3/3 (6.0)	nd	nd	nd	nd

dpi, days post-inoculation; ELISA, C-ELISA; HI, hemagglutination inhibition; nd, no data; H7N2/LP, A/*Anas platyrhynchos*/Spain/1877/2009; H7N1/HP, A/Chicken/Italy/5093/1999; H5N1/HP, A/Great crested grebe/Basque Country/06.03249/2006.

*GMT (log_2) are indicated in parenthesis. GMT include only positive birds.

†No data is available for contact quail in these groups.

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PLoS one

Highly (H5N1) and Low (H7N2) Pathogenic Avian Influenza Virus Infection in Falcons Via Nasochoanal Route and Ingestion of Experimentally Infected Prey

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Abstract

An experimental infection with highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) viruses was carried out on falcons in order to examine the effects of these viruses in terms of pathogenesis, viral distribution in tissues and viral shedding. The distribution pattern of influenza virus receptors was also assessed. Captive-reared gyr-saker (*Falco rusticolus* x *Falco cherrug*) hybrid falcons were challenged with a HPAI H5N1 virus (A/Great crested grebe/Basque Country/06.03249/2006) or a LPAI H7N2 virus (A/*Anas plathyrhynchos*/Spain/1877/2009), both via the nasochoanal route and by ingestion of previously infected specific pathogen free chicks. Infected falcons exhibited similar infection dynamics despite the different routes of exposure, demonstrating the effectiveness of in vivo feeding route. H5N1 infected falcons died, or were euthanized, between 5–7 days post-infection (dpi) after showing acute severe neurological signs. Presence of viral antigen in several tissues was confirmed by immunohistochemistry and real time RT-PCR (RRT-PCR), which were generally associated with significant microscopical lesions, mostly in the brain. Neither clinical signs, nor histopathological findings were observed in any of the H7N2 LPAI infected falcons, although all of them had seroconverted by 11 dpi. Avian receptors were strongly present in the upper respiratory tract of the falcons, in accordance with the consistent oral viral shedding detected by RRT-PCR in both H5N1 HPAI and H7N2 LPAI infected falcons. The present study demonstrates that gyr-saker hybrid falcons are highly susceptible to H5N1 HPAI virus infection, as previously observed, and that they may play a major role in the spreading of both HPAI and LPAI viruses. For the first time in raptors, natural infection by feeding on infected prey was successfully reproduced. The use of avian prey species in falconry husbandry and wildlife rehabilitation facilities could put valuable birds of prey and humans at risk and, therefore, this practice should be closely monitored.

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Introduction

Avian influenza (AI) is one of the most important biological threats, not only for poultry but also for other avian species and humans [1,2]. Susceptibility to AI viruses varies greatly among wild bird and poultry species, as well as their possible role as vectors or reservoirs [3,4]. Gallinaceous poultry are considered to be highly susceptible, whereas waterfowl have long been recognized as natural reservoirs, although they may show variable morbidity depending on the infective viral strain [5,6].

Although AI typically courses as an asymptomatic infection in wild birds, recent highly pathogenic avian influenza (HPAI) epidemics resulted in unprecedented high mortality rates for certain wild bird species. In the past, HPAI viruses were rarely found in birds of prey and were restricted to only a few isolated cases [7,8]. However, during recent H5N1 outbreaks, increasing number of birds of prey have been reported to be infected, probably as a result of improvements in sampling and diagnostic tools. It is worth highlighting that Hong Kong had a series of cases of natural infection of peregrine falcons (*Falco peregrinus*) with H5N1 in 2004, 2006 and 2008 [9–11], although other countries subsequently reported HPAI cases in different prey species, such as in Hodgson's hawk eagles (*Spizaetus nipalensis*) in Belgium [12], saker falcons (*Falco cherrug*) in Saudi Arabia [13] and, more recently in Saudi Arabia, houbara bustards (*Chlamydotis undulate macqueenii*),- which interestingly infected falcons that came into contact with them [14].

Even though the number of AI natural cases in raptor species has gradually increased, data prevalence is still scarce. However, some countries have performed active AI surveillance of these species. In Sweden, neither HPAI nor low pathogenic avian influenza (LPAI) infections were found in white-tailed sea eagle (*Haliaeetus albicilla*) or peregrine falcons by standard screening using real time RT-PCR (RRT-PCR), and serology [15]. Besides this, 7.7% of the Falconiformes tested between August 2005 and February 2006 in the United Arab Emirates were seropositive [16]. It should be noted that in the Middle East, in addition to the circulation of the H5N1 viruses, there is also evidence of extensive

circulation of LPAI viruses, mainly H9N2 viruses [17,18]. The co-circulation of H9N2 and H5N1 subtypes of AI in these species may increase the risk of generating reassortant viruses with pandemic potential [19].

Falconry is an ancient tradition in the Arabian Peninsula that has spread worldwide, resulting in a strong trade of all species of falcons around the world. Nowadays, falconry is most popular in European countries such as The United Kingdom, Germany and Spain, in that order [20]. It is well known that migration of infected wild birds is one of the mechanisms in the spreading of AI viruses [21], thus many falcon species may contribute to the movement of both HPAI and LPAI viruses within, or between countries. Wild birds of prey are at an increased risk of acquiring AI viruses because they regularly feed on avian carcasses and diseased avian prey [22–24]. In falconry, birds of prey are kept in captivity and come into close contact with humans. Although there is still no direct evidence of virus transmission from falcons to humans, birds of prey could represent a bridging species for AI viruses and, consequently, the practice of falconry may pose an enhanced risk of transmission to humans and poultry. However, a recent study by Kohls *et al.* [25] indicates that the AI virus prevalence of prey birds from falconry is generally low, and that falconry birds which come into contact with AI viruses through their prey do not necessarily become infected, as in most cases the falconer does not allow them to eat the whole prey. However, concerning ornithophagous free ranging raptors, the risk of infection would be higher, since these usually feed on the whole prey. There is no evidence to confirm this so far.

To date, scarce experimental infections have been performed in birds of prey. Lierz *et al.* [26] studied the effects of H5N1 HPAI virus infection by performing an experimental vaccination trial on captive gyr-saker falcon hybrids (*F. rusticolis* × *F. cherrug*) via the oculo-oronasal route. Recently, an experimental infection in American kestrels (*Falco sparverius*) with various doses of H5N1 HPAI virus inoculated via the intranasal and intrachoanal route was performed [27]. Both studies showed that these birds are extremely susceptible to the H5N1 HPAI virus.

Although it is evident that birds of prey can be infected with HPAI viruses, their susceptibility to LPAI viruses still remains unclear, and the pathogenicity of both HPAI and LPAI viruses to these species has not been described extensively. Moreover, raptor's putative role as reservoirs in AI ecology and their potential to shed viruses need to be investigated. In the present study, the pathogenesis of HPAI and LPAI viruses in ¾ gyr-saker (*Falco rusticolus* × *Falco cherrug*) hybrid falcons was determined. The birds were experimentally inoculated via the nasochoanal route and by ingestion of virus-infected preys. Viral load distribution in several tissues and the extent and duration of viral shedding were also evaluated. In addition, localization of influenza virus receptors in different tissues was also assessed in order to identify the target cells of AI viruses in this species.

Materials and Methods

Ethics Statement

This study was carried out in strict accordance with the recommendations of the Ethical Commission of Animal Experimentation of the Autonomous Government of Catalonia (*Comissió d'Experimentació Animal de la Generalitat* (CEA), Permit Number: 5567). The protocol was approved by the Ethics Committee of Animal and Human Experimentation of the *Universitat Autònoma de Barcelona* (*Comissió d'Ètica en l'Experimentació Animal i Humana* (CEEAH), Permit Number: 1066). All manipulations were performed under sodium pentobarbital anesthesia, and every effort was made to minimize suffering. Welfare information and end point criteria are included in Text S1.

Viruses

Two strains of AI virus were used: the HPAI isolate A/Great crested grebe/Basque Country/06.03249/2006 (H5N1) (H5N1 HPAI) and the LPAI isolate A/*Anas plathyrhynchos*/Spain/1877/2009 (H7N2) (H7N2 LPAI). The deduced amino acid sequence of the region coding for the cleavage site of the haemagglutinin molecule were PEIPKGSRVRR*GLF for the isolate H5N1 HPAI and PEIPKGR*GLF for the isolate H7N2 LPAI, being typical of HPAI and LPAI viruses, respectively. In addition, the H5N1 HPAI subtype demonstrated an intravenous pathogenicity index of 3.0 [28].

Stocks of the H5N1 HPAI and the H7N2 LPAI viruses were produced in 9-day-old embryonated specific pathogen free (SPF) chicken eggs. In both cases, the allantoic fluid was harvested at 48 hours post inoculation, aliquoted and stored at -80° C until use. Virus was diluted tenfold in phosphate buffer saline (PBS) for titration in 9-day-old embryonated SPF chicken eggs. The 50% egg lethal dose (ELD_{50}) for H5N1 HPAI, and the 50% egg infective dose (EID_{50}) for H7N2 LPAI, were determined using the Reed and Muench method [29].

Animals

Juvenile (5–10 weeks old) male captive-reared ¾ gyr-saker (*Falco rusticolus* × *Falco cherrug*) hybrid falcons were obtained from a breeder (Roc Falcon S.L). From 3 to 10 weeks of age, falcons were imprinted by a person from the research group in order to minimize further stress. In order to assess that optimal health conditions existed, a complete blood cell count (CBC) was performed and a peripheral blood smear evaluated to rule out the presence of hemoparasites in all birds. Results of the CBC and blood smears were unremarkable. Also, an anti-parasite treatment (Baycox®) was carried out. In addition, non-diluted serum samples were collected to ensure that birds were serologically negative for AI antibodies by a competitive enzyme-linked immunosorbent assay (C-ELISA) (ID-VET, Montpellier, France). Furthermore, oropharyngeal and cloacal swabs were collected in order to make certain that falcons were negative for AI virus by real time RT-PCR (RRT-PCR). Birds were weighed and tagged with numbered aluminum leg bands, and brought to biosafety level 3 (BSL-3) in CReSA facility, where they were randomly distributed within the experimental groups and separately housed in negative-pressured isolators with HEPA-filtered air. It is worth highlighting that isolators were environmentally adapted for falcons' welfare (Text S1). Whole chick-preys, supplemented with vitamins were provided twice a day. Birds were adapted to their new environment for 5 days before experimental infection.

Experimental design

The experimental design is summarized in Table 1. Seventeen falcons were distributed into five experimental groups (A to E). Groups A (n = 4) and B (n = 4) were challenged with 10^6 EID_{50} of the H7N2 LPAI virus, whereas groups C (n = 3) and D (n = 4) were challenged with 10^6 ELD_{50} of the H5N1 HPAI virus. Two falcons from group E were inoculated nasochoanally with PBS solution and served as negative controls.

Groups A and C were inoculated nasochoanally; in group A, one of the 4 falcons was not-inoculated and was referred to as the contact animal. Groups B and D were challenged via the natural feeding route with previously infected SPF chicks. Briefly, one-day-old SPF chicks, confirmed negative for AI virus by RRT-PCR, were inoculated via oculonasal route with either 10^6 EID_{50} of the

Table 1. Experimental design of the study.

GROUPS	INOCULUM	TITER/N° CHICKS	INFECTION ROUTE	NUMBER OF ANIMALS
A	H7N2 LPAI	10^6 EID_{50}	Nasochoanal	4 (3+1)
B	H7N2 LPAI	2 chicks/falcon	Feeding	4
C	H5N1 HPAI	10^6 ELD_{50}	Nasochoanal	3
D	H5N1 HPAI	5 chicks/falcon	Feeding	4
E	PBS	–	Nasochoanal	2

LPAI, low pathogenic avian influenza; HPAI, highly pathogenic avian influenza; PBS, phosphate buffer saline; EID_{50}, 50% egg infectious dose; ELD_{50}, 50% egg lethal dose. LPAI virus was A/*Anas plathyrhynchos*/Spain/1877/2009 (H7N2); HPAI virus was A/Great crested grebe/Basque Country/06.03249/2006 (H5N1).
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H7N2 LPAI virus or 10^6 ELD_{50} of the H5N1 HPAI virus. At 3 days post-infection (dpi), chicks were confirmed positive for AI virus by performing a RRT-PCR on oropharyngeal and cloacal swabs. Two whole H7N2 LPAI-infected chicks and five whole H5N1 HPAI-infected chicks were used to infect each falcon from groups B and D, respectively. In order to estimate the viral load ingested by the falcons, viral titration in Madin-Darby Canine Kidney (MDCK) cells was performed on tissue homogenates from LPAI-infected and HPAI-infected chicks. Two out of three homogenates of trachea, lung, kidney and small intestine from H7N2 LPAI-infected chicks contained from $10^{3.2}$ to $10^{7.2}$ $TCID_{50}$/g of tissue, whereas the viral load from homogenates of liver, lung, kidney and brain of all H5N1 HPAI-infected chicks reached titers from $10^{6.5}$ to $10^{7.4}$ $TCID_{50}$/g of tissue. Inocula titers of all the experimental groups were verified by performing a RRT-PCR of both the original non-diluted viruses and the diluted inocula.

Sampling

All falcons were monitored daily for the development of clinical signs, and oropharyngeal and cloacal swabs were obtained to measure viral shedding by RRT-PCR. Besides, blood samples were collected before euthanasia to detect AI antibodies by C-ELISA testing. As it was terminal, bleeding was done from the heart after previous anesthesia with intramuscular injection of ketamine/xylazine (10 g/kg body weight, Imalgene® 1000 and 1 g/kg body weight, Xilagesic® 2%). Mortality and mean death time (MDT) were calculated. Ethically euthanized and naturally dead falcons were necropsied to evaluate gross lesions and obtain samples for pathological and molecular studies. Negative control falcons and surviving infected falcons were euthanized at the end of the experiment (11 dpi for LPAI groups and 10 dpi for HPAI groups). Falcons were euthanized using intravenous sodium pentobarbital (100 mg/kg, Dolethal®,Vétoquinol, Cedex, France). Oropharyngeal and cloacal swabs, blood samples and tissue samples for molecular studies were stored at -80° C until further use.

Histopathology

Necropsies and tissue sampling were performed according to a standard protocol. After fixation in 10% neutral buffered formalin and paraffin embedding, tissue sections were processed routinely for haematoxylin/eosin (H/E) staining. The following tissues were examined: esophagus, crop, proventriculus-ventriculus, duodenum, jejunum-ileum, cecum/cecal tonsil, rectum, pancreas, liver, kidney, adrenal gland, gonad, nasal turbinates, trachea, lung, heart, breast muscle, skin, bone marrow, spleen, bursa of Fabricius, thymus, brain, spinal cord and sciatic nerve.

Virus detection by immunohistochemistry (IHC)

An immunohistochemical technique based on the Avidin-biotin complex immunoperoxidase system was performed as previously described [30,31]. The primary antibody was a mouse-derived monoclonal commercial antibody against nucleoprotein (NP) of influenza A virus (IgG2a, Hb65, ATCC). As a secondary antibody, a biotinylated goat anti-mouse IgG antibody (GaMb, Dako E0433, Glostrup, Denmark) was used. Tissues previously demonstrated to be positive against nucleoprotein of influenza A virus by IHC were used as a positive control. Tissues from sham-inoculated animals were incubated without the primary antibody and served as a negative control. The following score was used in order to grade the staining in tissues: no positive cells (−), single positive cells (+), scattered groups of positive cells (++), widespread positivity (+++).

Virus detection by RRT-PCR

Oropharyngeal and cloacal swabs were placed in 0.5 mL of Dulbecco's Modified Eagle's Medium (DMEM) with antibiotics. Additionally, tissue samples from trachea, lung, kidney and small intestine of H7N2 LPAI infected falcons, and from lung, kidney, duodenum/pancreas, liver and brain of H5N1 HPAI infected falcons were placed in 0.5 mL of PBS. Viral RNA was extracted with NucleoSpin® RNA Virus kit (Macherey-Nagel, Düren, Germany) following the manufacturer's instructions. A RRT-PCR assay was used to detect the viral *M* gene in Fast7500 equipment (Applied Biosystems, Foster City, CA, USA), using the primers and probe previously described [32], at a concentration of 400 nM for each primer and 300 nM for the TaqMan probe, the AgPath-ID one-step RT-PCR reagents (Applied Biosystems, Foster City, CA, USA) and 3 µL of eluted RNA in a total volume of 20 µL. The amplification conditions were as follows: reverse transcription at 48° C 10 min; initial denaturation at 95° C for 10 min and 40 PCR-cycles of 97° C for 2 sec and 61° C for 30 sec.

Serology

A C-ELISA test was carried out in order to detect antibodies against the A nucleoprotein of AI virus using the commercially available kit ID Screen® Influenza A Antibody Competition (ID-VET, Montpellier, France), performed according to the manufacturer's instructions.

Lectin histochemistry detection of influenza virus receptors

Lectin histochemistry was carried out in respiratory (nasal turbinates, trachea and lung) and digestive (proventriculus, duodenum, ileum, cecum and rectum) tracts of control falcons using the lectins *Maackia amurensis* agglutinin II (MAAII) and

Sambucus nigrans agglutinin (SNA), which show affinity for α-2,3 (avian type) and α-2,6 (human type) receptors, respectively.

Lectin histochemistry was performed using previously described procedures [33] with minor modifications. Briefly, 3 μm-thick sections were dewaxed and treated with 3% H_2O_2 in methanol to eliminate endogenous peroxidase activity, washed with TNT (0.1 M Tris HCl, 0.15 M NaCl, pH 7.5) and blocked with TNB (TNT plus blocking reagent) (Perkin Elmer, US) for 30 minutes at room temperature (RT). Tissue sections were then incubated with biotynilated SNA (10 μg/ml) and MAAII (15 μg/ml) (Vector Laboratories Inc, CA, US) in TNB at 4° C, overnight. After washing with TNT, sections were incubated with streptavidin-horse radish peroxidase (SA-HRP) 1:100 for 1 hour, and again incubated with SA-HRP for 30 min at RT. The reaction was developed with diaminobenzidine (Sigma-Aldrich, MO, US) at RT for 30 seconds followed by counterstaining with Mayer's haematoxylin. To rule out the non-specific binding of lectins, two sequential slides were used as negative controls. One slide was pretreated with neuraminidase, which cleaves both α-2,3 and α-2,6 residues, as previously described [33], and the other was incubated with PBS instead of the lectins. Negative controls consisted of the substitution of the lectin with a TNB buffer. Human, pig and mice tissue samples were used as positive controls because of previous publications reporting the lectin pattern of staining of these species [33–36]. For each slide, and in order to compare receptor expression patterns among the tissues included in this study, the relative intensity of receptor expression was scored based on the percentage of cells in a section showing positivity, and was graded as: no positive cells (−), single positive cells (+), scattered groups of positive cells (++), widespread positivity (+++).

Statistical analysis

Oral viral shedding obtained from RRT-PCR was analyzed by Kruskal-Wallis test for significant differences ($p<0.05$) between H7N2 and H5N1 groups and between routes of infection. The statistical tests were performed using the Statistical Package for the Social Sciences for Windows Version 17.0.

Results

Clinical signs and Mortality

There were no relevant differences between nasochoanally inoculated animals and animals challenged via the natural feeding route in both H5N1 HPAI and H7N2 LPAI groups. Falcons from both H5N1 HPAI challenged groups (groups C and D) showed first clinical signs at 5 dpi which consisted of depression, apathy, impaired respiration and, above all, slight neurological signs that in a matter of hours turned into moderate or severe, and included torticollis, head tilt, ataxia, circling, incoordination, leg/wing paralysis, opisthotonus and tremors (Movie S1). Some birds were also found recumbent and unresponsive. Following the endpoint criteria established in the ethical protocol, one falcon per group was ethically euthanized at 5 dpi. At 6 dpi, one falcon infected by feeding and showing severe neurological signs was also ethically euthanized, while at 7 dpi one falcon per group was found dead. Remaining falcons (one per group) survived until the end of the experiment at 10 dpi and did not develop clinical signs. To sum up mortality data, two out of three nasochoanally inoculated falcons, and three out of four falcons challenged by feeding died between 5 and 7 dpi, and the MDT was 6 dpi. Neither morbidity nor mortality was observed in the negative control group (group E) or in the H7N2 LPAI groups (groups A and B).

Gross findings

Findings associated with AI virus infection were only observed in some of the H5N1 HPAI infected falcons (groups C and D). Lesions were very similar regardless of the infection group and day of necropsy, being pancreas, proventriculus, ventriculus and brain the most affected organs. Regarding the nasochoanal group (group C), multifocal hemorrhagic necrosis in the pancreas was found in the falcon necropsied at 5 dpi (Figure 1), while the falcon found dead at 7 dpi showed multifocal petechia on the proventriculus-ventriculus junction mucosa, as well as brain and eyelid congestion. The surviving falcon from this group also showed multifocal petechia in the proventriculus-ventriculus junction mucosa at time of necropsy (10 dpi). Concerning the prey ingestion group (group D), the falcon necropsied at 5 dpi did not show significant lesions, whereas multifocal petechia on the proventriculus-ventriculus junction mucosa and brain and eyelid congestion were found in the falcon euthanized at 6 dpi. In this group, the falcon found dead at 7 dpi also showed multifocal petechia on the proventriculus-ventriculus junction mucosa and multifocal hemorrhagic necrosis in the pancreas. No gross lesions were observed in the H5N1 HPAI infected falcon of the prey ingestion group euthanized at 10 dpi.

Histopathological findings

Histological lesions and influenza A viral antigen (NP) were only observed in H5N1 HPAI challenged falcons (groups C and D) (Tables 2 and 3). Neither significant lesions nor positive IHC staining were observed in H7N2 LPAI challenged animals (groups

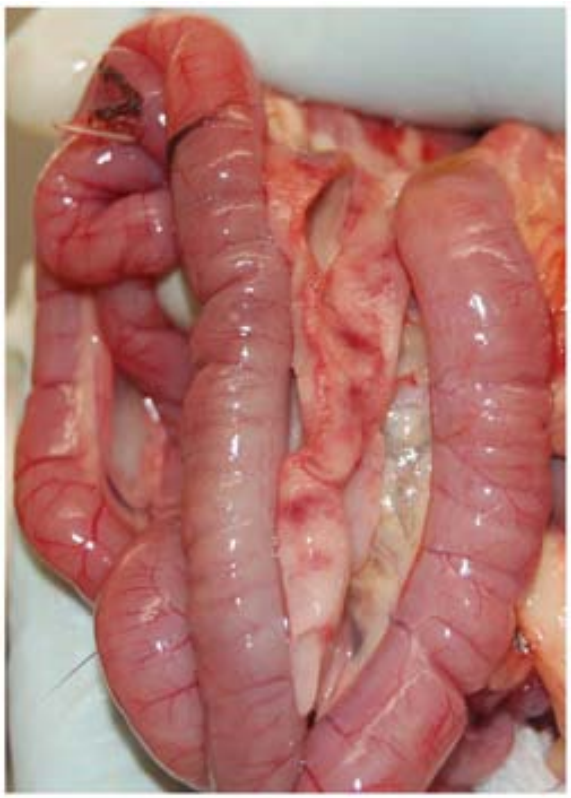

Figure 1. Pancreatic macroscopic lesions from a falcon experimentally infected with highly pathogenic avian influenza virus H5N1. Multifocal hemorrhagic necrosis in the pancreas of a falcon infected via the nasochoanal route with A/Great crested grebe/Basque Country/06.03249/2006 H5N1 HPAI virus (5 dpi).
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Table 2. Average distribution of nucleoprotein antigen, as determined by immunohistochemistry, in tissues sampled from falcons infected via the feeding route with A/Great crested grebe/Basque Country/06.03249/2006 (H5N1) HPAI virus.

TISSUE	2VN6	4AN6	3ES4	6TN14	PREDOMINANT CELL TYPES	ASSOCIATED LESION
	5 dpi	6 dpi	7 dpi	10 dpi		
Pancreas	−	−	−	−	−	NSL
Kidney	+	−	−	−	Collecting tubular epithelial cells	NSL
Nasal turbinates	nd	+	+	−	Lateral nasal gland epithelial cells	NSL
Trachea	−	+	−	−	Pseudostratified epithelial cells	Necrosis of pseudostratified epithelium. Mild focal tracheitis.
Lung	+++	−	nd	nd	Bronchial epithelial cells, goblet cells	NSL
Brain	+++	+++	++	+	Neurons, ependymal cells, glial cells, endothelial cells	Malacia in cortex. Necrosis of ependymal cells of ventricles and epithelial cells of choroid plexus. Perivascular cuffing. Endothelia hypertrophia. Chromatolysis of Purkinje cells.
Spinal cord	−	−	−	−	−	NSL

− = no positive cells; + = single positive cells; ++ = scattered groups of positive cells; +++ = widespread positivity.
NSL, no significant lesions; nd, not determined.
doi:10.1371/journal.pone.0032107.t002

A and B), or in the control birds. Tissues not present in Tables 2 or 3 appeared overtly normal on histopathological analysis and did not show positive IHC staining.

The most severely affected organ was the brain (Tables 2 and 3). The main findings consisted in moderate (5–6 dpi) to severe (7 dpi) multifocal areas of malacia in the cortex, present in all hemispheres of the brain, associated with spongiosis of the neuropil, chromatolysis, gliosis and caryolysis (Figure 2A). Vascular endothelial swelling was also observed, especially at 5–6 dpi. Evident severe necrosis of ependymal cells of the ventricles and epithelial cells of the choroid plexus was present in almost all falcons. The cerebellum frequently showed multifocal areas of moderate to severe chromatolysis of Purkinje neurons, sometimes associated with necrosis of the Purkinje cell layer and non-suppurative inflammatory infiltrate. Severe non-suppurative choroiditis was observed in two falcons of the prey ingestion group, particularly in the falcon found dead at 7 dpi and in the falcon euthanized at 10 dpi, as well as in the falcon found dead at 5 dpi of the nasochoanal group. Moderate multifocal areas of perivascular cuffing were present, being severe in the falcon from the prey ingestion group necropsied at 10 dpi. This falcon had the same histopathological findings as the neurologically affected falcons, whereas the falcon from the nasochoanal group necropsied at 10 dpi did not show any significant lesions in the brain. Significant microscopic lesions were seen in the spinal cord of the falcon from the nasochoanal group necropsied at 5 dpi, where severe malacia in the grey matter, and severe gliosis and necrosis of the ependym and neuropil surrounding the medullary canal were observed. Interestingly, almost all lung tissues showed positive IHC staining in bronchi, in particular bronchial epithelial cells and goblet cells. In general, antigenic staining was mainly nuclear and also often cytoplasmic in distribution and correlated well with histopathological findings (Figure 2B).

Avian influenza virus detection by RRT-PCR

Real time RT-PCR was performed on oropharyngeal and cloacal swabs, and on various tissues obtained at necropsy. In both H7N2 LPAI-infected groups (groups A and B) (Figure 3A), viral RNA was only detected on oropharyngeal swabs. Regarding H7N2 LPAI prey-infected falcons (group B) the amount of viral RNA detected orally was consistent and reached minimum cycle of threshold (Ct) values of 22.29 at 2 dpi. Besides, viral RNA was

Table 3. Average distribution of nucleoprotein antigen, as determined by immunohistochemistry, in tissues sampled from falcons infected via the nasochoanal route with A/Great crested grebe/Basque Country/06.03249/2006 (H5N1) HPAI virus.

TISSUE	1VN6	4CS1	1US18	PREDOMINANT CELL TYPES	ASSOCIATED LESION
	5 dpi	7 dpi	10 dpi		
Pancreas	+	+	−	Acinar cells	Lytic necrosis. Heterophilic infiltrate.
Kidney	+	−	−	Collecting tubular epithelial cells	NSL
Nasal turbinates	−	nd	−	−	NSL
Trachea	−	−	−	−	NSL
Lung	+	+	+	Bronchial epithelial cells, goblet cells	NSL
Brain	+++	+++	−	Neurons, ependymal cells, glial cells, endothelial cells	Malacia in cortex. Necrosis of ependymal cells of ventricles and epithelial cells of choroid plexus. Perivascular cuffing. Chromatolysis of Purkinje cells. Endothelial hypertrophy.
Spinal cord	++	−	−	Neurons, ependymal cells, glial cells	Malacia in grey matter. Necrosis of the ependyma and neuropil.

− = no positive cells; + = single positive cells; ++ = scattered groups of positive cells; +++ = widespread positivity.
NSL, no significant lesions; nd, not determined.
doi:10.1371/journal.pone.0032107.t003

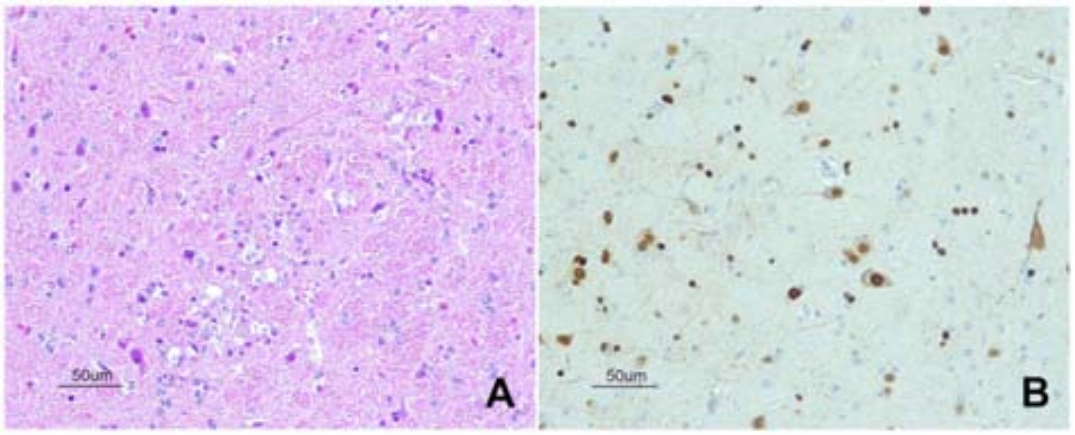

Figure 2. Brain tissue from an experimentally H5N1 HPAI virus-inoculated falcon dead at 5 dpi. A. Focal area of malacia in the cortex (HE stain). **B.** Immunohistochemical staining of influenza A virus antigen in brain tissue.
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already detected at 1 dpi in one animal, with a Ct value of 37.31. H7N2 LPAI viral RNA was detected in all falcons until the end of the experiment (11 dpi), although amounts of viral RNA were evidently declining. Regarding falcons inoculated via the nasochoanal route (group A), detection was similar to that observed in the feeding group, although quantities of viral RNA declined more rapidly. No viral RNA was detected in swabs from the contact animal during the whole experiment.

For H5N1 HPAI infected falcons (groups C and D) (Figure 3B), viral RNA detection had a different profile than for that of H7N2 LPAI infected animals. Regarding oropharyngeal swabs, both nasochoanally and feeding infected falcons showed viral RNA from 1 dpi until prior to death, peaking at 2 dpi (Ct 24.08). Falcons that survived until the end of the experiment (10 dpi) stopped shedding at 7 dpi. In contrast with H7N2 LPAI falcons, H5N1 HPAI infected animals showed some viral RNA detection in cloacal swabs. In particular, the falcon from the feeding group that was euthanized at 5 dpi showed low amounts of virus (Ct 33.15) at the time of necropsy, as well as two falcons from the nasochoanal group: the one that was euthanized at 5 dpi showed virus from 3 to 5 dpi (Ct 33.77, Ct 37.96 and Ct 29.21), and the one found dead at 7 dpi had a Ct value of 31.51 at 5 dpi.

Results of viral detection by RRT-PCR from the tissues are shown in Table 4. In general, all falcons that showed clinical signs and died, or were ethically euthanized had positive results for almost all the selected tissues. The highest amounts of viral RNA were detected in the brain. The surviving falcon's brain from the feeding group was positive for RRT-PCR.

The statistical analysis performed on the results of the oropharyngeal swabs until 7 dpi (both individual and mean Ct) showed no significant differences between routes of infection for both H5N1 HPAI and H7N2 LPAI groups ($p>0.05$), whereas amounts of viral RNA of H7N2 LPAI groups were significantly higher than in H5N1 HPAI groups ($p<0.05$).

Serology

With the exception of the contact bird, all H7N2 LPAI infected falcons were seropositive at the end of the experiment (11 dpi). Concerning H5N1 HPAI infected falcons, only serum samples from 10 dpi were seropositive. No seroconversion was observed in the negative control falcons (group E).

Lectin histochemistry detection of influenza virus receptors

Lectin immunohistochemistry was carried out in the respiratory and digestive tracts of control falcons in order to assess the distribution pattern of SNA (α-2,6) and MAAII (α-2,3). Regarding respiratory tract (Figure 4), moderate expression of α-2,6 was observed in ciliated epithelial cells and mucous gland cells of the respiratory tract of nasal turbinates and in salivary gland epithelium. Mild expression of α-2,6 receptors was observed in ciliated epithelial cells, mucous gland epithelium and goblet cells of the trachea. However, expression of receptors was predominantly α-2,3 (avian type) on the respiratory tract, being as follows: strong in bronchial epithelial cells; moderate in ciliated epithelial cells and goblet cells of the trachea; and mild in ciliated epithelial cells of the respiratory tract of nasal turbinates and in nasal gland epithelium, and in mucous gland epithelium of the trachea. Regarding digestive tract (Figure 5), strong α-2,3 expression was noted in enterocytes of the rectum, and mild α-2,3 expression was observed in goblet cells of the same region. Other cell types, such as endothelial cells, macrophages and lymphocytes, gave mild positive results for both α-2,3 and α-2,6 receptors in the cecum/cecal tonsil and rectum.

Discussion

This is the first experiment which demonstrates that falcons can be infected with both HPAI and LPAI viruses, not only via the nasochoanal route but also by feeding on infected prey. For both viruses, infected falcons exhibited similar infection dynamics despite the different routes of exposure, demonstrating that ingestion of infected-SPF chicks is as effective as direct nasochoanal route to produce infection. To the best of our knowledge, this is the first study demonstrating that the consumption of infected prey is a viable route of transmission for both HPAI and LPAI viruses in falcons. Other studies have addressed the role of feeding on influenza-infected prey in other animal species [23,24,37]. Infectious dose to which the falcons were experimentally exposed via feeding on infected-SPF chicks could be comparable to the infectious dose to which wild falcons would be exposed during feeding on infected wild preys. This situation could also be feasible in falcons raised for falconry or in wild falcons clinically-admitted in wildlife rehabilitation centers

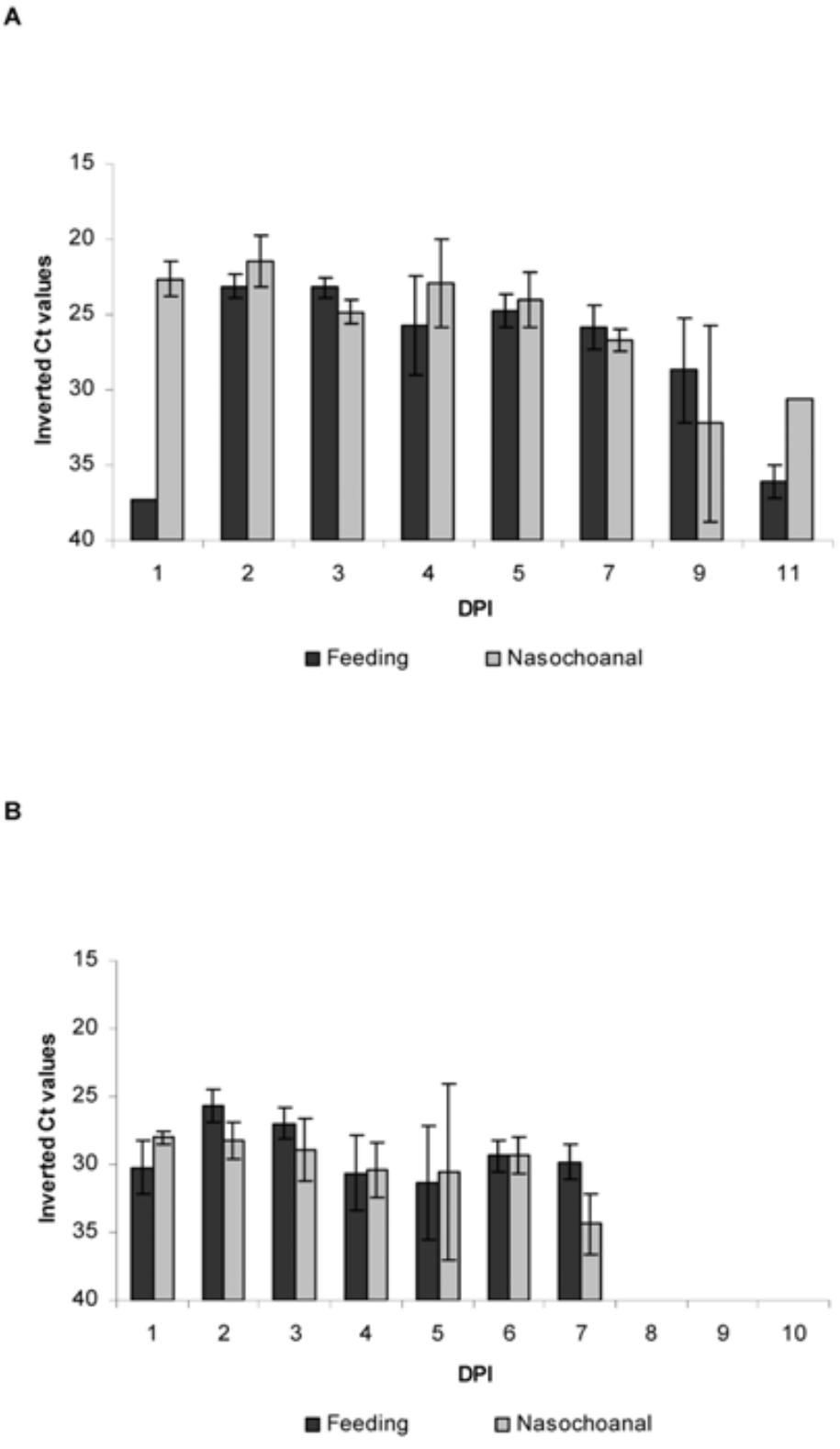

Figure 3. Oral shedding from experimentally infected falcons with avian influenza virus. Viral RNA shedding detected by RRT-PCR in oropharyngeal swabs of falcons infected via the feeding route or via the nasochoanal route. Ct, cycle of threshold. **A.** Falcons infected with A/*Anas plathyrhynchos*/Spain/1877/2009 (H7N2) LPAI virus and euthanized at 11 dpi. **B.** Falcons infected with A/Great crested grebe/Basque Country/06.03249/2006 (H5N1) HPAI virus.
doi:10.1371/journal.pone.0032107.g003

Table 4. Viral RNA in tissues of falcons infected with A/Great crested grebe/Basque Country/06.03249/2006 (H5N1) HPAI virus.

INFECTION ROUTE, ANIMAL ID	DAY OF DEATH	RNA in tissue, Ct value				
		Lung	Kidney-adrenal gland	Duodenum-pancreas	CNS	Liver
Feeding						
2VN6	5	24.23	25.42	25.58	15.82	undet
4AN6	6	28.77	undet	34.92	14.45	undet
3ES4	7	29.23	32.67	36.31	14.73	undet
6TN14	10	undet	undet	undet	19.22	undet
Nasochoanal						
1VN6	5	23.90	24.56	26.16	14.43	29,36
4CS1	7	26.48	29.38	28.45	17.84	undet
1US18	10	undet	undet	undet	undet	undet

Ct, cycle of threshold; CNS, central nervous system; undet, not detected by RRT-PCR.
doi:10.1371/journal.pone.0032107.t004

when fed on AI infected preys (either live-bird market or wild birds). Therefore, this practice should be closely monitored.

The high pathogenicity of the H5N1 HPAI strain used in the present study is in agreement with that obtained in other H5N1 HPAI experimental infections in falcons [26,27]. In our study, 5 out of 7 falcons died between 5 and 7 dpi; whether the two surviving falcons would have died in a longer experiment (at some point after the 10 dpi) remains unclear. Nevertheless, clinical signs of H5N1 HPAI infected animals were very acute and extremely severe, similar to those observed in Hall's study [27]. Such neurologic disorders would be feasible in natural infected falcons; these signs could be seen in free range birds under surveillance, and would certainly be noticed in animals under captivity. However, evident external lesions that are expected in AI-infected gallinaceous species, such as cutaneous hemorrhages on the legs [3], were not observed in falcons and, thus, may be overlooked during routine necropsy when AI is not suspected. Viral H5N1 HPAI RNA in tissues correlated well with IHC results, the brain being the most affected organ. Indeed, falcons only demonstrated severe neurological signs prior to death and only after necropsy and histopathological studies did some of the other tissues exhibit significant lesions. The feeding H5N1 HPAI infected falcon that survived until the end of the experiment could possibly have demonstrated clinical signs later on, since the virus was detected in the CNS by both IHC and RRT-PCR, and in the lungs by IHC.

Viral shedding was considered mainly oral for H5N1 HPAI infected falcons, being consistent and lasting for one week. In both experimental studies with H5N1 HPAI in falcons performed up to date [26,27] oral shedding seemed to be predominant over cloacal shedding. In addition, the only viral shedding route observed in H7N2 LPAI infected falcons was the oral one, which was significantly higher and lasted longer (up to 11 dpi) than in H5N1 HPAI infected animals. Moreover, the distribution pattern of influenza virus receptors seems to be in agreement with the pattern of viral shedding observed. The presence of avian-type receptors (α-2,3) in the nasal turbinates, trachea and bronchial epithelium, and their absence in other parts of the lung, support previous findings regarding domestic birds that AI viruses mainly localize in the upper respiratory tract

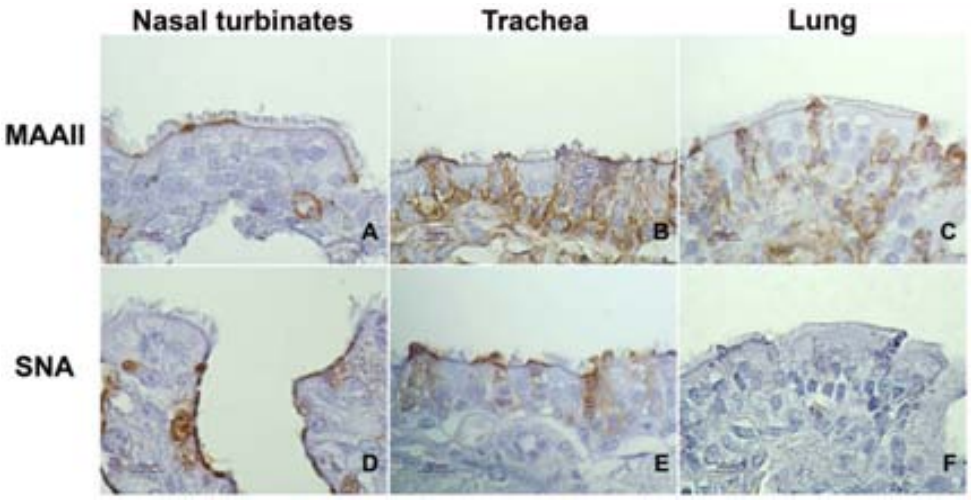

Figure 4. Distribution of α-2,3 and α-2,6 receptors in the respiratory tract of falcons demonstrated by means of MAAII and SNA lectin histochemistry. A. Nasal turbinates stained by MAAII lectin. **B.** Trachea stained by MAAII lectin. **C.** Lung stained by MAAII lectin. **D.** Nasal turbinates stained by SNA lectin. **E.** Trachea stained by SNA lectin. **F.** Lung stained by SNA lectin.
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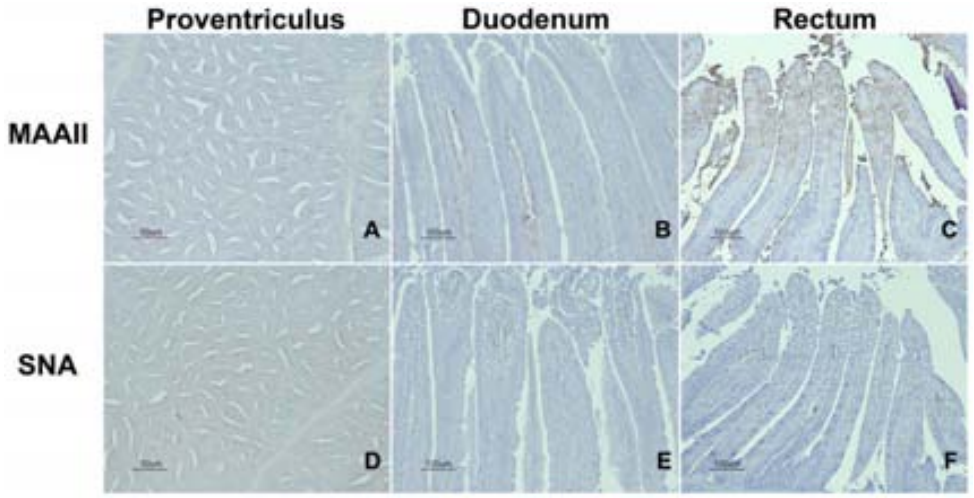

Figure 5. Distribution of α-2,3 and α-2,6 receptors in the digestive tract of falcons demonstrated by means of MAAII and SNA lectin histochemistry. A. Proventriculus stained by MAAII lectin. **B.** Duodenum stained by MAAII lectin. **C.** Rectum stained by MAAII lectin. **D.** Proventriculus stained by SNA lectin. **E.** Duodenum stained by SNA lectin. **F.** Rectum stained by SNA lectin.
doi:10.1371/journal.pone.0032107.g005

[5,38], and thus, successful oral shedding is detected. The scarce cloacal shedding observed in some H5N1 HPAI infected falcons is in accordance with the absence of avian-type receptors in the intestinal tract, which was only expressed in rectum.

Given that falcons can shed a considerable amount of AI virus before the appearance of overt clinical signs or death (if so), this species may contribute to viral transmission within the geographical limits in free-living birds, or to a local outbreak when reared in outdoor operations. Therefore, infected falcons shedding AI virus could represent a risk for humans and other valuable bird species when admitted in wildlife rehabilitation centers or during shipping for falconry trade.

Raptors are at the top of their food chain, representing a natural surveillance system that target those subjects more likely to have had HPAI virus exposure. However, the possible introduction of HPAI or LPAI viruses in raptor populations could have a negative impact on already threatened species. Therefore, surveillance could be an invaluable tool in studying the epidemiological situation of AI viruses in raptor and other related wild bird populations. Data obtained in the present study indicates that oropharyngeal swabs can be successfully used for virus detection in falcon surveillance programs, as is also recommended for other species [21,39]. In addition, not only brain, but also pancreas specimens are useful for AI virus detection and histopathological diagnosis.

In conclusion, our observations suggest that gyr-saker hybrid falcons are highly susceptible to infection with the H5N1 HPAI virus used in this study, and that they may play a major role in spreading AI viruses, given that a prolonged and consistent viral shedding has been demonstrated, especially with the H7N2 LPAI virus used in this study. Therefore, this species, whether wild or in captivity, should be included in passive surveillance programs, in order to prevent risk to humans and other wild bird species, and to minimize the threat of spreading, particularly of HPAI viruses within and among countries via animal trade or natural movements.

Supporting Information

Text S1 Welfare information and end point criteria established for the experimental infection.
(DOC)

Movie S1 Clinical signs in a falcon experimentally infected via the feeding route with highly pathogenic avian influenza virus H5N1. Severe neurological signs like torticollis, ataxia, circling, incoordination and opisthotonus.
(AVI)

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Author Contributions

Conceived and designed the experiments: NB RD NM. Performed the experiments: KB NB DS IC FXA NM. Analyzed the data: KB NB NM. Wrote the paper: KB NM. Reared and prepared the animals for the experimental infection: JGF KB Designed the negative-pressured isolators of the BSL-3 for the falcon's welfare: DS IC Reviewed the histopathological and immunohistochemical results: TC.

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