

Outcome of low pathogenicity avian influenza (H7N9) experimental infection of urban and rural house sparrows (*Passer domesticus*) is impacted by natural Avipoxvirus infection

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Abstract

The house sparrow (*Passer domesticus*), a ubiquitous but declining species, is a potential bridge host for introduction of avian influenza virus (AIV) into poultry farms. We investigated the influence of diet and urban vs rural habitat on susceptibility to and persistence of AIV infection. Wild captured sparrows were inoculated intratracheally with H7N9 LPAIV (A/Anas crecca/Spain/1460/2008), while control birds received saline. Swab and blood samples collected on 0, 1, 3, 4, 7, 10 and 14 days post-inoculation (dpi), and vascular feathers (1, 7 dpi) from 25% of individuals per group were tested by real-time RT-PCR. Although clinical signs were mild or absent, unexpected mortality (8.3%, 3/36) occurred starting at 4 dpi in urban and 7 dpi in rural sparrows. Viral shedding was observed between 1 and 4 dpi and seroconversion was confirmed in both inoculated groups. Natural *Avipoxvirus* (APV) infection was evidenced in all experimental groups, by histology, PCR and sequencing. Three distinct APV lineages were detected in oral swabs from urban and rural control birds, on 1 dpi. APV prevalence was higher and persisted longer in LPAIV-infected sparrows, with APV detection overlapping the peaks of LPAIV shedding in both urban and rural birds. These results confirm that urban and rural sparrows are similarly susceptible to experimental H7N9 LPAIV infection. Increased prevalence and persistence of APV during LPAIV infection suggest interaction between these viruses. Such interactions may represent an ecologically relevant but often overlooked component of avian influenza epidemiology and should be considered when evaluating sparrows as potential bridge hosts.

INTRODUCTION

House sparrows (*Passer domesticus*) are one of the most abundant passerine species worldwide and are closely associated with human activities, particularly poultry farms. Their high density around livestock, combined with frequent movements between farms, urban environments and natural habitats, make them potential bridge hosts for pathogen transmission between wild birds and domestic poultry. This may be especially relevant for avian influenza viruses (AIVs) if sparrows serve as intermediaries between the waterfowl reservoir and domestic birds [1].

Although wild waterfowl are the natural reservoirs of AIVs, there is growing evidence that passerines can also become infected and play a role in virus transmission [2, 3, 4]. Experimental studies have confirmed that sparrows and other passerines can transmit AIVs between the natural waterfowl reservoir and poultry farms [1, 5, 6]. House sparrows are susceptible to high pathogenicity AIVs (HPAIVs), including H5N1, and can shed and transmit these viruses, highlighting their potential role in the epidemiology of avian influenza at the interface between wildlife and domestic poultry [7].

However, most AIV infections detected in passerines correspond to low pathogenicity AIVs (LPAIVs), which generally cause asymptomatic or mild infections [8]. In poultry, LPAIV infections are typically characterised by low mortality and mild respiratory signs, unless aggravated by unfavourable environmental conditions or coinfections [9]. Similarly, LPAIV strains can be both mechanically carried and actively excreted by sparrows without causing obvious clinical signs, facilitating the persistence and spread of the virus in the environment and to other hosts [10, 11].

Habitat and diet are important ecological factors that influence immune function and susceptibility to infection in wild birds [12]. Urban environments can alter immune capacity and microbiota composition due to pollution, altered food resources, and chronic environmental stress [13]. Previous studies have documented differences

between urban and rural birds in immune responses and pathogen dynamics, including infections with West Nile virus, suggesting that environmental context can modulate host-pathogen interactions in passerines [14, 15]. Another example is *Avipoxvirus* (APV), a widespread avian pathogen that infects more than 230 bird species worldwide and typically causes proliferative cutaneous lesions [16]. The prevalence of clinical avian pox varies considerably among host species and populations, but surveys in house sparrows (*Passer domesticus*) have reported an approximate prevalence of 3% in Spain [17]. Although urban environments may facilitate transmission through increased host density and vector abundance, evidence for consistently higher APV prevalence in urban populations remains inconclusive [17].

As house sparrows are abundant in poultry environments, susceptible to AIV, and exhibit habitat and diet related effects on immune function, understanding how the ecological context determines AIV infection dynamics is essential for clarifying the role of this species in avian influenza epidemiology. In general, experimental infection studies typically focus on single-pathogen infections, while this is rarely the case for free-living species [18]. In this study, we investigated how habitat and diet (urban vs rural) influence the course of H7N9 LPAIV infection in house sparrows, while accounting for the presence of natural APV coinfections.

MATERIALS AND METHODS

Experimental design

All experimental procedures were authorised by the Ethical Committee on Animal Experimentation of the Catalan Government (11936). Capture of sparrows was authorized by the Ethical Committee for Animal Experimentation of the University of Castilla-La Mancha (OHPR202214) and the Junta de Comunidades de Castilla-La Mancha (avp22_123). Handling and sampling procedures were carried out in accordance with all applicable institutional and national guidelines for the care and use of animals in experimentation.

Animals

In September 2024 108 wild adult house sparrows, 54 from rural and 54 from urban environments were captured. Males and females were included in approximately equal proportions, and adults were selected to ensure prolonged exposure of individuals to a specific habitat and diet. Rural sparrows were captured close to a partridge farm located near a village, whereas urban sparrows were captured in a small city-centre park, both in Castilla-La Mancha, South-central Spain. Upon capture, morphometric measurements (body weight and tarsus length) were taken, individuals were banded and samples of blood, vascular feather and oral and cloacal swabs were collected and analysed. Sparrows were housed temporarily (15 days) in flight cages located in the experimental farm of the Institute for Game and Wildlife Research (Instituto de Investigación en Recursos Cinegéticos IREC). To ensure that the birds had not been previously exposed to AIV, we tested for AIV antibodies using a competitive ELISA test (Ingenasa-Eurofins, Madrid, Spain) and for matrix gene viral RNA by real-time RT-PCR as described previously [19, 20]. After confirming all birds were AIV-negative, they were transported to the Centre de Recerca en Sanitat Animal (CRESA) and housed in flight cages in two different rooms within the biosafety level 3 (BSL-3) facilities. Following one week of acclimation, the sparrows were randomly assigned to experimental groups and housed in separate flight cages according to their habitat of origin (urban vs rural). Control and infected sparrows were kept in separate rooms with negative-pressure HEPA filtered air, and in each room, urban and rural birds were housed in separate in 4.5 m² (9 m³) aviaries. During quarantine, transport and

throughout the experiment, birds were kept under species- and age-appropriate conditions, including temperatures of 20–25 °C and 12-hour photoperiods with 15-min gradual light transitions. Importantly, each group had *ad libitum* access to habitat-specific diets [21] (Additional file 1) as well as to water. In addition, sparrows were provided with adequate environmental enrichment, including grit, shelter, natural branch perches at different heights and a shallow, easy-to-clean tray for bathing. The aviary floor was covered with corrugated cardboard that was changed daily.

Virus

The H7N9 LPAIV isolate A/Anas crecca/Spain/1460/2008 (H7N9 hereafter) was obtained from the ongoing AIV surveillance program in wild birds carried out in Catalonia, northeastern Spain. The amino acid sequence at the HA0 cleavage site of the H7N9 isolate was PEIPKGR/GLF, a motif typical of LPAIV. The virus stock was produced and titrated in embryonated chicken eggs as per standard procedures [22].

The 108 sparrows (54 birds from urban and 54 from rural environments), were allocated into four groups. Within each habitat type, individuals were randomly assigned, maintaining sex ratios, to an infected group (36 birds per habitat of origin) or a control group (18 birds per habitat of origin). Birds in the infected groups were inoculated with 10^5 mean embryo infectious doses of the H7N9 strain in a total volume of 0.1ml via the choanal route, whereas control birds received the same volume of sterile phosphate buffered saline via the same route.

Sampling

All sparrows were monitored daily for clinical signs of infection until 14 days post inoculation (dpi). Birds were sampled at different dpi to assess body weight, viral shedding, lesions and seroconversion. For welfare purposes, each group (infected and control, rural and urban) was further divided into two subgroups that alternated on sampling days, reducing handling and minimizing stress. This design limited each bird to a maximum of three sampling events during the experiment. The samples collected varied by subgroup and dpi and are summarised in Fig. 1.

Thus, two infected subgroups of nine birds per habitat of origin were alternately sampled at 1, 3, 4, 7, 10, and 14 dpi. Of these subgroups, one ($n = 9$) was sampled for whole blood for serum collection, while the other was sampled for oral and cloacal swabs that were placed into RNA shield. At 1 and 7 dpi, a vascular feather was also collected into viral transport medium per bird. Individuals in the control groups were divided into subgroups of six birds and sampled on the same dpi, as described for the infected groups. All birds in the infected and control groups were weighed on 0, 3, 7, 10 and 14 dpi. All sparrows that died or were euthanised for ethical reasons during the experiment, along with 10% of the surviving sparrows euthanized at the experimental endpoint, were necropsied to evaluate macroscopic lesions and to collect tissue samples (lung, central nervous system, heart, spleen, kidney and liver) into RNA shield and a vascular feather into viral transport medium for molecular detection of AIV, histopathology and immunohistochemistry (IHC). All samples collected during the experiment, except formalin fixed tissues, were stored at -70°C until analysis.

Since sampling was performed on alternating subgroups for welfare purposes, as previously mentioned, results are considered at the group level rather than the individual level.

Pathogen detection

Viral genetic material was extracted from oropharyngeal and cloacal swabs, feather pulp samples and tissues of deceased/euthanised individuals (lung, central nervous system, heart, spleen, kidney and liver). Extraction was performed on the Kingfisher Flex extraction robot (Thermo Fisher Scientific, Waltham, Massachusetts, USA) using the INDICAL BIOSCIENCE kit that simultaneously recovers viral RNA and DNA (Indical Bioscience, Leipzig, Germany).

Detection of avian influenza viral RNA was carried out by real-time RT-PCR as described previously by Spackman et al. [23] and was performed using the Fast7500 kit (Applied Biosystems, Foster City, CA, USA). For APV detection, PCR was carried out in a total volume of 25 µl in Promega PCR Master Mix, 2X (Promega, Madison, WI, USA) reaction buffer (pH 8.5) containing 50 units/ml of Taq DNA polymerase, 400µM dATP, 400µM dGTP, 400µM dCTP, 400µM dTTP, 3mM MgCl₂ (supplemented to a final 4mM MgCl₂), 10 µM of each primer (P4b F 5'-CAGCAGGTGCTAAACAACAA-3' and P4b R, 5'-CGGTAGCTTAACGCCGAATA-3), adapted from Lee & Lee, 1997 [24]. Each PCR reaction was performed on a BioRad T100 Thermocycler (Bio-Rad Laboratories, Hercules, CA, USA) with approximately 25 ng of genomic DNA as template using the following conditions: an initial denaturing step at 94°C for 5 min, was followed by 45 cycles of 94°C for 30 s, 60 °C for 30 s and 72°C for 60 s. A final run at 72°C for 5 minutes completed the program. The reaction products were checked for the anticipated 578 bp amplicon by running a 1% agarose gel for band visualization of each sample. All samples were tested in duplicate. Extraction blanks and negative controls were included in all PCR and genome-amplification assays and were consistently negative.

Sequencing of APV PCR products was performed in an ABI Prism 3730 automated sequencer (Perkin Elmer Applied Biosystems, Foster City, CA) at Secugen, Madrid on the forward strand only. Sequences with a high identity (> 99.2%) to APV sequences recovered from the GenBank database using the BLAST algorithm were considered positive.

Histopathology and detection of AIV by immunohistochemistry (IHC)

After fixation in 10% neutral buffered formalin and embedding in paraffin, tissue sections were routinely processed for haematoxylin/eosin (H/E) staining. The following tissues were examined: oesophagus, duodenum, liver, kidney, gonad, nasal turbinates, lung, heart, muscle, bone marrow, spleen, cloacal bursa, brain and spinal cord.

An immunohistochemical technique (IHC) based on the avidin-biotin complex (ABC) immunoperoxidase system was performed as previously described [25, 26]. The primary antibody was a commercial mouse-derived monoclonal antibody against influenza A virus nucleoprotein (IgG2a, Hb65, ATCC). A biotinylated goat anti-mouse IgG antibody (GaMb, Dako E0433, Glostrup, Denmark) was used as a secondary antibody. Tissues previously shown to be positive for influenza A virus nucleoprotein by IHC were used as positive controls. Negative controls were tissues from mock-inoculated birds and tissues incubated without the primary antibody. The following scoring was used to measure staining in the tissues: no positive cells (-), isolated positive cells (+), scattered clusters of positive cells (++), widespread positivity (+++).

Haemagglutination inhibition (HI) assay for AIV

All 180 plasma samples, collected at 1, 3, 4, 7, 10 and 14 dpi (9 samples per infected group and 6 per control group; 30 samples per dpi) were tested for H7N9 LPAIV-specific antibodies in HI assay following standard procedures [27] and using H7N9 LPAIV (A/Anas crecca/Spain/1460/2008) as antigen. Plasma samples were incubated for 90 minutes at 56°C to inactivate non-specific inhibitors of haemagglutination, and serial dilutions up to 1:256 were tested against H7N9 LPAIV. Negative controls, consisting of virus-free serum incubations, were used to measure the non-specific haemagglutination activity of each sample.

Statistical analysis

All analyses were carried out using R software version 4.3.2 [28]. A scaled body mass index (SMI) was calculated per individual and sampling time based on body weight and tarsus length [29]. Descriptive statistics were calculated for each sampling day (dpi) and habitat category (urban vs rural).

To evaluate differences in body condition among experimental groups, SMI values were analysed using linear models including habitat of origin, treatment (H7N9 vs control), dpi (treated as categorical factor), sex, and their interactions. Additional subgroup analyses were conducted separately for infected and control individuals. *Post hoc* comparisons were performed using estimated marginal means with Tukey adjustment for multiple testing.

Prevalence of AIV and APV was estimated for each sampling day. APV detection was based on molecular presence/absence data. Categorical differences between habitats were assessed using Pearson's Chi-squared tests or Fisher's exact tests when appropriate. Temporal patterns of APV occurrence were further analysed using binomial generalized linear models including habitat, dpi and their interaction.

AIV infection dynamics were analysed using generalized linear models (GLMs) with binomial distribution and logit link function were fitted, with AIV positivity (combined oropharyngeal and cloacal detection) as response variable and habitat, dpi, and their interaction as predictors.

Viral shedding intensity was quantified using real-time RT-PCR cycle threshold (Ct) values from oropharyngeal and cloacal swabs.

Viral load was analysed using Gaussian linear models including habitat, dpi, sample type (Oropharyngeal vs cloacal), and their interactions.

Systemic infection was quantified as the number of PCR-positive organs per individual, calculated as the sum of positive detections across lung, central nervous system, heart, liver, kidney, and spleen. This variable was analysed using a Poisson generalized linear model with a log link and using habitat and dpi as predictors.

Survival analyses were conducted using Cox proportional hazards models. Time-to-event was defined as dpi until death. Survival curves were estimated using Kaplan-Meier methods.

Model assumptions were assessed through residual diagnostics. Statistical significance was set at $p < 0.05$.

RESULTS

Clinical signs, weight loss and mortality

None of the infected sparrows exhibited respiratory or neurologic signs during the experiment. However, some individuals showed moderate conjunctivitis and listlessness. Mortality was moderate in both groups and started on 4 dpi in urban and 7 dpi in rural sparrows, reaching 8.33% (3/36) by 14 dpi in both groups (Additional file 2). No mortality was observed in the control groups. Throughout the experiment, both urban and rural infected birds experienced weight loss, particularly at 3 dpi, when compared to control individuals sampled on the same day (Figure 2).

Analysis of SMI revealed a strong effect of habitat ($F_{1,661} = 26.18, p < 0.001$), indicating consistent significantly worse body condition in urban than rural sparrows across the experimental period (Figure 2). SMI also varied significantly across dpi ($F_{7,661} = 13.43, p < 0.001$), indicating temporal changes in body condition in all experimental groups. In contrast, neither infection status ($F_{1,661} = 0.01, p = 0.909$) nor sex ($F_{1,661} = 0.73, p = 0.392$) had a detectable effect on SMI.

Importantly, a significant habitat $\times$ dpi interaction was detected ($F_{7,661} = 2.16, p = 0.036$), reflecting the slower recovery of body condition after the initial impact of infection in the urban sparrows. The habitat $\times$ treatment interaction showed a marginal effect ($F_{1,661} = 3.05, p = 0.081$), suggesting a weak tendency for infection effects to vary between habitats. Neither the treatment $\times$ dpi interaction nor the three-way habitat $\times$ treatment $\times$ dpi interaction were significant (both $p > 0.80$).

Post hoc comparisons of estimated marginal means indicated that habitat differences in SMI were most pronounced among H7N9-infected birds during early infection. Rural infected birds showed significantly higher SMI than urban infected birds at inoculation ($p = 0.011$) and at 1 dpi ($p = 0.027$). No consistent differences between treatment groups were observed within habitats across time.

To further explore these patterns, infected and control birds were analysed separately. Among infected individuals, both habitat ($F_{1,449} = 34.51, p < 0.001$) and dpi ($F_{7,449} = 11.19, p < 0.001$) significantly affected SMI, with a marginal habitat $\times$ dpi interaction ($F_{7,449} = 1.98, p = 0.056$). In contrast, in control birds, SMI was influenced only by dpi ($F_{7,213} = 3.54, p = 0.001$), with no effect of habitat or interaction (both $p > 0.20$).

Mortality in the H7N9-infected groups started at 4 dpi in urban sparrows and at 7 dpi in rural sparrows, reaching 8.33% (3/36) mortality rate by 14 dpi in both groups (Additional file 2). No mortality was observed in the control groups.

Detection of AIV by real-time RT-PCR

In both groups, H7N9 AIV shedding was primarily detected from 1 to 4 dpi, with oropharyngeal excretion being the most frequent route. Cloacal shedding was sporadic in both groups, although it appeared earlier and more frequently in rural individuals, while in urban birds it was only detected at 3 dpi (Figure 3, table 1). Viral RNA detection in feather pulp was limited and only observed at 7 dpi in a small proportion of individuals from both groups (Table 1).

Table 1. Prevalence of avian influenza virus H7N9 detected by real-time RT-PCR in oropharyngeal swabs, cloacal swabs, and feather pulp in intratracheally inoculated rural and urban sparrows at 1,3,4, 7 and 14 dpi. Birds in the control groups did not excrete virus by any of the routes studied throughout the experiment. NB. Prevalence is reflected at group level. Different individuals were tested on each dpi to reduce impact of handling stress.

Group	Sample	1 dpi	3 dpi	4 dpi	7 dpi	14 dpi
Rural	Oropharyngeal	55.56% (5/9)	55.56% (5/9)	88.89% (8/9)	0% (0/9)	0% (0/9)
	Cloacal	33.33% (3/9)	0% (0/9)	55.56% (5/9)	0% (0/9)	12.5% (1/8)
	Feather pulp	0% (0/9)	-	-	22.22% (2/9)	-
Urban	Oropharyngeal	66.67% (6/9)	77.78% (7/9)	55.56% (5/9)	12.5% (1/8)	0% (0/9)
	Cloacal	0% (0/9)	22.22% (2/9)	0% (0/9)	0% 0/9	0% (0/9)
	Feather pulp	0% (0/9)	-	-	11.11% (1/9)	-

A mixed-effects analysis of viral load confirmed significantly higher loads in oral than in cloacal swabs ($t = 4.63$, $p < 0.001$). A significant dpi $\times$ sample type interaction indicated that viral loads declined more rapidly in oral swabs over time ($t = -3.18$, $p = 0.002$). No effects of habitat or any habitat-related interactions were detected (all $p > 0.69$), indicating similar viral shedding dynamics in urban and rural sparrows.

At later stages of infection, a higher proportion of urban sparrows remained tissue-positive than rural sparrows. At 14 dpi, viral RNA was still detectable in tissues of both groups, with positive individuals detected more frequently among urban sparrows (3/9; 33.33%) than rural sparrows (1/11; 9.1%). Notably, the only rural bird testing positive at this time point died at 14 dpi, while the three urban positive birds were euthanised at the end of the experiment. When considering all tissues analysed from birds that died during the experiment and those euthanised at 14 dpi, tissue positivity was also more frequent in urban (5/12; 41.67%) than rural sparrows (3/13; 23.08%) (Figure 4; Table S2a,b).

Systemic viral dissemination, measured as the number of positive organs per individual, decreased significantly over time ($\beta = -0.170 \pm 0.025$ SE, $z = -6.74$, $p < 0.001$). Birds that died during the experiment exhibited higher levels of tissue positivity than individuals euthanised at 14 dpi (Figure 4; Table S2). However, habitat had no effect on systemic dissemination ($\beta = -0.094 \pm 0.164$ SE, $z = -0.57$, $p = 0.566$), indicating that despite the higher frequency of tissue-positive birds observed among urban sparrows, the overall progression and systemic spread of infection were similar in urban and rural sparrows.

Haemagglutination inhibition (HI) assay

In both infected groups, HI antibodies against H7N9 LPAIV were detected from 7 dpi onwards (Figure 5), with no seroconversion observed in control birds.

Overall, seroconversion occurred in both rural and urban sparrows, although the temporal distribution of antibody titres differed across groups. At 7 dpi, 44.44% of both rural and urban sparrows had already developed high antibody titres ($>1:256$). By 10 dpi, high titres ($>1:256$) were less frequent in rural sparrows (11.11%) than in urban sparrows, where 22.22% of individuals had titres $>1:256$ and an additional 11.11% had titres of 1:128. By 14 dpi, antibody levels had declined in most tested sparrows, with only one rural individual showing a moderate titre (1:64) and one urban sparrow maintaining a high titre ($>1:256$).

Among seropositive individuals, most birds had evidence of viral shedding at some point during the experiment, with this proportion being higher in rural sparrows (83.33%) than in urban sparrows (66.67%).

Histopathological findings

Necropsies were performed on the birds that died or were euthanised for ethical reasons during the experiment, in addition to the scheduled necropsies at 14 dpi. During the necropsy of deceased sparrows, no notable macroscopic lesions were observed.

Microscopically, the infected urban sparrow that died at 7 dpi showed loss of cilia and necrosis of the submucous glands of the nasal turbinates. In addition, lesions in the soft palate typical of APV infection were observed, including epithelial hyperplasia, ballooning degeneration of keratinocytes and intracytoplasmic (Bollinger) inclusion bodies. Mild to moderate periportal inflammation was also present in the liver.

The infected rural sparrow that died at 9 dpi showed small multifocal areas of hepatocyte necrosis and mild to moderate periportal inflammation. Other tissues were too autolytic to assess for additional lesions. The infected rural sparrow that died at 10 dpi showed hydropic degeneration of hepatocytes.

None of the sparrows subjected to histopathological analysis at 14 dpi had any microscopic lesions. Immunohistochemistry did not reveal AIV antigen in any of the tissues examined.

Detection of APV coinfection

We detected APV infection in individuals from both the infected and control groups across both habitats of origin, although prevalences differed according to H7N9 infection status. Specifically, urban H7N9 inoculated sparrows showed a prevalence of APV of 22.22% (2/9) at 4 dpi and 11.11% (1/9) at 10 dpi. Rural H7N9 inoculated sparrows exhibited APV prevalences of 11.11% (1/9) at 3 dpi, increasing to 22.22% (2/9) at 4 dpi, peaking at 33.33% (3/9) at 7 dpi, and declining to 11.11% (1/9) at 10 dpi. In contrast, control groups displayed only isolated detections, with one positive bird per habitat at 1 dpi (1/6; 16.67%) (Additional file 4, Figure 6).

Among APV-positive birds, three distinct lineages were identified. The A2 lineage was detected only in rural birds ($n = 2$ sequences), whereas two B1 (canarypox) genotypes were recovered, one exclusively from urban birds and a second exclusively from rural H7N9-infected birds. APV sequence diversity was highest in the rural H7N9-infected group, from which all three lineages were recovered.

The A2 lineage was represented by a sequence recovered from a rural control bird that was identical to clade A2, originally described from a pigeon sampled in 1975 (GenBank Acc. No. AM050385). A second sequence, recovered from a rural H7N9-infected bird at 1 dpi, showed 98.9% nucleotide identity to this A2 lineage.

The first B1 (canarypox) genotype corresponded to a lineage initially detected in a Norwegian great tit in 1972 (GenBank Acc. No. AY453174). All APV sequences recovered from urban birds belonged to this genotype. Three sequences obtained from H7N9-infected urban birds sampled at 3, 4 (including the bird that died), and 7 dpi were >99.2% identical to the reference sequence. In addition, sequences recovered from the urban control bird sampled at 1 dpi and two H7N9-infected urban birds sampled at 4 dpi were >99.3% identical to the same genotype.

A second B1 (canarypox) genotype was detected exclusively in rural H7N9-infected birds. Sequences recovered from birds sampled at 4 and 7 dpi were >99.3% identical to reference sequence AM050375.

Statistical analysis confirmed that APV detection probability was not influenced by habitat ($\beta = -0.140 \pm 1.310$ SE, $z = -0.11$, $p = 0.915$), dpi ($\beta = -0.059 \pm 0.126$ SE, $z = -0.47$, $p = 0.641$), or their interaction ($\beta = -0.032 \pm 0.198$ SE, $z = -0.16$, $p = 0.872$), indicating no statistical support for effect of habitat or time after AIV infection on APV occurrence.

DISCUSSION

This study examined the experimental infection of urban and rural house sparrows with H7N9 LPAIV under controlled conditions while maintaining a habitat-specific diet. By comparing infection outcomes, including viral shedding, seroconversion, systemic dissemination, mortality, and interactions with naturally circulating APV, we aimed to assess how ecological context influences sparrow susceptibility to LPAIV infection. Results showed modest effects of habitat and diet on LPAIV infection dynamics in house sparrows, together with evidence of interaction with APV co-infection.

Habitat-related differences such as a lower body condition index (SMI) and slower recovery after infection in urban sparrows, is in line with previous work showing that urban sparrows often exhibit reduced body condition and altered life-history strategies compared to rural populations [30, 31]. Such differences may result from the impact of ecological pressures associated with urban environments, including food quality, chronic stressors, and anthropogenic disturbance. From an eco-physiological perspective, infection imposes energetic trade-offs between immune activation and other physiological processes [32], potentially constraining immune responsiveness in individuals in poorer baseline condition [33].

House sparrows were susceptible to H7N9 LPAIV infection, as shown by viral shedding, and seroconversion. Although LPAIV infections are generally considered mild and self-limiting, the observed mortality, 8.3% by group (Additional file 2), was notable and, in the context of experimental LPAIV infections, is higher than the results previously reported, as infections are typically subclinical and associated with negligible mortality in passerines [8, 35, 36]. This suggests that additional factors inherent to wild-caught birds, including prior exposure histories and concomitant infections such as APV, may influence individual outcomes [17]. However, survival analyses revealed no significant habitat effect on mortality risk, and only one deceased individual was confirmed APV coinfecting, indicating that mortality cannot be attributed solely to co-infection. The LPAIV strain used here has previously been tested in red-legged partridges (*Alectoris rufa*), in which no mortality and only limited shedding and seroconversion were observed [34], highlighting the importance of host species and ecological context in shaping infection outcomes. These findings are consistent with the generally low pathogenicity of LPAIV in passerines [37], while also aligning with reports that some LPAIV strains can occasionally cause systemic infection or more severe disease [38, 39]. They also agree with evidence that house sparrows are susceptible to highly pathogenic AIVs such as H5N1 clade 2.3.4.4b, with systemic infection and mortality [6, 40].

Viral excretion patterns were broadly similar between urban and rural sparrows, suggesting that habitat-associated differences did not substantially influence susceptibility to infection or the ability to shed virus. In both groups, oral shedding predominated over cloacal shedding, consistent with the known respiratory tropism of H7N9 and previous observations in passerine hosts [3]. Such patterns may be relevant in synanthropic species acting as ecological connectors between wildlife and livestock [41]. The predominance of oral excretion indicates that respiratory secretions are likely the main route of virus release in house sparrows, regardless of habitat. Although viral RNA was occasionally detected in cloacal samples, including in one rural bird at 14 dpi,

such late detections were uncommon and may reflect individual variation in viral clearance or host immune responses rather than a distinct excretion pattern [5].

Detection of viral RNA in feather pulp and multiple internal organs indicates that H7N9 infection in house sparrows can progress to systemic dissemination [38]. The greater tissue burden observed in birds that died during the experiment suggests an association between widespread viral distribution and disease severity [38]. Conversely, the lack of immunohistochemical evidence of viral antigen in some real-time RT-PCR-positive tissues at later time points may reflect the greater analytical sensitivity of real-time RT-PCR relative to IHC. Together, these findings are consistent with transient systemic spread followed by gradual viral clearance. Although viral RNA persisted in tissues of a greater proportion of urban birds at the end of the experiment, habitat was not associated with systemic dissemination overall, indicating broadly similar infection progression in urban and rural sparrows.

The observed antibody kinetics suggest subtle differences in the host response between populations, with evidence of a shorter-lived detectable humoral response in rural sparrows[42]. This pattern could reflect variation in viral clearance, the duration of antigenic stimulation, or other habitat-associated factors influencing immune function. However, given the limited magnitude of the differences observed, these findings should be interpreted cautiously.

The temporal overlap between peaks in APV detection and AIV infection suggests a potential interaction between the two pathogens. One possible explanation is that LPAIV infection altered host immune function increasing detectability of pre-existing subclinical APV infections or facilitating viral reactivation. Alternatively, APV itself may have influenced host susceptibility or responses to AIV. Similar pathogen-pathogen interactions have been described in other avian systems, like West Nile virus and coccidia, in which concurrent infections can modify infection dynamics, host competence, and disease outcomes [17, 43]. Given the relatively high prevalence of APV reported in wild sparrow populations in Spain [17], such interactions could represent an ecologically relevant but often overlooked component of avian influenza epidemiology.

Working with wild-caught, non-specific-pathogen-free (SPF) birds inevitably introduces variability due to prior exposure histories and latent infections. However, this limitation also represents a strength, as it provides an ecologically realistic insight into host-pathogen dynamics that cannot be captured in SPF systems. Similar complexity is expected in natural populations, particularly under current widespread circulation of H5N1 HPAIV.

Overall, this study demonstrates that house sparrows are susceptible to H7N9 LPAIV infection, capable of viral shedding, seroconversion, systemic infection, and, in some cases, suffer mortality. While habitat of origin significantly structured baseline physiological condition and temporal dynamics of infection, it did not consistently translate into differences in viral shedding, systemic dissemination, or survival outcomes in our experiment. Subtle habitat-associated effects on early infection dynamics and immune kinetics, however, suggest that environmental context may still influence host-pathogen interactions under more complex ecological conditions. The welfare-mediated sampling design may have lacked sufficient statistical power to detect these effects.

Given the ubiquity and synanthropic nature of house sparrows, and their frequent contact with humans, livestock, and wildlife, their potential role as bridge hosts in avian influenza ecology remains relevant and warrants further investigation [2, 3, 36].

CONCLUSIONS

House sparrows are susceptible to infection with the H7N9 LPAIV virus, which results in viral shedding, seroconversion and, occasionally, systemic dissemination and mortality under experimental conditions.

Despite clear differences in baseline physiological status between urban and rural populations, habitat of origin did not significantly influence viral shedding, systemic infection or survival, but subtle habitat-associated variations in early infection dynamics and immune responses suggest modulation of acute-phase responses by the environmental context. In addition, we evidence interaction of LPAIV infection dynamics with natural APV infections, highlighting the importance of individual conditions and pathogen interactions over habitat effects in determining disease progression.

Declarations

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

All experimental procedures were approved by the Ethical Committee on Animal Experimentation of the Catalan Government (Approval No. 11936). Capture of sparrows was authorized by the Ethical Committee of the University of Castilla-La Mancha (Approval No. OHPR202214) and the Junta de Comunidades de Castilla-La Mancha (Permit No. avp22_123). Handling and sampling procedures were carried out in accordance with all applicable institutional and national guidelines for the care and use of animals in experimentation.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

COMPETING INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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AUTHORS' CONTRIBUTIONS

SMM participated in animal capture, sampling, transport and the experimental infections; collected biological samples and performed laboratory and data analyses; drafted the manuscript and revised and edited all versions of the manuscript. **ASC** participated in animal capture, sampling, transport collected biological samples from the control group during the experiment. **RV** performed the laboratory analyses. **MJVM** participated in the experimental infections and performed the laboratory analyses. **RAJW** performed laboratory analyses and data analysis related to *avipoxvirus* and critically reviewed the manuscript. **KB** contributed to the study design, project proposal and funding acquisition; critically reviewed the manuscript. **NM** contributed to the study design, project proposal and funding acquisition; critically reviewed the manuscript. **UH** contributed to the study design, project proposal and funding acquisition; participated in animal capture, sampling, transport and the experimental infections, collected biological samples, and critically reviewed all versions of the manuscript. All authors read and approved the final version of the manuscript.

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Figures

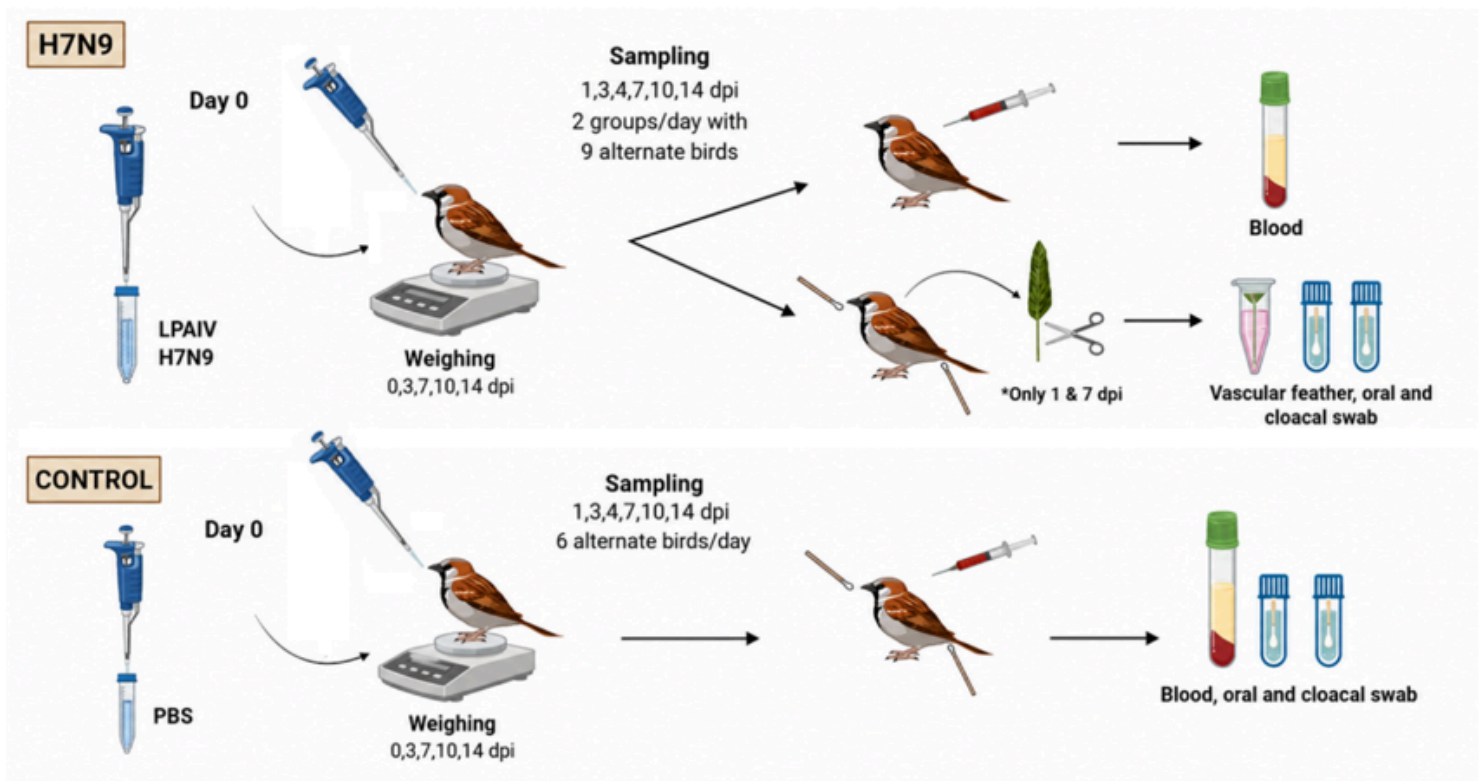


Figure 1

Summary of the procedures performed in the groups of rural and urban inoculated sparrows and controls on the different sampling days post- inoculation (dpi).

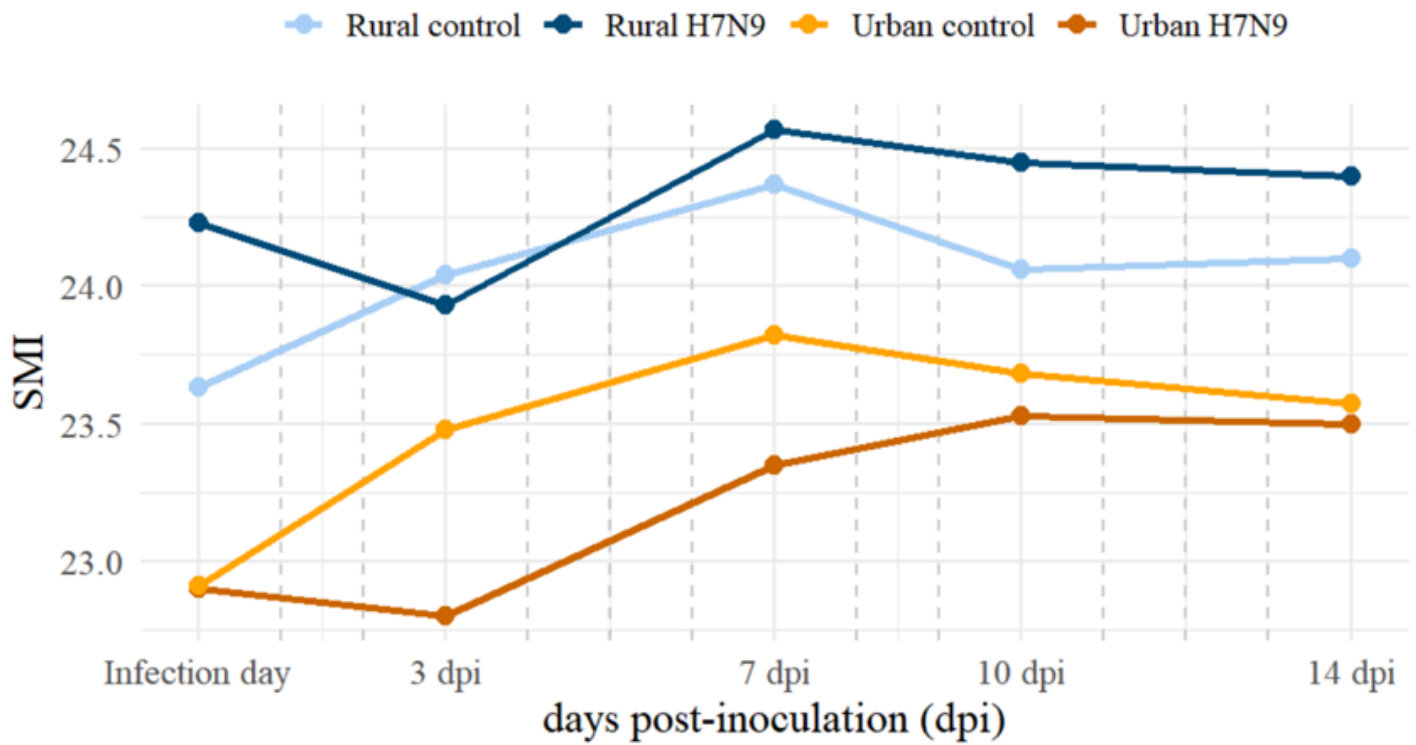
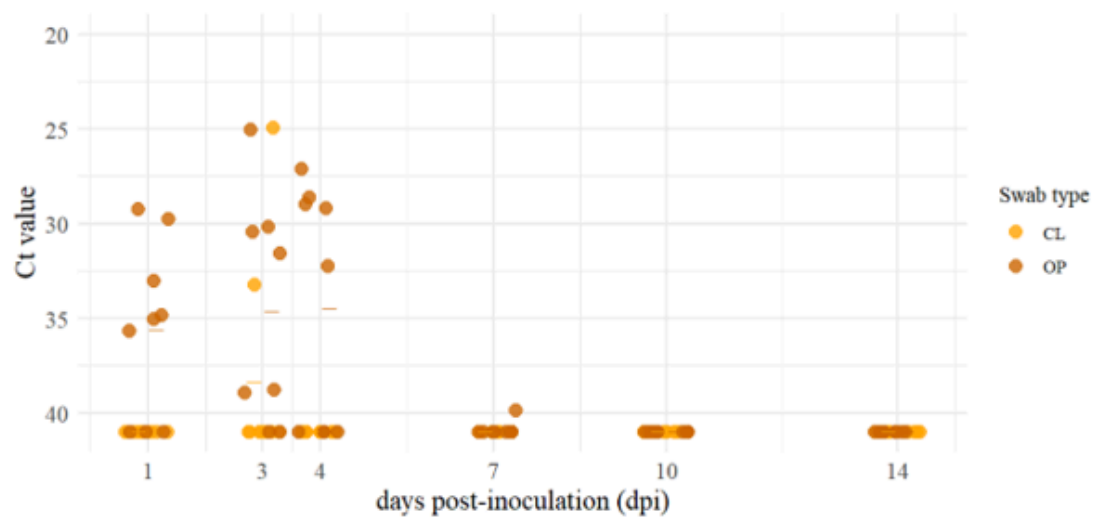


Figure 2

Changes in scaled body mass index (SMI) on the day of infection and at 3, 7, 10 and 14 days post-inoculation (dpi) in H7N9 LPAIV infected urban and rural sparrows and controls.

a) *Urban*



b) *Rural*

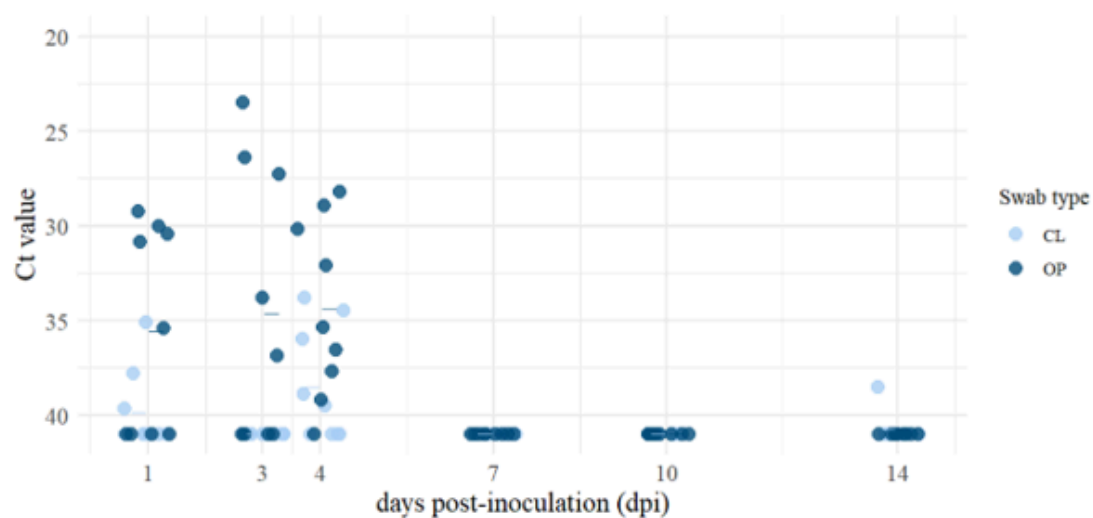


Figure 3

Detection of H7N9 LPAIV RNA and CT in positive samples from oropharyngeal (OP) and cloacal swabs (CL) as proxy for shedding in experimentally infected urban (a) and rural (b) sparrows.

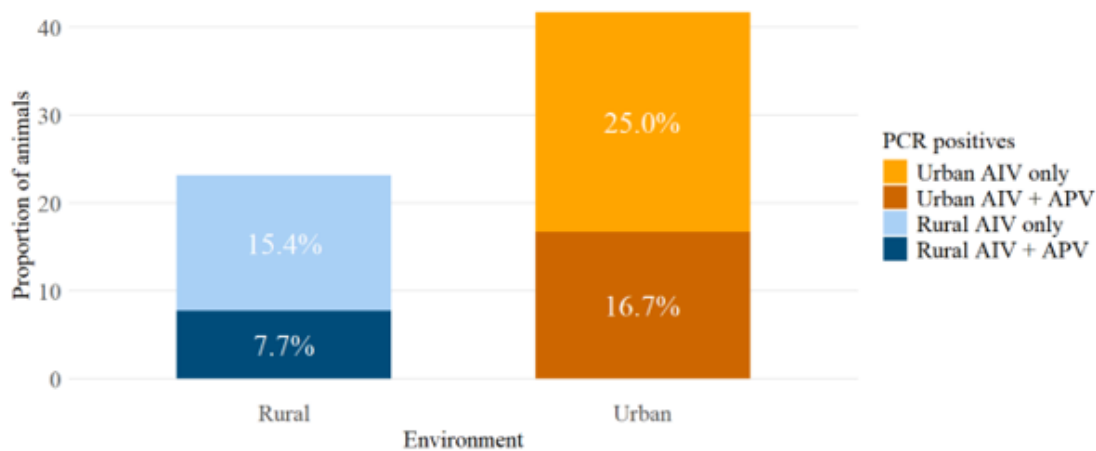


Figure 4

Proportion of naturally deceased or euthanised (at 14 dpi) rural and urban sparrows with a positive signal for H7N9 LPAIV in tissues, and the proportion of sparrows in which avipoxvirus (APV) co-infection was detected in swab samples. Colours indicate individuals positive only for AIV (light orange and light blue) and those co-infected with AIV + APV (dark orange and dark blue).

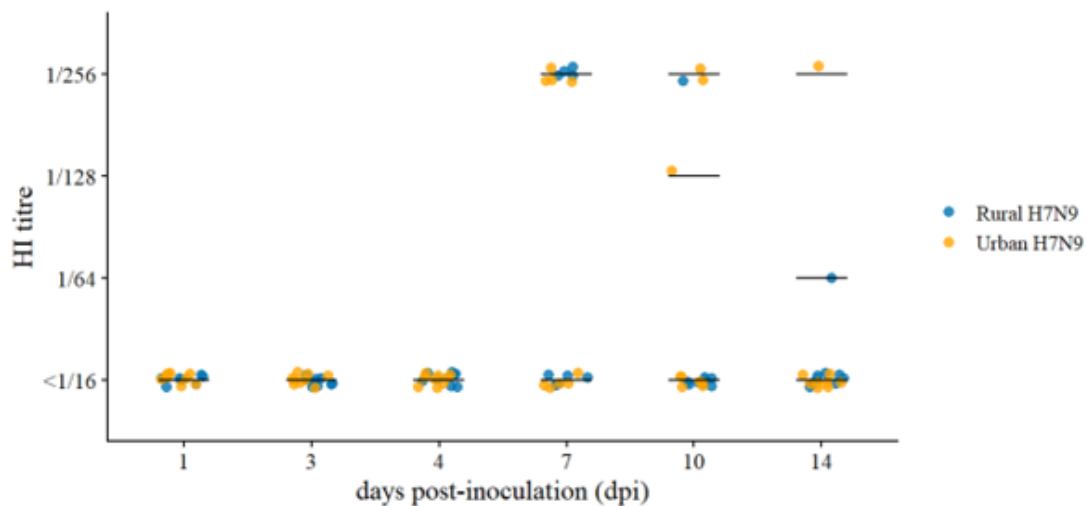


Figure 5

Hemagglutination-inhibiting antibody titres against H7N9 LPAIV in experimentally infected rural and urban sparrows at 7, 10 and 14 days post-inoculation (dpi).

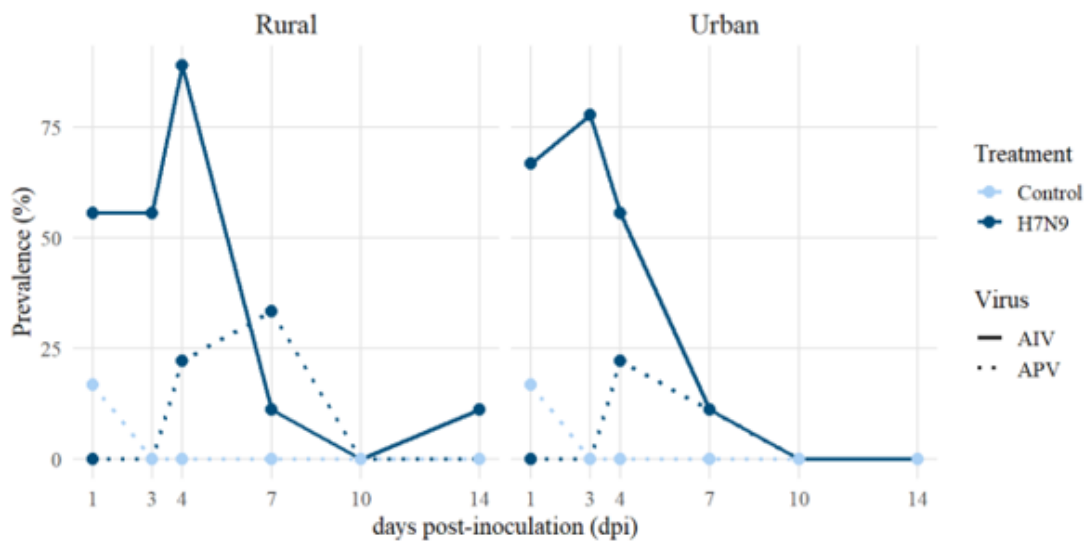


Figure 6

Prevalence (%) of avipoxvirus (APV) and avian influenza virus (AIV) in experimentally H7N9 LPAIV infected rural and urban sparrows and controls from 1 to 14 days post-inoculation (dpi).

Supplementary Files

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- [ADDITIONALFILE2FigureS1.KaplanMeiersurvivalcurvesofhousesparrowsfromurbanandruralorigin.pdf](#)
- [ADDITIONALFILE3TableS2.DistributionofAIVviralRNAintissuesofH7N9infectedurbanandruralsparrows.pdf](#)
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