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Experimental infection of domestic geese (Anser anser var. domesticus) with H5N8 Gs/GD and H7N1 highly pathogenic avian influenza viruses

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Abstract

Prior to the emergence of the Asian-origin H5 Goose/Guangdong/1/96 (Gs/GD) lineage, highly pathogenic avian influenza viruses (HPAIV) had rarely caused high mortalities in domestic geese. In 2016/2017 European epidemics, H5N8 Gs/GD clade 2.3.4.4 Group B produced an unprecedented number of outbreaks in waterfowl holdings. In this study, the pathogenesis of H5N8 HPAIV in comparison with H7N1 HPAIV and the role of domestic geese in the epidemiology of these viruses were evaluated. Local and commercial geese (*Anser anser var. domesticus*) were intranasally inoculated with 10^5 ELD_{50} of A/goose/Spain/IA17CR02699/2017 (H5N8) or A/Chicken/Italy/5093/1999 (H7N1) and monitored daily during 15 days. H5N8 was highly virulent to domestic geese, reaching 100% mortality by day 10 post-infection. Systemic microscopic necrotizing lesions associated to widespread AIV-antigen by IHC techniques were detected, being the most severely-affected the central nervous system. High viral loads by qRT-PCR were present in all samples collected: oral and cloacal swabs, plasma and tissues, and moderate levels in pool water. Domestic geese were also susceptible to H7N1 infection, as demonstrated by seroconversion and detection of viral RNA in tissues and plasma in few geese, but all lacked clinical signs. Viral shedding was confirmed in only some geese and restricted to oral route, but levels were high and still detected at the end of the study. Overall, H7N1 present a lower letality and shedding than H5N8 in geese, however the viral shedding indicate that these species could play a role in the epidemiology of Gs/GD and other lineages of HPAIVs.

Keywords: highly pathogenic avian influenza; Gs/GD lineage; domestic geese; pathogenicity; shedding.

Highlights

- H5N8 Gs/GD clade 2.3.4.4 Group B is highly virulent to domestic geese.
- The severity of H5N8 was associated with multisystemic replication.
- H7N1 can infect domestic geese but is avirulent to this species.
- Domestic geese could play a role in the epidemiology of Gs/GD HPAIVs.

1. Introduction

Wild aquatic birds have been considered the natural reservoirs of avian influenza (AI) viruses (AIV) so far. In nature, most of the 16 hemagglutinin (HA) and 9 neuraminidase (NA) combinations of AIVs have been isolated in wild Anseriformes (e.g. ducks, geese, swans) and Charadriiformes (e.g. gulls, terns and waders), implying a pivotal role of these species in the epidemiology of AI (Webster *et al*., 1992; Olsen *et al*., 2006). Intermittently, the contact between infected wild birds and/or their droppings results in the spillover of some H5 and H7 LPAI viruses to poultry. In gallinaceous species, low pathogenic AIVs (LPAIV) can mutate into highly pathogenic AIVs (HPAIV), which cause important economic losses in the poultry sector (Monne *et al.*, 2014).

Chicken farming is the leading producer in poultry sector with approximately the 90% of world poultry meat and egg production (FAO, 2020). Even so, rearing minor avian species such as domestic waterfowl represents a significant part of the national agriculture in different countries of the world (Hugo, 1995). Descendants of the wild Greylag goose (*Anser anser,* Western breeds) and the Swan goose (*Anser cygnoides,*

Eastern breeds), were one of the first birds to be domesticated. Used as a multi-purpose poultry species, most goose breeds are raised for their meat, but also for feathers and fatty livers, in several production systems that range from backyards to specialized commercial farms. Moreover, domestic geese are used for controlling weeds in several crops, kept as guard birds or pets (FAO, 2002; Hugo *et al*., 1995). The detection of HPAIVs in domestic geese populations have been generally lower in comparison with gallinaceous species and associated to low mortalities (Alexander & Brown, 2009). Moreover, the few studies performed in domestic geese indicate that most HPAIVs appear to be avirulent for this species under experimental conditions (Narayan *et al.,* 1969; Röhm *et al.,* 1996). However, data clearly demonstrates that domestic geese have played a main role in the emergence, perpetuation and interspecies transmission of HPAIVs belonging to the Asian-origin goose/Guangdong H5 lineage (Gs/GD) (Henning *et al*., 2009; Sonnberg *et al.,* 2013). In addition, natural or experimental infections with several Gs/GD HPAIVs may result lethal in domestic geese. The clinical outcome ranges from subclinical to severe (100% mortalities) depending on viral factors, including the clade and genetic group of the virus, and host factors, including the species, breed, and age at infection (Perkins & Swayne, 2002; Webster *et al*., 2002; Zhou *et al*., 2006; Smietanka *et al*., 2013; Berhane *et al*., 2016; Xiang *et al*., 2017; Pantin-Jackwood *et al*., 2017; Grund *et al*., 2018).

In 2016, a novel reassortant H5N8 B (Gochang-like) HPAIV was simultaneously detected in dead wild birds in UVs-Nuur Lake (Tyva Republic) and in Qinghai Lake (China) (Lee *et al.*, 2017; Li *et al.,* 2017). Subsequently, the virus spread into Russia, Middle East, Europe and Africa (Ghafouri *et al*., 2017; Selim *et al*., 2017; Globig *et al*., 2018; Marchenko *et al*., 2018; Twabela *et al*., 2018). In 2016-2017, H5N8 B HPAIVs

caused unprecedented outbreaks in numerous European countries in both domestic and wild birds, with evidence for local virus amplification and gene exchange with LPAIVs (Pohlmann *et al.,* 2017; Poen *et al*., 2018). To date, the 2016-2017 H5N8 B HPAIV is the responsible of the largest epidemic by a HPAIV ever reported in the continent (Alarcon *et al*., 2018). During the 2016-2017 European epidemics, a high number of outbreaks were recorded in waterfowl holdings (mainly ducks and to a lesser extent in geese) (Napp *et al*., 2018). The elevated proportion of outbreaks in domestic waterfowl suggests that particularities of the production in these species are associated with higher probability to H5N8 Group B HPAIV infection. However, it could also reflect the acquisition of an increased affinity, tropism and/or virulence towards waterfowl species. Grund *et al.* (2018) reported that the increased virulence of H5N8 Group B HPAIV in domestic ducks in comparison to H5N8 Group A HPAIV was associated with the neuro- and hepato- tropism characteristics of the former. Despite domestic geese were the third domestic species most affected during the European epidemics, the pathobiological features of H5N8 Group B HPAIV in this species have not been characterized to date. In late 2019 and early 2020, H5N8 Group B HPAIVs have been detected again in several countries from Central and Eastern Europe in different avian species, including geese (King *et al*., 2020).

Taking into consideration the large number of outbreaks in geese holdings caused by Gs/GD H5N8 clade 2.3.4.4 Group B HPAIV, the comparatively lower isolation rates of HPAIVs others than those belonging to Gs/GD lineage and the lack of direct comparison of different HPAIVs in this species, the aims of this study were to: 1) perform a profound investigation of the differential pathobiology of a H5N8 Gs/GD Group B and H7N1 HPAIVs in domestic geese, and 2) evaluate the susceptibility and

potential role of local and commercial breeds of geese in the epidemiology of these HPAIVs.

2. Materials and methods

2.1. Viruses

The viruses used in this study were: A/Chicken/Italy/5093/1999 (H7N1), isolated in 1999-2000 during an Italian epidemic that mainly affected Veneto and Lombardia regions (kindly provided by Dr. Ana Moreno from the *Instituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna*) and A/Goose/Spain/IA17CR02699/2017 (H5N8 clade 2.3.4.4. group B), isolated in Catalonia (Northeastern Spain) during the 2016/2017 European epizootics. Both viruses are highly pathogenic based on the aminoacid sequences at the HA0 cleavage site: PEIPKGSRVRR↓GLF (H7N1) and PLREKRRKR↓GLF (H5N8). Virus stocks were produced in 10 days-old SPF embryonated eggs. The allantoic fluid was obtained at 24-48 hpi, filtered and aliquoted at -75ºC until use. Serial ten-fold dilutions of the filtered viruses in PBS were used for titration in 10 days-old SPF embryonated eggs. The mean egg lethal doses (ELD_{50}) were determined by Reed and Muench method (Reed & Muench, 1938).

H5N8 and H7N1 were subjected to full-genome characterization using next-generation sequencing methods. Briefly, viral RNA was extracted from virus-containing allantoic fluid using RNeasy Mini Kit (Qiagen, Valencia, CA, USA), following manufacturer's instructions. The whole-genome sequencing was carried out using an Ilumina Miseq platform. A RNAseq library (Illumina, San Diego, CA, US) was constructed and

checked using LabChip. A 250 Nano run of Miseq was performed (Illumina, San Diego, CA, US). Sample reads yielding a QC score >20 were accepted for further filtering. Reads were mapped against reference genomes, and a consensus sequence for every segment was assembled using a tailor-made script. The consensus full genome sequences corresponding to the eight segments of local H5N8 are available in Genbank under accession numbers MK494920 to MK494927 (H5N8).

The nucleotide sequences of the local H5N8 HPAIV were subjected to BLAST analyses in Global Initiative on Sharing All Influenza Data (GISAID) database (Shu & McCauley, 2017). The closest strains updated to GISAID until the isolation of A/Goose/Spain/IA17CR02699/2017 (H5N8) (2017.03.02) were annotated. Sequences available in GISAID until 2018.12.31 were downloaded and used for multiple sequence alignment in BioEdit 7.0. The nucleotide homologies between Spanish isolate and sequences available in databases were evaluated by the ClustalW method in BioEdit 7.0. The phylogenetic trees for each gene of AIVs (based on the nucleotides of the coding sequence) were constructed in MEGA X, using the Neighbor-joining algorithm, the Tamura-Nei model and 1000 bootstrap replicates to evaluate the confidence of the internal branches of the tree (Felsenstein, 1985; Saitou & Nei, 1987; Tamura & Nei, 1993; Kumar et al., 2018).

The presence of specific amino acids in particular positions in AIV proteins previously associated with host tropism, transmissibility and/or virulence of AIVs in mammals and avian species in the local H5N8 were evaluated using BioEdit 7.0. The amino acid identity and the differences in the amino acid sequence in the 8 conserved internal and

non-structural viral proteins (PB2, PB1, PA, NP, M1, M2, NS1, NS2) between H5N8 and H7N1 HPAIVs were determined.

2.2. Animals and facilities

A total of 29 geese (*Anser anser var.domestica*) of approximately 3-5 months of age were used in this study. Two breeds were included: 18 birds of the *Empordanesa* breed, a local geese breed present in backyards in Spain, and 11 birds of the G35-line geese, which is a commercial breed raised in specialized farms. At arrival, the birds were individually identified and placed in different negative-pressured HEPA-filtered boxes present in BSL-3 facilities in *Centre de Recerca en Sanitat Animal* (*Programa de Sanitat Animal*, IRTA). Water pools over the minimum size required by the Spanish Royal Decree 53/2013, which lays down the basic obligations and general principles concerning the animal protection in experimentation, were included in the boxes. In total, 4 enclosures were present. The birds were kept 5 days for acclimatization. Prior to infection, serum samples were obtained from all birds to ensure that they were seronegative to IAVs by a cELISA (ID-VET, Montpellier, France). Furthermore, oral swabs (OS) and cloacal swabs (CS) were collected from all birds and confirmed to be negative to AIV by one-step qRT- using the primers and probe (Spackman *et al*., 2002) as well as conditions of amplification previously described (Busquets *et al*., 2010)... During the experimental procedures, food and water were provided *ad libitum*. The experimental design was approved by the ethical commission of *Institut de Recerca i Tecnologia Agroalimentàries* (IRTA) and the Government of Catalonia (*Departament de Territori i Sostenibilitat, Direcció General de Polítiques Ambientals i Medi Natural*) under reference code CEEA 57/2017- 10185.

2.3. Experimental design and sampling

At arrival, 29 geese were randomly separated into five groups: four challenged groups and one negative group. For each HPAIV (H7N1 and H5N8), 5 commercial and 8 local geese were challenged via the intranasal route. The viruses were diluted in PBS in order to inoculate 10^5 ELD_{50} in a final volume of 0.05 mL (0.025 ml inoculated in each nostril). 1 commercial and 2 local geese were used as non-inoculated negative control birds, and due to space limitations, they were euthanized prior to infection in order to collect samples (as described below).

All birds were monitored daily for clinical signs until 15 dpi. A standardized OIE clinical scoring system was used (OIE, 2010). Endpoint criteria included severe listless, reluctance to movement and neurological disorders (i.e. head-shaking). Moribund geese were anesthetized using ketamine/xylazine (20 mg/kg body weight, Imalgene 100 and 5 mg/kg body weight, Rompun 20 mg/ml) via the intramuscular route, euthanized with intravenous pentobarbital (140 mg/kg body weight, Euthasol 400 mg/ml) and scored as dead. The clinical signs, mortality and MDT were recorded for each virus and breed.

Programmed necropsies were performed at 4 dpi and at the end of the study (15 dpi) in order to evaluate gross lesions and collect tissues for pathological studies and viral detection and quantification. 3 birds (2 local and 1 commercial) inoculated with H7N1 and H5N8 HPAIVs were randomly selected and sacrificed at 4 dpi. At the end of the study, 2 survivor geese of each breed were necropsied. All birds found dead as well as those euthanized for ethical reasons during the experiment were included. In order to evaluate viral shedding, OS and CS were obtained from all birds at 1, 3, 6 and 10 dpi. 1,5 ml of pool water were collected from all pools at the same time points. Furthermore,

approximately 0.75 ml of total blood in a 1:1 ratio with anticoagulant (Alsever's solution, Sigma-Aldrich, Missouri, USA) was extracted from the medial brachial vein of all geese at 3, 6 and 10 dpi. At the end of the study, serum samples were obtained from all survivor birds. All samples were appropriately conserved at -75ºC until further use.

2.4. Pathological examination and immunohistochemical testing

Tissues collected at necropsies were immersed in 10% buffered formalin for fixation during 48 hours and embedded in paraffin wax. These samples included skin, thymus, ocular conjunctiva, pectoral muscle, nasal cavity, trachea, lung, central nervous system, heart, spleen, liver, kidney, proventriculus, gizzard, pancreas, duodenum, cecum, colon and bursa of Fabricius. Microtome sections of 3 µm of thickness (Leica RM2255, Nussloch, Germany) from formalin-fixed, paraffin-embedded (FFPE) tissues were processed, stained with Hematoxylin and eosin (H/E) and then examined under light microscopy. An immunohistochemical (IHC) technique was performed on the same tissues. Sections were dewaxed and treated with 3% H_2O_2 in methanol at room temperature for 30 minutes to eliminate endogenous peroxidase activity. After a wash of distilled water and a wash of PBS, samples were pretreated with 0.1% protease at 37ºC during 8 minutes, followed by a wash of PBS for 5 minutes. A mouse-derived monoclonal commercial antibody against NP of IAVs (ATCC, HB-65, H16L-10-4R5) was used as a primary antibody (1:500 in dilution solution). The slides were incubated overnight at 4ºC. Three washes with PBS were performed. The samples were then incubated with an anti-mouse secondary antibody conjugated to an HRP-Labelled Polymer (Dako, immunoglobulins As, Denmark) at room temperature for 45 minutes. Following the incubation time, three washes with PBS were carried out. The antigen–

antibody reaction was visualized using the chromogen 3,3'-diaminobenzidine tetrahydrochloride (DAB) (0.05% DAB diluted in PBS + 100 µl H_2O_2) for 8 minutes. Sections were counterstained with Mayer's haematoxylin and examined under light microscopy. The positivity in the tissues was semi-quantitatively assessed taking into consideration the percentage of NP-positive and negative cells in the tissue. The samples were classified as follows: no positive cells (-), <10% positive cells (+), 10-40% positive cells (++), >40% positive cells (+++) in a tissue section. Positive and negative controls were used. The positive control was a central nervous system from a chicken experimentally infected with H7N1 HPAIV (Chaves *et al.,* 2011), and the negative control consisted in the same tissue from non-inoculated geese incubated with PBS instead of the primary antibody.

2.5. AIV RNA detection and quantitation

Swabs were placed in 0.5 ml of sterile PBS enriched with Penicillin-Streptomycin (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and Nystatin (Sigma-Aldrich, Missouri, USA) at a final concentration of 6%. Blood was centrifuged at 1000 g for 10 minutes and plasma was collected. Thin sections of spleen, central nervous system and lung obtained during necropsies were placed in 1 ml of RNAlater Stabilization Solution (Invitrogen, Carlsbad, CA, USA). After overnight conservation at 4º, RNAlater was removed from samples and 30 mg of each tissue were weighted, homogenized in 400 µl of Nuclease-free water using a pestle, centrifuged for 3 minutes and the supernatant collected.

Viral RNA was extracted from OS and CS, pool water, plasma and from homogenized RNAlater-stabilised tissues using Nucleospin RNA virus kit (Macherey-Nagel, Düren,

Germany), following manufacturer's instructions. A highly conserved region of IAVs M1 gene was detected by one-step Taqman RT-PCR in Fast7500 equipment (Applied Biosystems, Foster City, CA, USA), using the primers and probe (Spackman *et al*., 2002) as well as conditions of amplification previously described (Busquets *et al*., 2010). To extrapolate the genome equivalent copies (GEC) present in the swabs, a standard curve obtained by amplification of the same region of the M1 gene was included in the qRT-PCR technique. Briefly, the amplified region was ligated in pGEM-T vector (Promega, Madison, Wisconsin, USA). The ligation product was purified using MinElute Reaction Cleanup Kit (Qiagen, Valencia, CA, USA) and transfected into electrocompetent *E.coli* cells (Thermo Fisher Scientific, Waltham, Massachusetts, USA) by electroporation. The recombinant plasmid was purified from transformed colonies using NucleoSpin Plasmid (Macherey-Nagel, Düren, Germany) and quantified in Biodrop (Biodrop μLite, Cambridge, England). GEC were calculated using DNA Copy Number Calculation (Thermo Fisher Scientific, Waltham, Massachusetts, USA). The limit of detection of the technique was 1,89 log GEC in OS and CS, 2,37 log GEC in plasma and water and 1,79 log GEC in tissue samples.

2.6. Seroconversion

To evaluate seroconversion, serums were tested by a cELISA test that detects antibodies against the NP of IAVs (ID Screen® Influenza A Antibody Competition Multi-species, ID-VET, Montpellier, France). The technique was performed following manufacturer's instructions.

3. Results

3.1. Viral sequence homology, phylogenetic analyses and amino acid characterization

The complete genomes of H7N1 and H5N8 were fully sequenced. All genome segments of the Spanish H5N8 isolate presented high nucleotide identity (99.5-99-9%) to H5N8 HPAIVs previously isolated in Europe and Asia during 2016/2017 (Table 1).

Based on the topology of the HA gene phylogenetic tree, Spanish H5N8 isolate clustered within the genetic clade 2.3.4.4 group B of Gs/GD lineage, closely related with H5N8 B isolated in wild and domestic birds in Europe, Asia and Africa in 2016, 2017 and 2018 (Figure 1A). High homogeneity among H5N8 B 2016/2017 European isolates was found in NA (Figure 1B), M and NS (Sup. Figure 1A-B). However, based on PB2, PB1, PA and NP tree topology (Sup. Figure 1C-F), European isolates separated into five different clusters (named CL1, CL2, CL3, CL4, CL5). Spanish H5N8 B fell in all gene segments into CL2, which includes H5N8 B HPAIVs isolated from several European countries (France, Italy, Poland, Germany, Hungary, Croatia and Macedonia).

H5N8 HPAIV presented mutations related to increased pathogenicity, transmissibility and inhibition of host responses in avian and mammal species in all proteins (Sup. Table 1). High amino acid identity between H5N8 and H7N1 HPAIVs was found in PB2, PB1, PA, NP and MP proteins, but not in NS proteins (Table 2). Both HPAIVs present amino acids in particular positions associated to virulence and/or transmission of HPAIVs in chickens and/or ducks (PB2: 123E; PB1: 3V, 38Y, 436Y; PA: 237E, 383D, 515T, 672L; NP: 105V, 184K; M1: 43M; NS: 106M, 125D, 149A). Only 5 differing amino acids between the proteins sequence of H7N1 and H5N8 HPAIVs associated with a biological function were detected, and all were found in H5N8 HPAIV. This

virus presented 375N and 42S-55E-103F in PB1 and NS1 proteins, respectively, which are associated with increased virulence and/or inhibition of host immune response in mammals, and 103F and 114S in NS1 inhibit host gene expression in chicken cells (Table 2).

Clinical signs and mortality

Clinical signs were only observed in H5N8 HPAIV-inoculated geese, being listless and neurological signs the most frequently observed. Until 5 dpi, no evident clinical signs or mortality were recorded. At 5 dpi, one local goose was found dead without previous evident clinical signs. At 6 dpi, two geese (one local and one commercial) presented severe nervous signs, including ataxia and head shaking, and were consequently euthanized. Furthermore, two geese (one of each breed) were found dead without showing prior clinical signs. At 7 dpi, two local geese presented severe listless and incoordination. The remaining geese showed mild listless and tremor. At 9 dpi, one commercial goose was found dead. At 10 dpi, the remaining geese (one local and one commercial) were euthanized for ethical reasons, reaching a mortality of 100% in both breeds (Figure 2).

The MDT was 6,2 and 7 dpi in local and commercial geese inoculated with H5N8 . No differential susceptibility between local and commercial breeds to H5N8 HPAIV infection was present, considering mortality was 100% in both breeds and the minor differences in MDTs. Due to the low differences between breeds, no statistical analyses were carried out. Neither evident clinical signs nor mortality were recorded in H7N1 HPAIV-inoculated geese along the experimental period (Figure 2).

Gross lesions

Evident macroscopic lesions were only detected in the group of domestic geese inoculated with H5N8 HPAIV, being the pancreas the most affected organ. Macroscopic examination of H5N8 HPAIV-inoculated geese euthanized at 4 dpi revealed moderate congestion of the nasal turbinates and intestines. Moreover, two geese presented splenomegaly. However, specific lesions were not detected until 5 dpi, when the geese found dead presented multifocal areas of hemorrhage and necrosis in pancreas (Figure 3A), and tracheal congestion. Similar lesions in pancreas were observed in the necropsies performed in the severely-affected geese from 6 to 10 dpi. At 6 dpi, two geese also presented multifocal areas of necrosis in the liver associated with a moderate hepatomegaly. At 6 dpi, one geese presented multifocal petechiae in bursa of Fabricius, and another exhibited petechiae in gizzard. From 6 to 10 dpi, moderate to severe congestion in several organs, such as cecal tonsil, subcutaneous tissue and central nervous system, as well as necrotic areas in heart, were also frequently observed. At 10 dpi, one goose presented moderate friability of the liver, severe congestion of the intestinal mucosa and marked multifocal hemorrhages in central nervous system (Figure 3B).

Regarding H7N1 HPAIV-inoculated geese, birds necropsied at 4 and 15 dpi presented non-specific gross lesions, including mild to moderate congestion of the nasal turbinates and intestines. One goose also presented splenomegaly at 4 dpi.

Histopathological findings

Microscopic observation of tissues revealed evident lesions in mostly all H5N8 HPAIV-inoculated geese tissues, independently of the tested breed. The main microscopic

findings were multifocal to diffuse areas of necrosis and hemorrhages associated with inflammatory cell infiltration of variable severity. NP-positive cells in H5N8 HPAIV-inoculated geese were observed in mostly all collected tissues and correlated well with pathological findings. NP-positive staining was mostly detected in parenchymal cells, epithelial cells and inflammatory cells (Table 3).

The most severely affected organ was the central nervous system, followed by pancreas, liver, spleen, thymus and heart. In central nervous system, non-suppurative encephalitis characterized by multifocal areas of necrosis, spongiosis of the neuropil, chromatolysis, karyolysis, gliosis and diffuse congestion associated to widespread AIV antigen were present in cerebral cortex at 5 dpi, which correlated with the onset of mortality. In the cerebellum, necrosis of Purkinje cells associated to AIV antigen was also observed. Severe lesions and high viral antigen were observed in central nervous system in all birds until 7 dpi, and declined by 9 dpi (Figure 4A/B). At 10 dpi, the geese presented perivascular cuffing in brain, and was the only tissue that presented AIV-positive cells. Acinar cells lytic necrosis in pancreas was observed in nearly all geese, in association with low (4 dpi) to intense (6 dpi) AIV antigen detection in necrotic areas and surrounding acinar pancreatic cells (Figure 4C/D). Starting at 4 dpi and peaking from 5 to 7 dpi, severe multifocal areas of lytic necrosis and hemorrhages associated to high amounts of viral antigen were detected in liver (Figure 4E/F). In thymus and spleen, multifocal areas of necrosis and inflammatory infiltrate as well as moderate amounts of viral antigen were present from 4 to 9 dpi, reaching the maximum levels by 6-7 dpi (Figure 4G/H and I/J, respectively). In heart, multifocal areas of degenerated and/or necrotic myocardiocytes and mononuclear cell infiltration associated to low-moderate amounts of AI antigen were present from 5 to 9 dpi (Figure 4K/L). Mild focal areas of

necrosis and hemorrhages associated to low viral antigen were detected in other organs from 4 to 7 dpi, including kidney and bursa of Fabricius. In nasal cavity and lung, diffuse congestion associated to low amounts of viral antigen were also present. Single positive cells without evident microscopic lesions were detected in proventriculus and lamina propria of small intestine at 6 dpi and in 6-7 dpi, respectively. In general, higher amounts of AIV-positive cells were present in H5N8 HPAIV-inoculated local geese than in the commercial breed. No evident microscopic lesions or AIV-antigen positive cells were observed in H7N1 HPAIV-inoculated or negative control geese.

Viral shedding

High viral RNA excretion was present in H5N8 HPAIV-inoculated geese in both OS and CS from 3 dpi to 6 dpi (Figure 5A/B). Virus was firstly detected at 1 dpi only in OS from two commercial geese. At 3 dpi, moderate levels of viral RNA were present in nearly all local and commercial geese in both OS and CS samples. At 6 dpi, all birds presented viral RNA from OS and CS. On that day, viral RNA reached the maximum levels in OS in both breeds, and it was maintained at levels similar to 3 dpi in CS also in both breeds. At 10 dpi, only one local goose presented detectable levels of viral RNA, present in OS.

Regarding H7N1 HPAIV-inoculated geese, a very low number of birds presented viral shedding and it was principally restricted to samples from OS (Figure 5C/D). At 3 dpi, one local goose presented high levels of viral RNA from OS and in lower amounts from CS. At 6 dpi, the same local goose and one commercial goose tested positive to viral RNA only from OS. Lastly, at 10 dpi, viral RNA was detected again only from OS in one local goose and in two commercial geese. The local and one commercial geese

positive at 10 dpi were negative the previous days of sampling. We did not detect qualitatively important differences concerning viral shedding between local and commercial geese neither in H5N8 nor H7N1 HPAIVs-inoculated geese.

Viral RNA in pool water was detected in both enclosures of H5N8-inoculated geese and in the local geese inoculated with H7N1 HPAIVs. Viral RNA in the case of H5N8 HPAIV-inoculated groups was firstly detected at 6 dpi in both local (4,37 log GEC) and commercial geese (4,33 log GEC) groups, and the levels were constant until 10 dpi (4,28 log GEC, 4 log GEC, respectively). In contrast, viral RNA in the case of H7N1 HPAIV-challenged group was only present at 10 dpi in local geese, and was near to undetectable levels (2,49 log GEC).

Viral RNA in plasma

High levels of viral RNA were detected in plasma from almost all H5N8 HPAIV-inoculated local and commercial geese at 3 dpi, and all were positive at 6 dpi (Figure 6A). In general, the levels in plasma correlated well with levels present in swabs. At 10 dpi, no viral RNA was detected in plasma. Viral RNA in plasma of H7N1 HPAIV-inoculated geese was only detected in one local goose from 3 to 6 dpi and in one commercial goose at 3 dpi. The levels of viral RNA were generally lower and the decay was considerably faster than in H5N8 HPAIV-inoculated geese (Figure 6B). We did not detect differences concerning viral RNA in plasma between local and commercial geese neither in H5N8 nor in H7N1 HPAIV-inoculated geese.

Viral RNA in tissues

Since we did not find evident differences in mortality and in the main organs affected between local and commercial geese, the quantification of viral RNA in brain, spleen and lung in both breeds are described together. In H5N8 HPAIV-inoculated groups (Figure 7A), high amounts of viral RNA were detected in the spleen and lung at 4 dpi in all geese euthanized for pathological purposes. Low levels were present at that time in brain. Viral RNA in brain notably increased in all found dead and moribund geese from 5 to 10 dpi, reaching titers higher than 9 log GEC at 6, 7 and 9 dpi. At 10 dpi, high levels of virus were still detected in brain samples. Viral RNA in spleen and lung were detected in all birds and remained high at the different time points, reaching the maximum levels at 7 dpi. At 10 dpi, low levels were present in lung, and were undetectable in spleen.

Regarding H7N1 HPAIV-inoculated geese (Figure 7B), moderate amounts of viral RNA were detected in spleen and in lung in one geese at 4 dpi, but not in brain. At 15 dpi, both geese tested positive to AIV RNA in brain samples. One bird also presented AIV RNA in lung, but near the limit of detection of the technique. No viral RNA was detected in negative control birds.

Seroconversion

All birds were seronegative prior to infection. At 15 dpi, 33,3% (2/6) and 75% (3/4) of H7N1 HPAIV-inoculated local and commercial survivor geese, respectively, seroconverted. All birds that seroconverted presented viral RNA in plasma and/or tissues and/or presented viral RNA in swabs. H5N8-inoculated geese could not be tested since all birds succumbed to infection.

4. Discussion

Before the emergence of Gs/GD H5 lineage, the isolation of HPAIVs in domestic geese had been more sporadic than in gallinaceous species and generally not associated to high mortalities (Alexander & Brown, 2009). Several studies demonstrate that domestic geese have played an important role in the emergence, evolution, perpetuation and interspecies transmission of HPAIVs belonging to Gs/GD H5 lineage (Sonnberg *et al.,* 2013), but the information about their potential role in the epidemiology of other HPAIVs is lacking. In 2016-2017, the HPAIV H5N8 belonging to clade 2.3.4.4 Group B of Gs/GD lineage caused an unprecedented number of outbreaks in domestic duck and geese holdings in Europe (Napp *et al*., 2018). Here, a local and a commercial breed of domestic geese were intranasally inoculated with a H5N8 HPAIV (Gs/GD Spanish H5N8 clade 2.3.4.4 B strain) and H7N1 HPAIV (Italian strain). To our knowledge, this is the first study performing a profound direct comparison of the pathobiological features of different lineages of HPAIVsin domestic geese.

Our data clearly demonstrated that domestic geese were highly susceptible to H5N8 HPAIV infection. Starting at 5 and lasting until 10 dpi, the mortality rate in the domestic geese inoculated with H5N8 HPAIV reached the 100%. Previous studies have reported a wide variation of susceptibility of domestic geese to Gs/GD H5 HPAIVs. The lethality ranges from 0 to 100% depending on the subtype, clade and genetic group of the Gs/GD H5 HPAIV, dose of inocula, species (*Anser anser, Anser cygnoides*), breed and age at infection (Perkins & Swayne, 2002; Webster *et al*., 2002; Zhou *et al*., 2006; Smietanka *et al*., 2013; Berhane *et al*., 2016; Xiang *et al*., 2017; Pantin-Jackwood *et al*., 2017; Grund *et al*., 2018). In the present study, two factors could influence the

pathogenicity of H5N8 HPAIV in geese, most probably by reducing it. First, we challenged domestic geese with a dose of inoculum comparatively lower than the generally used for experimental infections in waterfowl species (10^5 ELD_{50} *versus* 10^6 ELD_{50} or higher). Second, we used geese of approximately 3 to 5 months of age, while most studies have performed the experimental infections in younger birds (Perkins & Swayne, 2002; Webster *et al*., 2002; Zhou *et al*., 2006; Smietanka *et al*., 2013; Berhane *et al*., 2016; Xiang *et al*., 2017; Pantin-Jackwood *et al*., 2017; Grund *et al*., 2018). The high mortality rates observed in our study despite the factors mentioned above demonstrate that H5N8 HPAIVs belonging to clade 2.3.4.4 Group B of Gs/GD lineage circulating in Europe in 2016-2017 were highly virulent to domestic geese. In comparison with the mortalities reported in previous studies, H5N8 clade 2.3.4.4 Group B HPAIVs appear to be more virulent to domestic geese than H5N8 clade 2.3.4.4 Group A HPAIVs (Grund *et al*., 2018; Pantin-Jackwood *et al*., 2017).

Grund *et al.* (2018) showed that all domestic ducks inoculated with European-isolated H5N8 B HPAIV succumbed to infection when inoculated by the intra-muscular route, but the mortality rates in those inoculated by the natural route (oro-nasal) decreased to the 20%. Similarly, the mortalities in domestic ducks were below 20% in the study of Slomka *et al.* (2019). Herein, the mortalities after intranasal inoculation reached the 100%, indicating that domestic geese are more susceptible to H5N8 B HPAIVs circulating in Europe in 2016-2017 than domestic ducks. The higher severity of clinical signs after inoculation with Gs/GD H5 HPAIVs in domestic geese over domestic ducks has been demonstrated in other experimental studies comparing side by side both species (Perkins & Swayne, 2002; Pantin-Jackwood *et al.,* 2017).

The pathogenicity of Gs/GD H5 HPAIVs in a wide range of avian and mammal species has been associated with the strong neurotropism characteristics of this lineage. Domestic geese infected with Gs/GD H5 HPAIVs usually exhibit neurological signs and microscopic lesions and viral antigen/RNA are detected in the central nervous system with or without mortality associated (Perkins & Swayne, 2002; Webster *et al*., 2002; Zhou *et al*., 2006; Smietanka *et al*., 2013; Berhane *et al*., 2016; Xiang *et al*., 2017; Pantin-Jackwood *et al*., 2017). In our study, the main clinical signs observed in the severely-affected geese were neurological, including tremor, ataxia and head shaking. The histological lesions of the tissues coincided with the clinical manifestations: birds infected with H5N8 HPAIV showed multifocal to diffuse areas of necrosis in the central nervous system associated to widespread presence of AIV antigen demonstrated by IHC. These findings correlated well with the onset of mortality at 5 dpi and were common until the end of the study. As expected, viral RNA quantification in brain was in concordance with IHC results, being the organ that presented the highest viral loads. Thus, our results demonstrate that Gs/GD H5N8 2.3.4.4 Group B HPAIV is highly neurovirulent to domestic geese. Despite neurological dysfunction was considered the main cause of the high mortalities, lesions in other organs could have an important effect in the infection outcome. Our data indicated that H5N8 HPAIV presented a multisystemic tropism in domestic geese. Multifocal areas of hemorrhages in the pancreas were commonly observed during the macroscopic examination of the birds. Microscopically, the birds exhibited large necrotic and inflammatory lesions and high levels of AIV antigen presence in pancreas, liver, and to a lesser extent in spleen and thymus. The intensity of the lesions produced by H5N8 HPAIV in these organs could lead to multi-organ failure, and compromise the cellular immunity in case birds

survive to infection. Similarly, Grund *et al*. (2018) reported that the H5N8 clade 2.3.4.4 Group B HPAIV also presented an intense hepato-tropism in domestic ducks.

Previous reports have demonstrated that HPAIVs other than those belonging to Gs/GD H5 lineage can readily infect domestic waterfowl and replicate in different internal organs, but in the majority of cases they produce a subclinical infection to mild disease (Wood *et al*., 1995; Aldous *et al*., 2010; Hiono *et al*., 2016; Nakayama *et al*., 2019). Specifically, the experiments performed in domestic geese of Nayaran *et al.* (1969) and Röhm *et al.* (1996) showed that H5N9 and H7N7 HPAIVs, respectively, did not produce mortalities in this species. However, experimental inoculation of particular H7 HPAIVs has caused severe clinical signs and mortality in ducks, in variable proportion depending on the species, virus strain and route of inoculation (Shi *et al*., 2018; Scheibner *et al*., 2019). These studies demonstrate the potential virulence of particular lineages of HPAIVs for domestic waterfowl. Therefore, the low mortalities reported in domestic waterfowl may have been the result of low exposure rather than to low virulence of the viruses for these species. During the epidemics caused by the HPAIV H7N1 in Italy in 1999/2000, several flocks consisting in mixed poultry species reported high mortalities. However, domestic waterfowl were generally unaffected. In contrast, a particular outbreak was characterized by mortality and nervous signs in domestic ducks and geese and by means of IHC techniques, the authors demonstrated AIV antigen in pancreas and in the central nervous system in these birds (Capua & Mutinelli, 2001). In the present study, domestic geese were susceptible to infection with the Italian H7N1 HPAIV as demonstrated by the seroconversion in several individuals. However, none of the birds showed evident clinical signs, gross or microscopic lesions, and all of them survived. Therefore, our results support the theory that most lineages of HPAIVs appear

to be avirulent to domestic geese under experimental conditions. However, we detected AIV RNA in plasma and in the three collected organs (central nervous system, pancreas and spleen) at different time-points in several geese inoculated with H7N1 HPAIV. In addition, viral RNA was detected in the spleen of one goose that presented splenomegaly at 4 dpi. Interestingly, one goose still presented detectable levels of viral RNA in brain and lung at the end of the study. Even though, the detection of viral RNA in tissues and plasma was inconsistent and at lower levels in comparison with those obtained from H5N8 HPAIV-inoculated geese, and all birds lacked AIV-positive cells by IHC techniques. These results provide evidence for a poor capacity of H7N1 HPAIV to produce a robust systemic infection in domestic geese. Therefore, differences related to the viral isolate, to the challenged dose, or to underlying factors in the birds (e.g. immunosupression, concomitant pathogens) could have facilitated the systemic dissemination of H7N1 HPAIV in that particular flock during the Italian 1999/2000 epidemics and consequently contributed to the mortality. The effect of co-infections in the pathogenicity of HPAIVs in poultry have been reviewed previously (Samy and Naguib, 2018). Specifically, co-infections of HPAIVs with other pathogens have been naturally detected in domestic waterfowl (Mansour *et al*., 2018). The age of the birds could be another pivotal factor. Previous studies have shown important differences in infection outcome to HPAIVs between domestic ducks of different ages, being the younger ducks more susceptible to infection and prone to show more severe clinical signs and mortality (Löndt *et al*., 2010; Pantin-Jackwood et al., 2007). The lack of mortality detected in domestic geese in the present study in comparison with that detected after the inoculation of the same H7N1 virus in ducklings (20% mortality) in the study of Scheibner *et al.* (2019) could be attributed to differences in age.

In the present study, we also evaluated the potential role of domestic geese in the epidemiology of the selected HPAIVs. Previous reports indicate the potentially important role of domestic geese in the epidemiology of Gs/GD H5 HPAIVs, as determined by high viral shedding and transmission to contact birds, and in some cases in a subclinical way (Zhou *et al*., 2006; Berhane *et al*., 2016; Xiang *et al*., 2017; Pantin-Jackwood *et al*., 2017). In our study, H5N8 HPAIV-inoculated geese shed large amounts of virus by the oropharynx (ranging from 2,16 to 7,75 log GEC) and the cloaca (ranging from 2,43 to 7,52 log GEC), suggesting that fecal-oral and oral-oral routes as well as respiratory tract exposure by means of inhalation of aerosols or large dopplers could play major roles in the transmission of H5N8 HPAIVs in domestic geese populations. Previous studies observed that Mandarin ducks inoculated with H5N8 HPAIV (clade 2.3.4.4 Group A and B) presented higher viral shedding (especially via the cloaca) than those infected with H5N1 (Clade 2.2 and 2.3.2.1) and H5N6 (clade 2.3.4.4 Group C) HPAIVs (Kang *et al.,* 2017; Son *et al.,* 2018). These findings support high replication rate and shedding from infected waterfowl into the environment which may favor more efficient transmission and spread between some waterfowl and geographic locations.

We detected a moderate viral load in the pool water at the later stages of infection. The detection in water suggests that sharing contaminated water can play an important epidemiological role as a source of H5N8 HPAIV infection. For instance, access to outdoor water and percentage of surface occupied by ponds are risk factors in the introduction of Gs/GD H5 HPAIVs to poultry flocks (Desvaux *et al.,* 2011). The detection of a high viral load in plasma indicates that blood (e.g. as a result of fighting

between infected geese) may represent an additional source of environmental contamination.

Despite the absence of clinical signs and mortality until 5 dpi, high viral shedding was already detected at 3 dpi. This, together with the high viral loads detected in all samples including water, suggest the possible involvement of domestic geese as an amplifying host, resulting in high environmental contamination. Thus, domestic geese could potentially facilitated the transmission of H5N8 B HPAIV between waterfowl holdings and spill back to wild birds, either directly or through a common source of water during the 2016-2017 H5N8 B European epidemics.

The high and/or prolonged viral excretion reported in several studies suggest that waterfowl could play a role in the dispersal of HPAIVs other than those belonging to Gs/GD lineage in case the infection is established. The study conducted by Pantin-Jackwood *et al.* (2016) demonstrated that mallards experimentally inoculated with a battery of H5 and H7 HPAIVs strains transmitted the virus to contact mallards. In addition, lesser scaups (*Aythya affinis*) infected with two North-American H7 lineages of HPAIVs shed virus up to 14 days after infection (Stephens *et al*., 2019). Domestic geese inoculated with H7N7 isolated in Germany also excreted titers similar to those in chickens up to 7 days (Röhm *et al*., 1996). H7 HPAIVs derived from the Asian H7N9 lineage also appears to be in process of adaptation to waterfowl, with ducks shedding virus for several days after experimental inoculation (Nakayama *et al*., 2019). However, the literature studying the potential role of domestic waterfowl in the epidemiology of non-Gs/GD HPAIVs is still scarce. In our study, a low number of H7N1 HPAIV-inoculated geese shed virus during the experiment and was mostly restricted by the oral

route, suggesting a high degree of adaptation of the virus to gallinaceous species. Similarly, few birds presented detectable levels of viral RNA in plasma. In addition, levels were close to the limits of detection of the technique in pool water. However, the levels detected in the positive OS were comparable with those collected from H5N8 HPAIV-inoculated geese (up to 6,32 log GEC). In addition, the detection of viral RNA in swabs in two geese at 10 dpi that were negative the prior days of sampling could be indicative of secondary transmission from experimentally infected geese in which productive infection took place (i.e. by aerosols), or from contaminated environment, including the pool, food or other fomites;,. Since geese did not present any evident clinical signs through the study, we suggest that domestic geese may play, to some extent, a role in the perpetuation and transmission of different lineages of HPAIVs to more susceptible avian species without infection in geese being noticed. However, the overall risk in spread of H7N1 by domestic geese is considered to be low.

Several mutations in viral gene segments that lead to amino acid substitutions in AIV proteins have been associated as markers of adaptation, and/or to increased virulence and transmissibility of HPAIVs in birds. PB2, PB1, PA, HA, NP, and NS gene segments are all responsible for Gs/GD H5N1 HPAIV pathogenicity in ducks (Hulse-Post *et al*., 2007; Sarmento *et al*., 2010; Song *et al.,* 2011; Hu *et al*., 2013; Kajihara *et al.*, 2013). No information is available in geese. The H7N1 and H5N8 HPAIVs strains used in the present study presented numerous amino acid differences in NS protein (67.3% identity) and to a lesser extent in PB2, PB1, PA, NP and M proteins (≥95% identity). Both HPAIVs presented amino acid substitutions associated with increased virulence in ducks (PB1: 436Y; PA: 237E; M1: 43M) (Hulse-Post *et al*., 2007; Hu *et al.,* 2013; Nao *et al*., 2015).. Therefore, the differences observed between viruses in our

study could also be associated to molecular markers of adaptation and virulence to waterfowl species that are still unidentified.

Some studies demonstrate wide differences in the susceptibility to HPAIVs between chicken breeds/lines (Sironi *et al*., 2008; Blohm *et al*., 2016; Lee *et al*., 2016; Matsuu *et al.,* 2016; Park *et al.,* 2019), whereas in ducks the differences appear to be less evident (Saito *et al*., 2009; Pantin-Jackwood *et al.,* 2013). In the present study, we did not detect evident differences in susceptibility between the local and commercial breed. However, the high mortalities of both, local and commercial geese, after infection with H5N8 HPAIV makes them suitable sentinels for the presence of the virus in the domestic-wild interface (local breed) and its introduction into commercial holdings (commercial breed). Domestic geese should be also targeted in active surveillance programs to early detect the circulation of HPAIVs of the Gs/GD lineage since they presented high viral loads of virus in different samples (OS, CS and blood) before the presence of evident clinical signs. The susceptibility of local geese to H5N8 and, to a lesser extent, to H7N1 HPAIVs is of particular interest. This breed is mostly reared in backyards, usually mixed with other domestic poultry species under minor biosecurity measures, which facilitates the exchange of HPAIVs between wild and domestic avian species and the potential generation of novel HPAIV reassortants with unknown biological characteristic to avian and mammal species.

The results of the present study demonstrate that domestic geese are susceptible to H5N8 and H7N1 HPAIVs. However, we demonstrate the lower infectivity, virulence and excretion of the H7N1 HPAIV strain in domestic geese in comparison with the Gs/GD lineage H5N8 strain when compared side by side. Since viral shedding were

detected in both H7N1 and H5N8-inoculated geese, and HPAIVs (including H5N8) continue to evolve and acquire new biological characteristics, an enhanced monitoring in a broad range of avian species, including backyard and commercial geese must be guaranteed in order to avoid the perpetuation of HPAIVs in the domestic-wild interface.

Disclosure statement

The authors declare that they have no competing interests.

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Tables

Table 1. Closest strains to H5N8 isolated in Spain, identity (%) and isolation date.

Genome segment	Closest strain (complete segment)	Nucleotide Id (%)	Isolation date
HA	A/goose/Hungary/55128/2016 (A/H5N8)	99,8%	16.11.11
NA	A/Indian Runner Duck/Czech Republic/749-17/2017 (H5N8)	99,6%	17.01.16
PB2	A/Anas platyrhynchos/Belgium/1899/2017 (A/H5N8)	99,8%	17.02.27
PB1	A/Anas platyrhynchos/Belgium/1899/2017 (A/H5N8)	99,7%	17.02.27
PA	A/duck/France/161108h/2016 (A/H5N8)	99,9%	16.11.28
NP	A/duck/France/161108h/2016 (A/H5N8)	99,7%	16.11.28
MP	A/chicken/Kalmykia/2643/2016 (A/H5N8)	99,5%	16.11.21
NS	A/domestic goose/Germany-BY/R677/2017 (A/H5N8)	99,5%	17.01.25

Table 2. Amino acid identity and differing amino acids in the viral proteins sequence between A/Goose/Spain/IA17CR02699/2017 (H5N8) and A/Chicken/Italy/5093/1999 (H7N1) HPAIVs. The amino acids previously associated with a phenotype are highlighted in black.

Protein	Aa Id (%)	Differing amino acids (aminoacid in H5N8 vs aminocid in H7N1)
PB2	98,9	K80R, M90I, N127H, I255V, S286G, V451I, R508M, R555K
PB1	98,5	A110T, G154D, R168K, S216G, G261S, T374N, **N375S**, K586R, V606I, S694N, K745E
PA	98,6	G59E, I61T, H96N, N115K, N184S, E252K, L261M, S453C, I459M, Y503F
NP	99,2	I201V, T350A, S377N, S403A
M1	98	V33A, L144F, I165M, N207S, R230K
M2	98,9	V50I
NS1	67,3	M6I, L7T, F14Y, Y17H, V18I, R21K, F22L, A23S, D24M, Q25R, E26D, G28C, L33D, **S42A**, R44K, N48S, G53D, I54C, **E55R,** T56V, R59M, A60E, Q63K, R67D, E70K, E71S, S73T, A76N, M79I, T80A, V81I, S86A, S87P, L90I, T94S, L95I, M98I, D101E, **F103Y**, K108R, A112T, **S114G,** C116M, I117V, N127R, V129I, V136I, I137L, R140Q, A143T, I145V, L146S, E153D, G158A, L163I, F166M, T170S, D171T, V180I, T191S, V192I, V194A, T197N, L198I, R204G, N205I, S206R, N207D, D209N, R211G, S213P
NS2	81,8	M6I, L7T, M14Q, G22E, E26V, G36E, S37R, L40I, Y48S, G63A, K64T, E67D, Q68E, G70S, E81A, V83C, H85N, R86I, K88T, I89K, M100L, Q111S

Table 3. Distribution of NP-positive cells and associated microscopic lesions in tissues collected from local and commercial geese experimentally inoculated with HPAIV H5N8. No positive cells (-), <10% positive cells (+), 10-40% positive cells (++), >40% positive cells, nd: not determined. L: local geese; C: commercial geese.

Tissue	4 dpi		5 dpi		6 dpi		7 dpi		9 dpi		10 dpi		NP+ cells	Microscopic lesions
	L	C	L	C	L	C	L	C	L	C	L	C		
Central nervous system	- (0/2)	- (0/1)	+++ (1/1)	nd	+++ (2/2)	+++ (2/2)	+++ (2/2)	nd	nd	+ (1/1)	+ (1/1)	+ (1/1)	Neurons, glial cells, ependymal cells, Purkinje cells	Multifocal areas of necrosis, diffuse congestion, perivascular cuffing (10 dpi)
Pancreas	+ (1/2)	- (0/1)	++ (1/1)	nd	++ (2/2)	+++ (2/2)	++ (2/2)	nd	nd	+ (1/1)	- (0/1)	- (0/1)	Acinar cells, macrophages	Multifocal areas of necrosis with inflammatory infiltrate
Liver	+ (2/2)	- (0/1)	++ (1/1)	nd	++ (2/2)	++ (2/2)	++ (2/2)	nd	nd	+ (1/1)	- (0/1)	- (0/1)	Hepatocytes, Kupffer cells, macrophages	Multifocal areas of necrosis and hemorrhages with inflammatory infiltrate
Spleen	+ (2/2)	- (0/1)	+ (1/1)	nd	++ (2/2)	++ (2/2)	++ (2/2)	nd	nd	+ (1/1)	- (0/1)	- (0/1)	Lymphoid cells, macrophages,	Multifocal areas of necrosis and hemorrhages with inflammatory infiltrate
Thymus	+ (2/2)	- (0/1)	++ (1/1)	nd	++ (2/2)	+ (2/2)	++ (1/2)	nd	nd	+ (1/1)	- (0/1)	- (0/1)	Lymphoid cells, macrophages,	Multifocal areas of necrosis with inflammatory infiltrate
Heart	- (0/2)	- (0/1)	+ (1/1)	nd	+ (2/2)	++ (2/2)	+ (2/2)	nd	nd	+ (1/1)	- (0/1)	- (0/1)	Myocardyocytes	Multifocal areas of necrosis with inflammatory infiltrate
Skin	- (0/2)	- (0/1)	- (0/1)	nd	+ (1/2)	+ (1/2)	- (0/2)	nd	nd	- (0/1)	- (0/1)	- (0/1)	Feather follicles	No apparent lesions
Nasal turbinates	+ (1/2)	- (0/1)	+ (1/1)	nd	+ (1/2)	+ (1/2)	++ (2/2)	nd	nd	- (0/1)	- (0/1)	- (0/1)	Respiratory epithelial cells, inflammatory cells	Diffuse congestion and edema with inflammatory infiltrate
Lung	+ (1/2)	- (0/1)	+ (1/1)	nd	+ (1/2)	+ (1/2)	+ (1/2)	nd	nd	- (0/1)	- (0/1)	- (0/1)	Macrophages	Diffuse congestion
B.Fabricius	+ (1/2)	- (0/1)	+ (1/1)	nd	+ (1/2)	- (0/2)	+ (1/2)	nd	nd	- (0/1)	- (0/1)	- (0/1)	Lymphocytes, macrophages	Bursal depletion, focal hemorrhagic areas
Kidney	- (0/2)	- (0/1)	- (0/1)	nd	+ (1/2)	- (0/2)	+ (1/2)	nd	nd	- (0/1)	- (0/1)	- (0/1)	Tubular epithelial cells, inflammatory cells	Congestion, focal hemorrhagic areas, focal necrosis of tubular cells
Proventriculus	- (0/2)	- (0/1)	- (0/1)	nd	+ (1/2)	+ (1/2)	- (0/2)	nd	nd	- (0/1)	- (0/1)	- (0/1)	Epithelial cells of the gastric glands	No apparent lesions
Small intestine	- (0/2)	- (0/1)	- (0/1)	nd	+ (1/2)	+ (1/2)	+ (2/2)	nd	nd	- (0/1)	- (0/1)	- (0/1)	Lymphoid cells	No apparent lesions

Figure captions

Figure 1. Neighbor-joining phylogenetic trees of HA (A) and NA (B) gene segments. The Spanish H5N8 is highlighted with a black dot. Bootstrap values ≥70% (700/1000 replicates) are shown. The different clusters are presented: 1 (orange), 2 (blue), 3 (green), 4 (yellow), 5 (grey). The clusters are based in the different genotypes identified in Poland, Germany, Italy and the Netherland reported in the study of Swiezton & Smietanka (2018). Scale bar indicates nucleotide substitution per site.

Figure 2. Survival curves of domestic geese experimentally inoculated with H7N1 o H5N8 HPAIVs at a dose of 10^5 ELD_{50}.

Figure 3. Diffuse hemorrhagic areas in pancreas (A) and central nervous system (B) found in geese experimentally inoculated with H5N8 HPAIV.

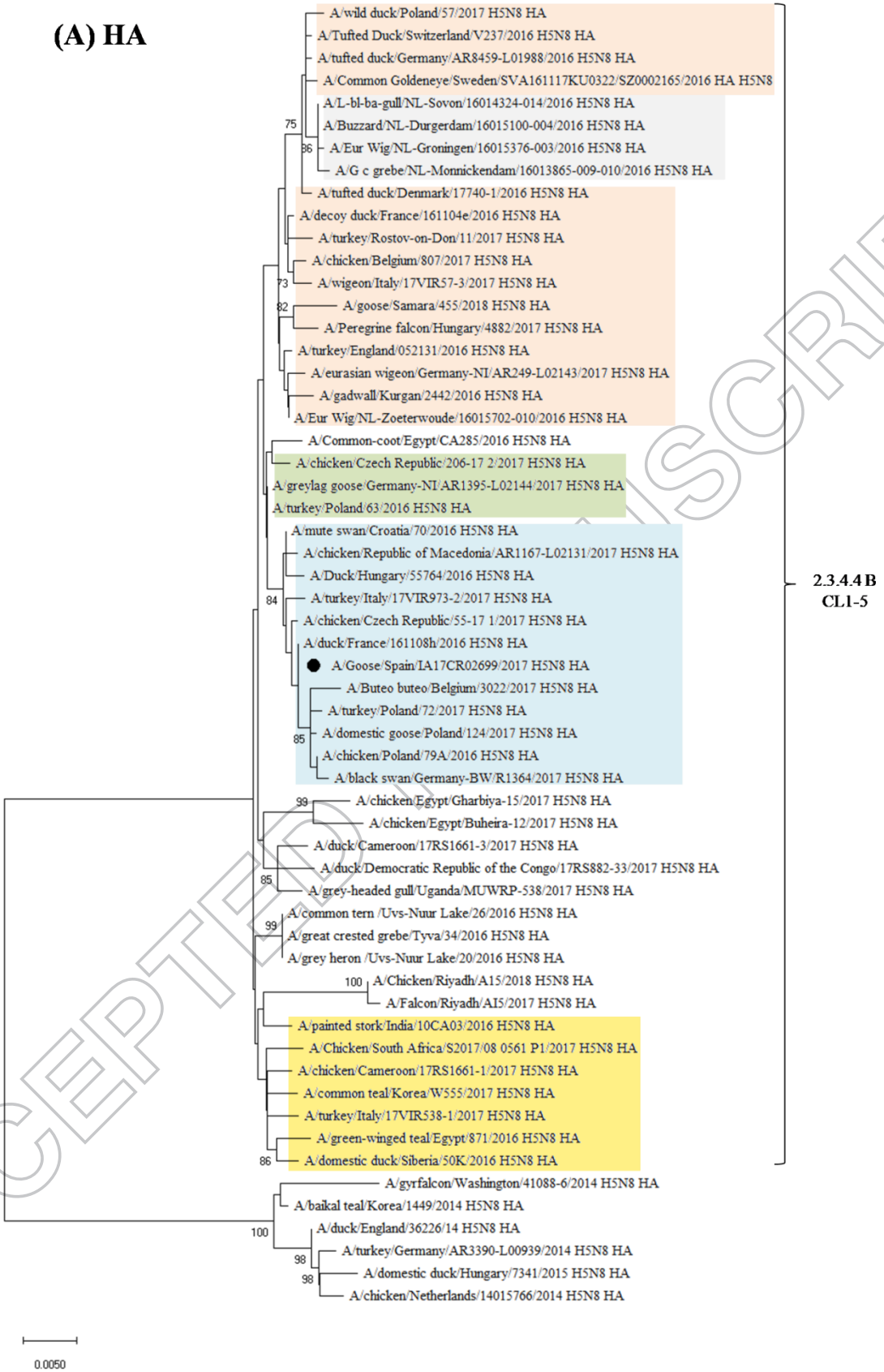
Figure 4. Serial sections of different organs of geese experimentally inoculated with H5N8 HPAIV stained with conventional HE staining and IHC techniques against NP nucleoprotein, respectively (20x). CNS A/B: diffuse areas of spongiosis and gliosis of cerebral parenchyma (A) and NP-positive neurons and glial cells (B). Pancreas C/D: diffuse areas of lytic necrosis of acinar pancreatic cells (C) and NP-positive cells in necrotic areas and surrounding acinar pancreatic cells (D). Liver E/F: multifocal areas of necrosis in liver parenchyma (E) and diffuse NP-positive hepatocytes, Kupfer cells and inflammatory cells (F). Thymus G/H: areas of necrosis in medulla (I) and NP-positive lymphoid cells (J). Spleen I/J: multifocal areas of mild necrosis and diffuse congestion (G) and presence of inflammatory cells positive to NP (H). Myocardium

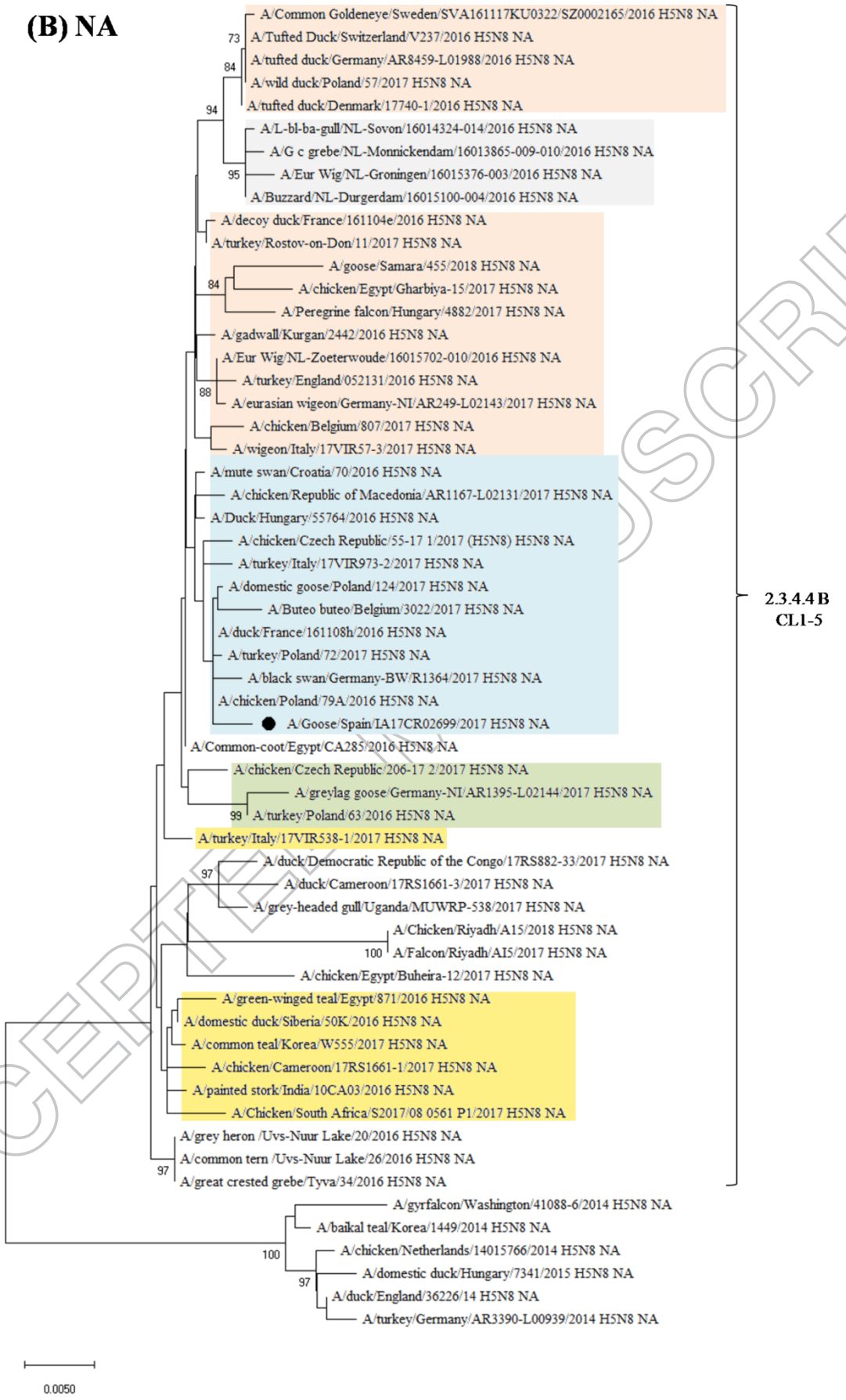
K/L: multifocal necrosis of myocardiocytes with mild inflammatory infiltrate (K) and NP-positive myocardiocytes and inflammatory cells (L).

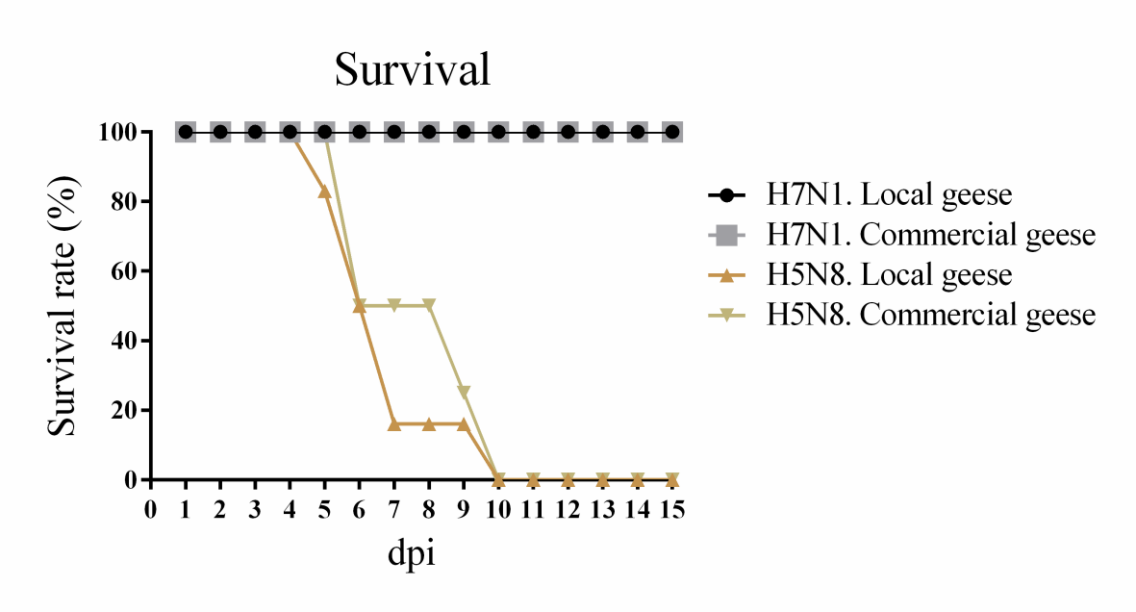
Figure 5. Viral titers expressed as log GEC in OS and CS obtained from domestic geese (local and commercial) inoculated with H5N8 (A, B) or H7N1 (C, D) HPAIVs at different time points post-inoculation. The ratios above the columns represent the number of birds shedding virus out of the total sampled. Dotted line represents limit of detection of the technique. Represented as Mean ± SEM. GEC: Genome equivalent copies; Dpi: day post-infection.

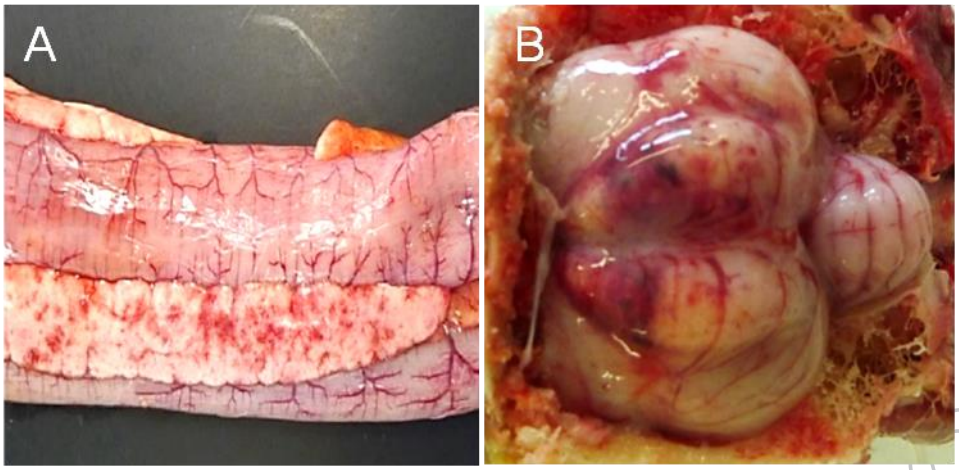
Figure 6. Viral titers expressed as log GEC in plasma obtained from domestic geese (local and commercial) inoculated with H5N8 (A) or H7N1 (B) HPAIVs at different time points post-inoculation. The ratios above the columns represent the number of birds showing viremia out of the total sampled. Dotted line represents limit of detection of the technique. Represented as Mean ± SEM. GEC: Genome equivalent copies; Dpi: day post-infection.

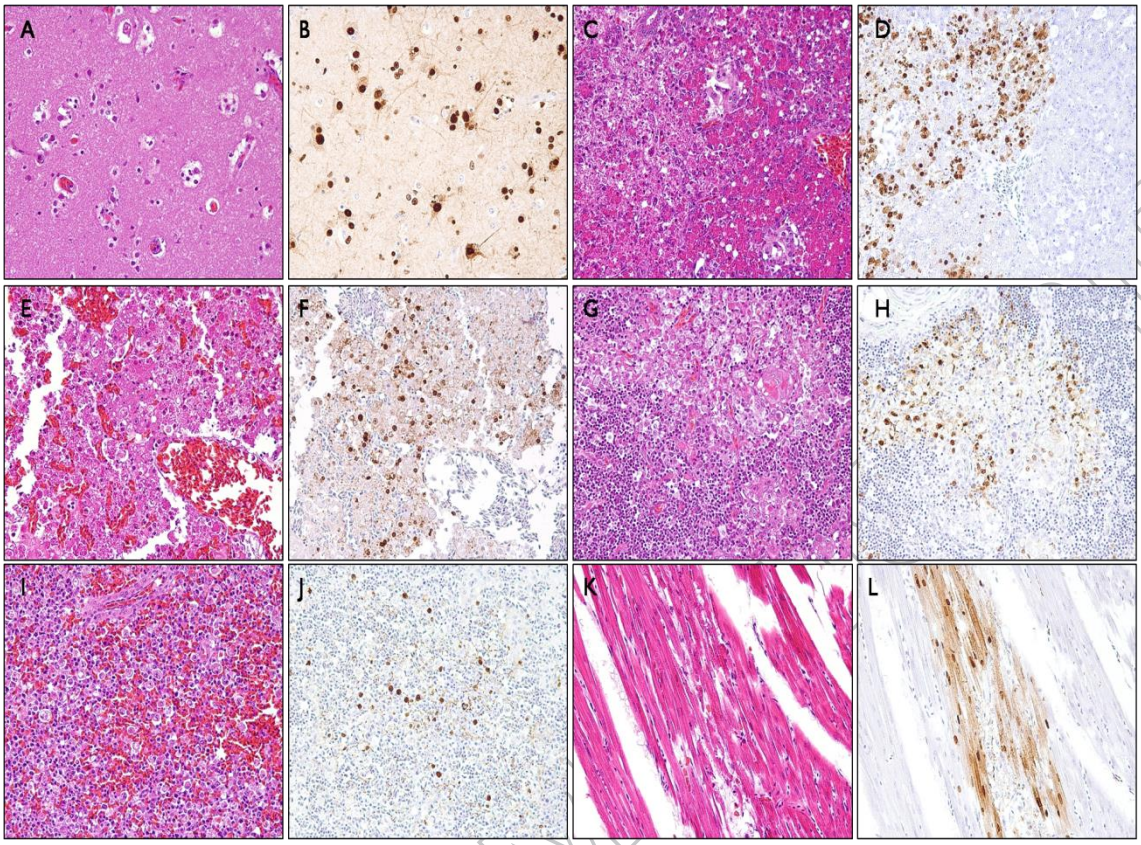
Figure 7. Viral titers expressed as log GEC in tissues (brain, spleen and lung) obtained from domestic geese (local and commercial) inoculated with H5N8 (A) or H7N1 (B) HPAIVs at different time points post-inoculation. The ratios above the columns represent the number of birds were viral RNA was detected out of the total sampled. Dotted line represents limit of detection of the technique. Represented as Mean ± SEM. GEC: Genome equivalent copies; Dpi: day post-infection.

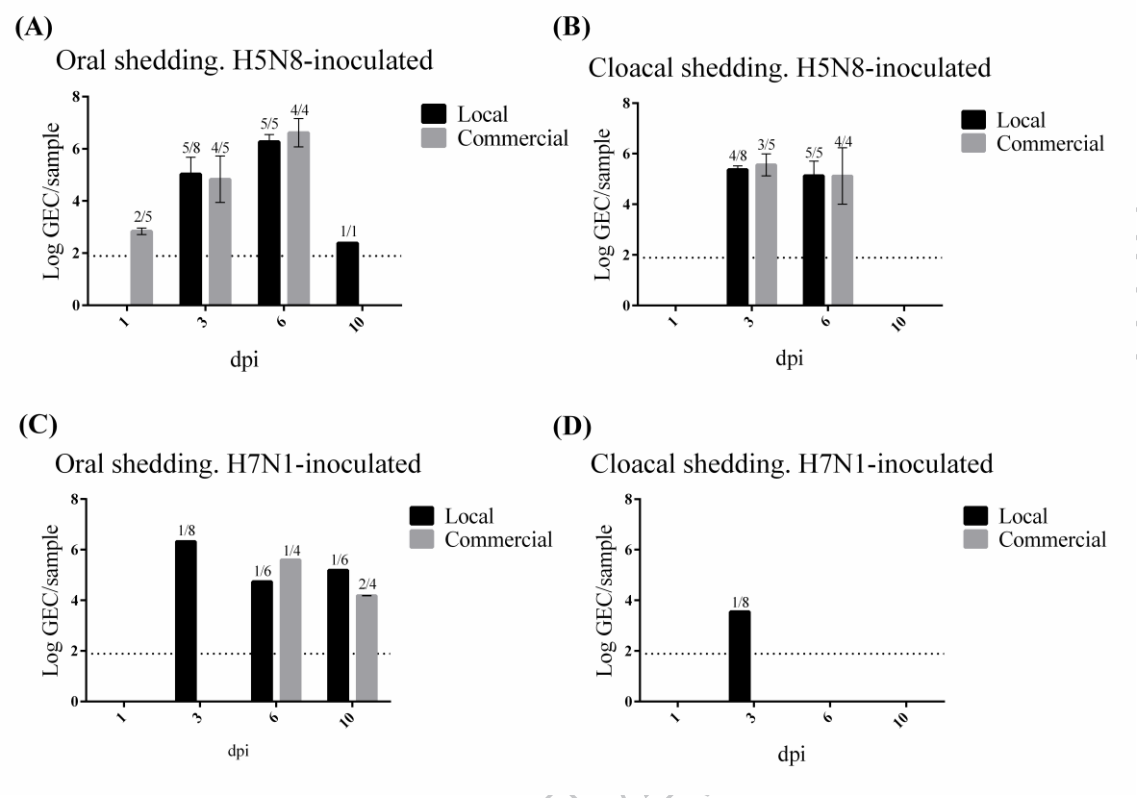


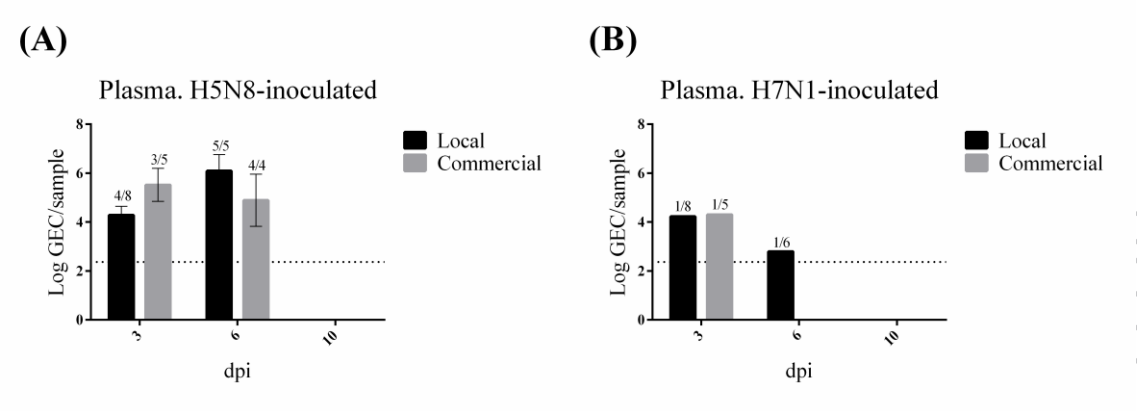


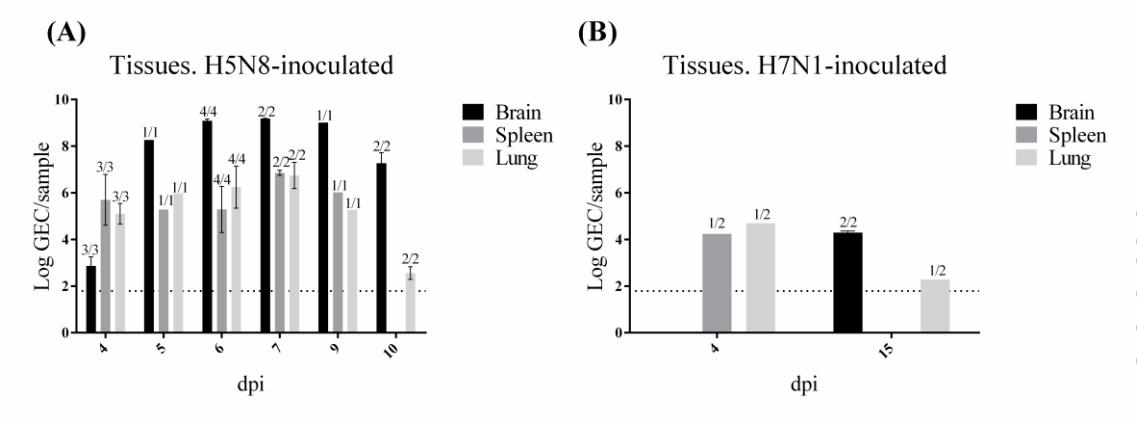








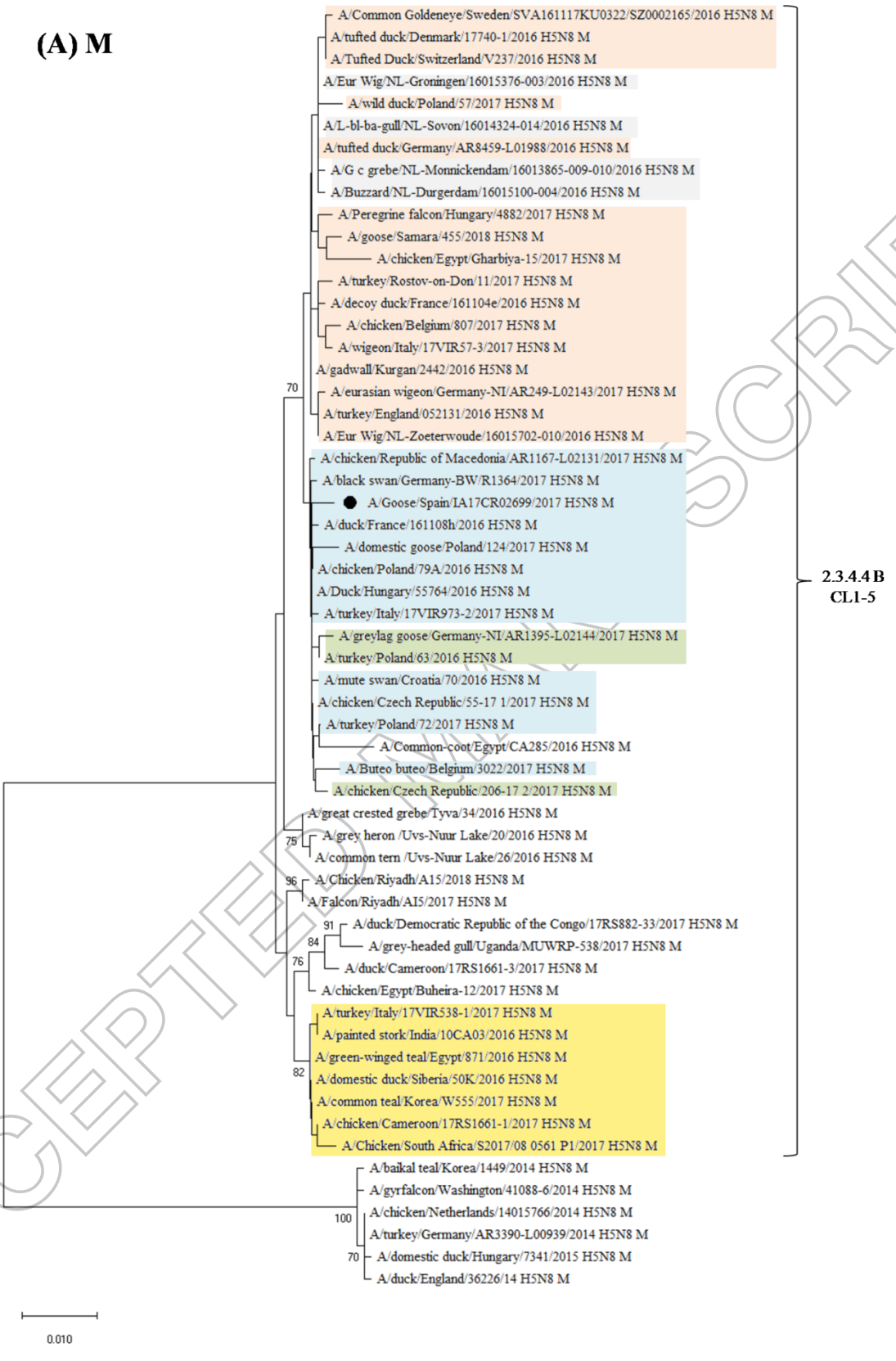


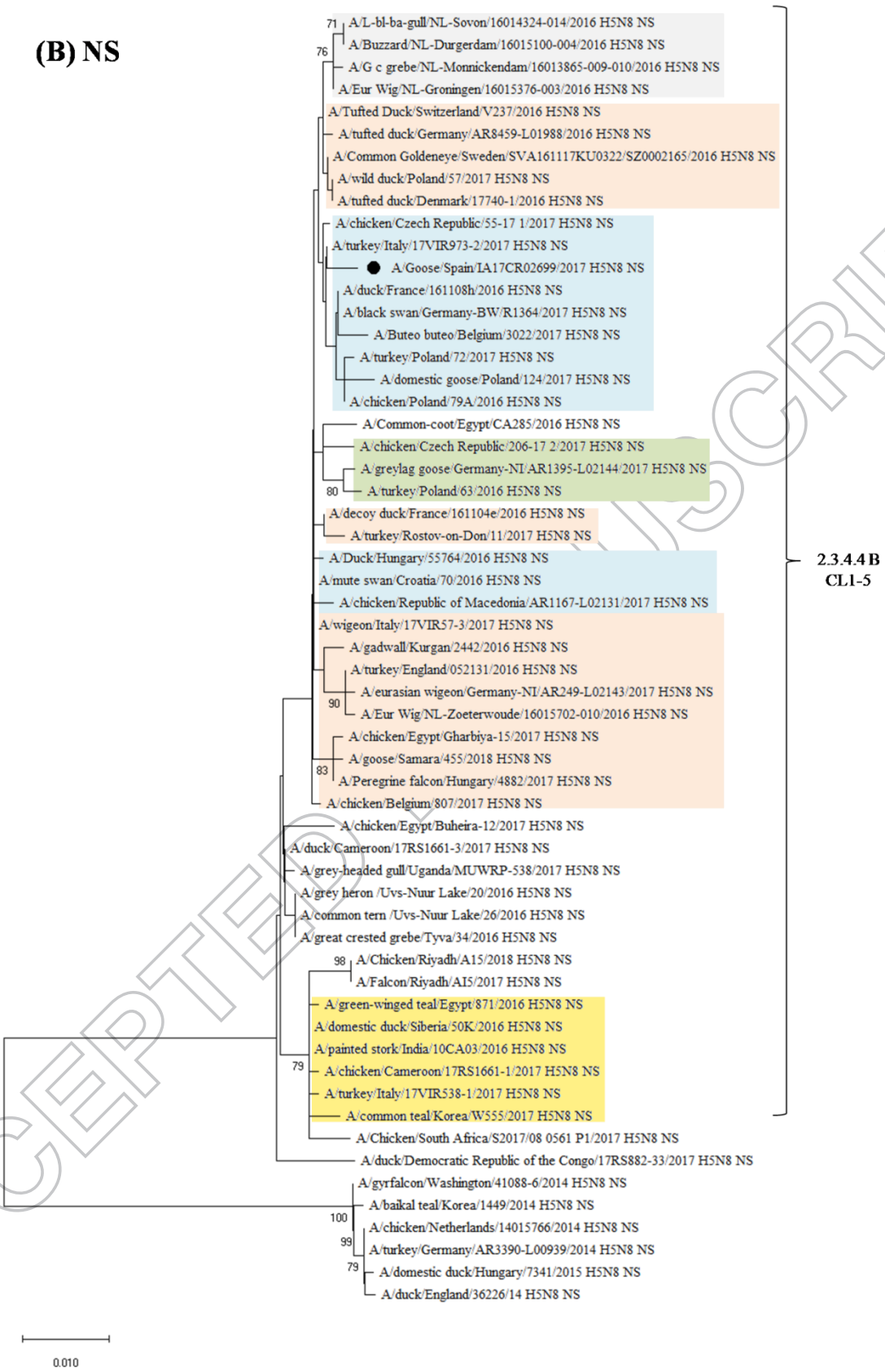


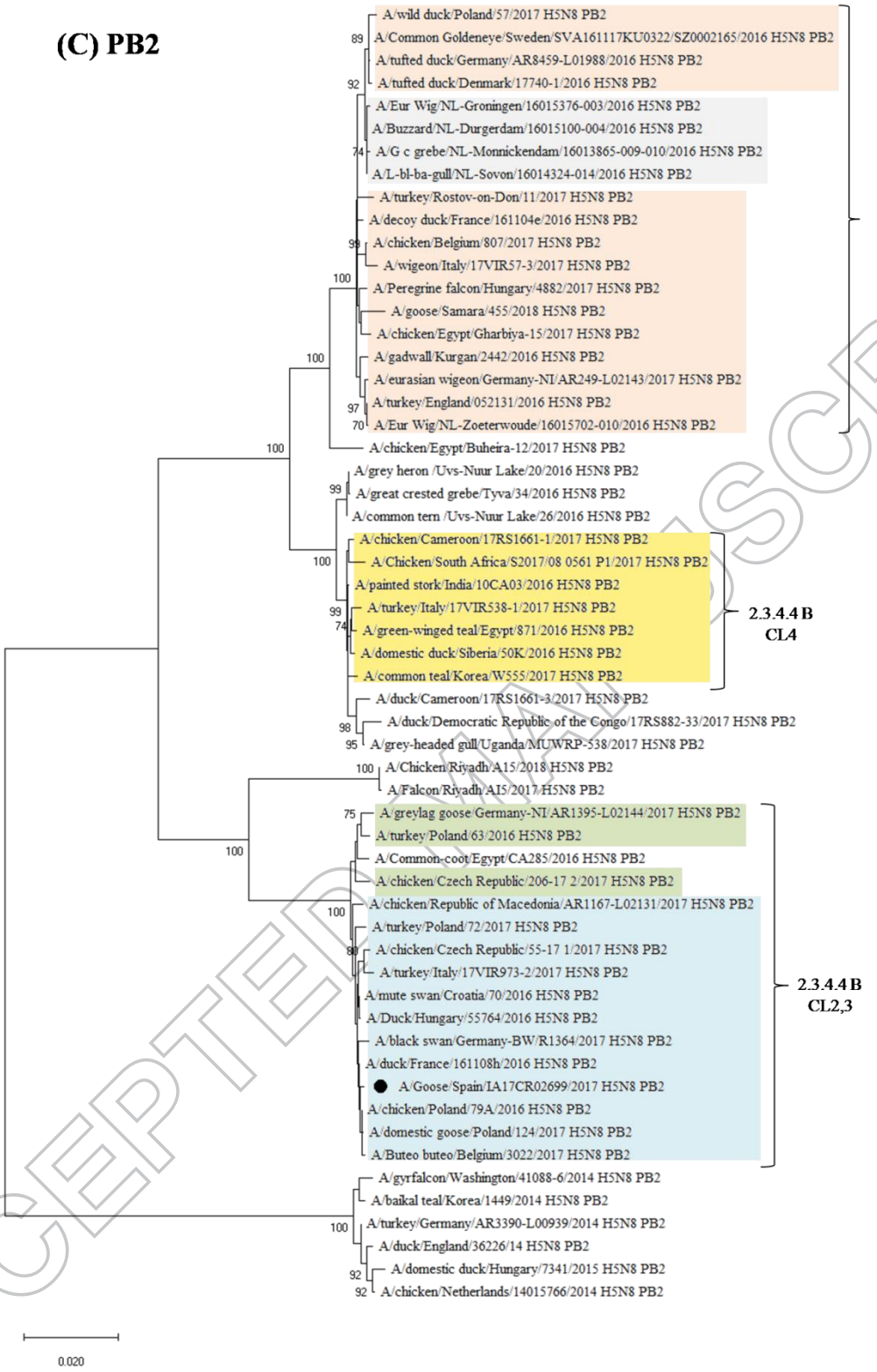
Supplemental online material

Supplemental Figure 1. Neighbor joining phylogenetic tree of MP (A), NS (B), PB2 (C), PB1 (D), PA (E) and NP (F) gene segments. The Spanish H5N8 is highlighted with a black dot. Bootstrap values ≥70% (700/1000 replicates) are shown. The different clusters are presented: 1 (orange), 2 (blue), 3 (green), 4 (yellow), 5 (grey). The clusters are based in the different genotypes identified in Poland, Germany, Italy and the Netherland reported in the study of Swiezton & Smietanka (2018). Scale bar indicates nucleotide substitution per site.

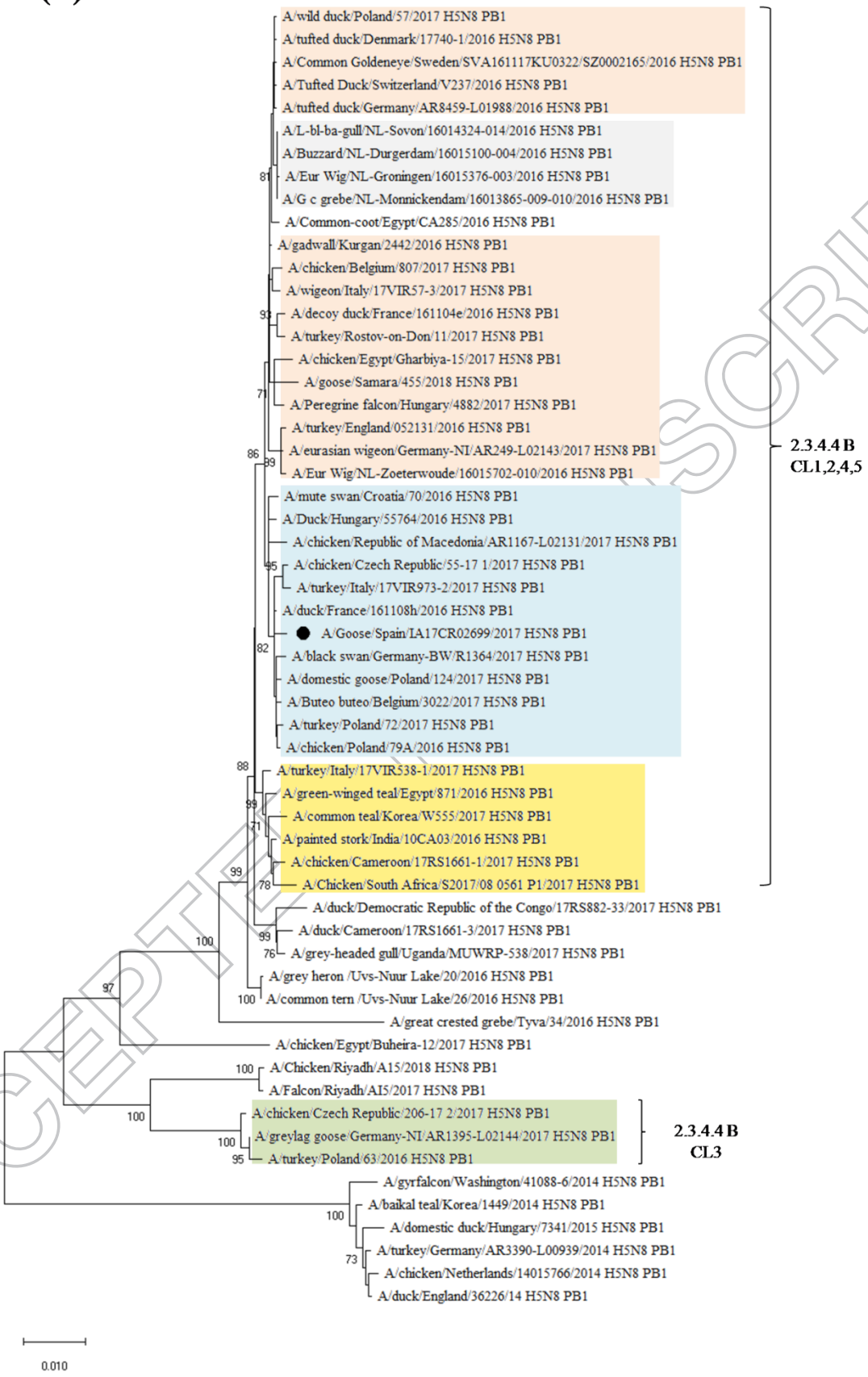
(A) M



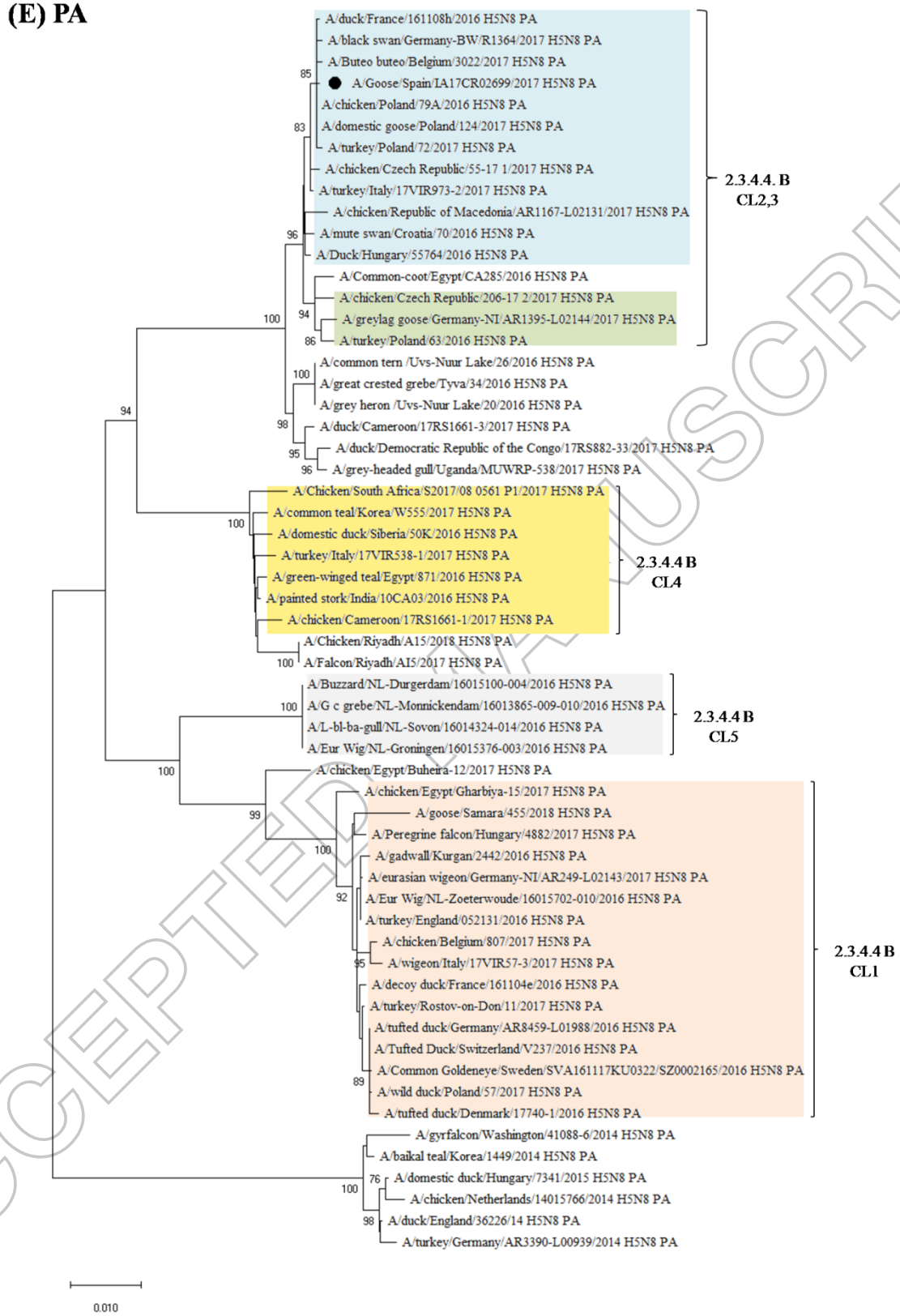




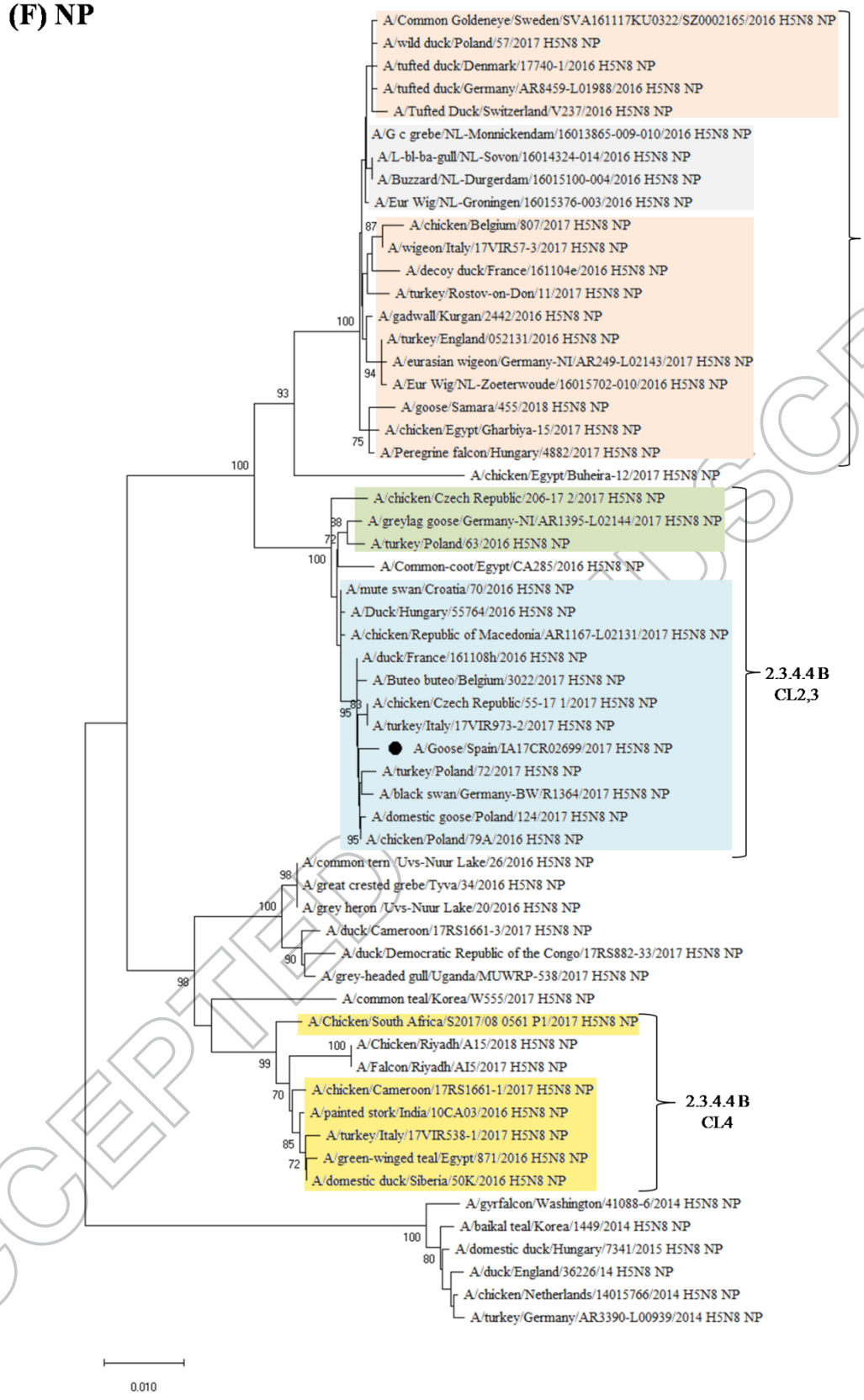
(D) PB1



(E) PA



(F) NP



Supplemental Table 1. Amino acid substitutions present in Spanish H5N8 isolate reported to change the virulence, transmissibility and/or host tropism of IAVs in avian and/or mammal species. [1]H5 numbering (A/Vietnam/1203/04 (HPAI)). [2]N2 numbering (A/Aichi/2/1968 (H3N2)).

Protein	Mutation	Phenotype	Subtype	Reference
PB2	K123E	Increased virulence in chickens	H7N7	Maruyama *et al.,* 2013
PB1	C38Y	Increased polymerase activity and virulence in chickens	H5N1	Suzuki *et al*., 2014
	H436Y	Increased virulence in mallard ducks	H5N1	Hulse-Post *et al*., 2007
PA	K237E	Increased polymerase activity and pathogenicity in mallards	H5N1	Hu *et al*., 2013
	N383D	Increased virulence in ducks (higher effect with PA S224P)	H5N1	Song *et al*., 2011
	A515T	Increased polymerase activity and virulence in mallard ducks	H5N1	Hulse-Post *et al*., 2007
	F672L	Increased transmissibility in chickens	H9N2	Zhong *et al*., 2014
HA[1]	S107R	Increased virulence in chickens and mice (+ HA T108I)	H5N1	Wessels *et al*., 2018
	T108I	Increased virulence in chickens and mice (+ HA S107R)	H5N1	Wessels *et al*., 2018
NP	M105V	Increased virulence in chickens	H5N1	Tada *et al*., 2011
	A184K	Increased virulence in chickens	H5N1	Wasilenko *et al*., 2009
NA[2]	I117T	Reduced susceptibility to oseltamivir and zanamivir	H5N1	Kode *et al*., 2019
	I314V	Reduced susceptibility to oseltamivir (+ NA I117V)	H5N1	Hurt *et al*., 2007
M1	I43M	Increased virulence in chickens and ducks	H5N1	Nao *et al*., 2015
NS1	L103F	Inhibition of host gene expression (+ I106M, P114S, G125D and N139D)	H9N2	Rodriguez *et al*., 2018
	I106M	Inhibition of host gene expression (+ L103F, P114S, G125D and N139D)	H9N2	Rodriguez *et al*., 2018
	P114S	Inhibition of host gene expression (+ L103F, I106M, P114S and N139D)	H9N2	Rodriguez *et al*., 2018
	G125D	Inhibition of host gene expression (+ L103F, I106M, P114S and N139D)	H9N2	Rodriguez *et al*., 2018
	V149A	Increased virulence in chickens, decreased interferon response	H5N1	Li *et al*., 2006

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