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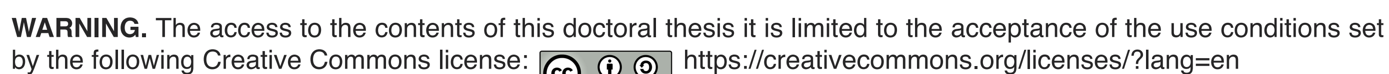

Interactions of avian influenza virus with the host and the environment

Albert Perlas Puente

PhD Thesis

Bellaterra, 2023

Interactions of avian influenza virus with the host and the environment

Tesi doctoral presentada per **Albert Perlas Puente** per optar al grau de Doctor en Veterinària dins del programa de doctorat de Medicina i Sanitat Animals del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona, sota la direcció de la **Dra. Natàlia Majó i Masferrer**, el **Dr. Martí Cortey Marquès** i el **Dr. Antoni Ramis Salvà**.

Bellaterra, 2023

La Dra. NATÀLIA MAJÓ i MASFERRER, el Dr. MARTÍ CORTEY MARQUÈS i el Dr. ANTONI RAMIS SALVÀ, professors i investigadors del Departament de Sanitat i d'Anatomia Animals de la Facultat de Veterinària de la Universitat Autònoma de Barcelona

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Dr. Martí Cortey Marquès

Dr. Antoni Ramis Salvà

Albert Perlas Puente

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Disseny de portada: Esther Carbonell (**esthercarbonellarquer.com**)

“Murphy's law doesn't mean that something bad will happen. It means that whatever **can** happen, will happen” – Cooper

Epp!! Llegeix el resum de la tesi ni que sigui abans d´anar directament als agraïments no?? Bé, la veritat és que cal reconèixer que aquesta és la millor part d´una tesi i que jo també he llegit primer els agraïments de totes les tesis que han passat per les meves mans. Per fi em toca fer-ho a mi, ja pensava que no arribaria mai el meu moment. No em vull deixar a ningú, així que intentaré ser sistemàtic. Començaré per abans de la tesi, el durant i finalment el després.

Pel que fa a l'abans, vull agrair directament a la Facultat de Veterinària de la Universitat Autònoma, per tot el que ha representat per mi. Sense l´ajuda de la seva gent, no hauria arribat mai fins aquí i és on he viscut els millors anys de la meva vida. Encara no em crec que així arribat el moment d´acomiadar-se. També m´agradaria donar les gràcies als professors del Màster de Zoonosi i Una Salut que en van guiar per trobar el camí cap a la investigació, en concret la Marga Martin que va apostar per mi per obtenir la beca PIF que m´ha permès realitzar el doctorat.

Durant la meva etapa de doctorand moltes persones han contribuït al fet que pogués arribar al final del camí. Els primers dies, quan encara anava perdut pel CReSA, la Mònica em va acollir a AP i el Raül em va ensenyar com ser un bon doctorant en grip aviària. Al llarg del camí els meus directors han sigut una peça fonamental i he estat sempre molt content dels directors que he tingut. El Toni sempre atent i gestionant els temes administratius, a vegades tan complexos. El Martí, un pilar imprescindible sense el qual no hagués entès mai l´evolució d´aquests virus i disposat a obrir-me les portes de casa seva per treballar a primera línia del Mediterrani. I òbviament, la Natàlia, el meu referent durant aquests 4 anys i mig, tot i ser directora del centre sempre ha estat allà, donant-me la flexibilitat i llibertat necessària per formar-me com a investigador. A part dels meus directors, també vull agrair el Jordi i la Kate sense els quals la qualitat dels meus estudis seria molt menor i dels que he après una barbaritat. I al Ferran que és una llàstima que hagi arribat al final, ja que és un plaer treballar al seu costat. Si abans he agraït directament a la facultat, ara ho voldria fer al CReSA. Crec que és un lloc increïble per fer ciència, on tots els obstacles a la investigació que existeixen en aquest país es veuen superats per la qualitat humana dels seus treballadors. Tot i que no hem d´oblidar la lluita contra la precarietat a la investigació, com hem fet aquests anys amb Doctorands en Lluita. Moltes gràcies al dream team de viro NBS2 pels bons moments i l´ajuda aquests

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Per acabar amb el durant, crec que es mereixien un paràgraf a part, el grup de doctorands. Sense vosaltres això hauria sigut més difícil, heu fet d´aquest viatge una cosa inoblidable. Continueu cuidant el grup i fent pinya en els bons i mals moments, no oblideu que al final estem tots en el mateix vaixell. No diré noms perquè no acabaríem mai, però si vull fer menció especial al Miguel, el Álvaro i la Uxia que m´han acompanyat fins al final i hem compartit els nervis i dubtes dels últims dies.

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Abbreviations:

AIV: avian influenza virus

C. NEGRA : castellana negra

C. PRAT : catalana del Prat

cRNA: complementary RNA

cRNA: complementary RNA

DEG: differential expressed gene

DEG: differentially expressed genes

dpi: days post-inoculation

E. OILOA: euskal oiloa

ECE: embryonated chicken eggs

EMP: empordanesa

eRNA: environmental RNA

F. AMET: flor d'ametller

FDR : false discovery rate

GO: gene Ontology

Gs/GD: A/Goose/Guangdong/1/1996

HA: hemagglutinin

HPAI: high pathogenic avian influenza

HPAIV: HPAI virus

IFITM : IFN-induced transmembrane protein

IFITM: interferon-induced transmembrane

IFN-I: type I interferon

IHC: immunohistochemistry

IRF7: interferon regulatory factor 7

ISG: interferon stimulated gene

ISG: interferon-stimulated genes

JAK-STAT: janus kinase-signal transducer and activator of transcription

LGP2: laboratory of genetics and physiology 2

LPAI: low pathogenic avian influenza

LPAIV: LPAI virus

M1: matrix protein 1

M2: matrix protein 2

MAVS: mitochondrial antiviral signalling protein

MDA5: melanoma differentiation-associated protein 5

NA: neuraminidase

NEP: nuclear export protein

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells

NLR: NOD-like receptor

NOD: nucleotide-binding oligomerization domain

NP: nucleoprotein

NS1: non-structural protein 1

NS2: non-structural protein 2

PA: polymerase acidic protein

PAMP: pathogen-associated molecular pattern

PAR: photosynthetic active radiation

PB1: polymerase basic protein 1

PB2: polymerase basic protein 2

PCoA: principal Coordinates Analysis

PENED: penedesenca

PKR: IFN-I-dependent double-stranded RNA-activated protein kinase

PRR: pattern recognition receptor

qPCR: quantitative polymerase chain reaction

RdRp: RNA-dependent RNA-polymerase

RIG-I: retinoic acid-inducible gene-I

RIN: RNA integrity number

RLR: RIG-I-like receptor

rRT-PCR: real-time RT-PCR

SA: sialic acid

SNP: single nucleotide polymorphism

SPF: specific pathogen free

TCID50: 50% tissue culture infection dose

TLR: toll-like receptor

TNF: tumor necrosis factor

vRNA: viral RNA

vRNP: viral ribonucleoprotein

Abstract:

Avian influenza is a disease with a clear One Health component, as it has relevant elements related to the environment, animal, and human health. Here, we have focused our attention on the interaction of avian influenza virus with the bird host and the environment. To unravel the complex mechanisms involved in those interactions we have analysed in a multidisciplinary way different factors such as the within-host viral diversity, the host's response to the virus, and the viral persistence in water.

In the third chapter, we analysed the viral-host interactions by means of a dual host and pathogen RNA-Seq analysis using samples from chickens experimentally inoculated with two different strains of high pathogenic avian influenza virus. First, we have classified the inoculated chickens as resistant or susceptible. Then, we performed an RNA-Seq analysis from the virus and the host side. From the virus side, few changes at the quasi-species level were detected, between the original inoculum and the oral swabs at three days post-inoculation in susceptible birds. These few changes pointed to some degree of viral adaptation to win viral fitness in these chickens, as changes were located at the RNA-dependent RNA-polymerases in both strains, but not in segments known to interact with the host immune response. However, in addition to the results obtained from the viral-compartmentalization analysis, we suggest that stochastic founder events associated with multiple bottlenecks during avian influenza virus dissemination could impair viral diversity and positive selection. Even if the comparison of the viral evolution between resistant and susceptible birds was not possible due to the low number of viral particles in resistant chickens, their shorter viral shedding made nucleotide differences in quasi-species of resistant birds unlikely. In contrast, huge differences between susceptible and resistant birds were seen at the host transcriptomic level. While high pathogenic avian influenza susceptible chickens showed huge differential expressed genes at 3 dpi, compared to control chickens, resistant chickens did not. Nevertheless, promising differential expressed genes were identified in resistant and susceptible chickens, showing opposite regulations between the two outcomes. These differentials expressed genes were related to NF-κB and MAPK cascades, showing a significant and distinctive pattern. They were

downregulated in resistant chickens compared to control chickens, while upregulated in susceptible chickens compared to control chickens, suggesting a detrimental role of these pathways if not optimally regulated. An in-deep analysis of these pathways should be a future objective, to disentangle which elements are positive in the fight against avian influenza virus, and which others are used by avian influenza virus to replicate in the host (e.g., viral RNA nuclear export), focusing on why some birds can regulate these pathways more effectively than others. Furthermore, our focus was on an individual differential expressed gene; a serine protease named *PLAU* known to convert plasminogen into plasmin. *PLAU* was downregulated in resistant chickens and upregulated in susceptible ones (always compared to control chickens), thus suggesting a role of *PLAU* in the chicken outcome against high pathogenic avian influenza viruses. We hypothesised that this role could be related to an increase in the synthesis of plasmin in susceptible chickens, increasing, in turn, the activation of the hemagglutinin to infect new cells, and causing coagulopathies and inflammatory disorders related to plasmin-induced fibrinolysis.

In chapter four, we analysed the persistence of a low pathogenic avian influenza virus in water, using artificial streams that simulated the environment where avian influenza reservoirs live and transmit the disease, in Mediterranean climates. Detection of viral RNA by quantitative PCR was possible until the end of the experiment (15 days); this highlights the validity of environmental RNA for active avian influenza virus surveillance under certain conditions. Moreover, quantitative PCR results revealed that the combination of sediments and cold water was the condition with a significantly higher protective effect on low pathogenic avian influenza viral RNA. Even though viral RNA detection was high, the results showed infectious persistence times lower than other experimental procedures. This suggested that factors analysed for the first time in our study, such as the effect of day/night cycles of sunlight and water movement, could be related to these striking differences. Although low persistence times were detected (maximum 2 days post spike), we hypothesise that transmission in Mediterranean climates can still be possible, in wild birds that aggregate at specific wetlands during short periods. Furthermore, these results were related to the environmental conditions reproduced in the artificial streams; if other conditions, such as those from northern

latitudes, were mimicked, different results might have been obtained, with longer persistence times expected due to lower temperatures and lower insolation times during the winter season.

Taken together, the present dissertation showed the importance to use multidisciplinary methodologies to disentangle the complex interactions that avian influenza viruses have with the host and with the environment. Opening the door to future perspectives, where the One Health approach will not be just a series of good intentions but a true way of working.

La gripe aviar es una enfermedad con un claro componente de "Una sola salud", ya que tiene elementos relevantes relacionados con el medio ambiente, la salud animal y la salud humana. Aquí hemos centrado nuestra atención en la interacción del virus de la gripe aviar con el huésped aviar y el medio ambiente. Para desentrañar los complejos mecanismos implicados en esas interacciones hemos analizado de forma multidisciplinar diferentes factores como la diversidad viral dentro del hospedador, la respuesta del hospedador al virus y la persistencia viral en el agua.

En el tercer capítulo, hemos analizado las interacciones entre el virus y el hospedador mediante un análisis de RNA-Seq dual de hospedador y patógeno utilizando muestras de pollos inoculados experimentalmente con dos cepas diferentes del virus de la gripe aviar de alta patogenicidad. En primer lugar, hemos clasificado los pollos inoculados como resistentes o susceptibles. A continuación, hemos realizado un análisis de RNA-Seq desde el lado del virus y del huésped. Desde el lado del virus, se detectaron pocos cambios a nivel de cuasiespecie, entre el inóculo original y los hisopos orales a los tres días post-inoculación en las aves susceptibles. Estos pocos cambios apuntaban a un cierto grado de adaptación viral para ganar aptitud viral en estos pollos, ya que los cambios se localizaban en las ARN-polimerasas dependientes del ARN en ambas cepas, pero no en los segmentos conocidos por interactuar con la respuesta inmunitaria del huésped. Sin embargo, además de los resultados obtenidos del análisis de compartimentación viral, sugerimos que los eventos estocásticos asociados a múltiples cuellos de botella durante la diseminación del virus de la gripe aviar podrían perjudicar la diversidad viral y la selección positiva. Aunque la comparación de la evolución viral entre las aves resistentes y las susceptibles no fue posible debido al bajo número de partículas virales en los pollos resistentes, su menor diseminación viral hizo improbables las diferencias de nucleótidos en las cuasiespecies de aves resistentes. En cambio, se observaron enormes diferencias entre las aves susceptibles y las resistentes a nivel transcriptómico del huésped. Mientras que los pollos susceptibles a la gripe aviar altamente patógena mostraron enormes genes expresados de forma diferencial a los 3 dpi, en comparación con los pollos de control, los pollos resistentes no lo hicieron. Sin embargo, se identificaron prometedores genes expresados de forma diferencial en los pollos resistentes y susceptibles, mostrando regulaciones opuestas entre los dos resultados. Estos genes expresados diferencialmente estaban relacionados con

las cascadas NF-κB y MAPK, mostrando un patrón significativo y distintivo. Fueron regulados a la baja en los pollos resistentes en comparación con los pollos de control, mientras que fueron regulados al alza en los pollos susceptibles en comparación con los pollos de control, lo que sugiere un papel perjudicial de estas vías si no se regulan de forma óptima. Un análisis en profundidad de estas vías debería ser un objetivo futuro, para desentrañar qué elementos son positivos en la lucha contra el virus de la gripe aviar, y qué otros son utilizados por el virus de la gripe aviar para replicarse en el huésped (por ejemplo, la exportación nuclear del ARN viral).. Además, nos centramos en un gen individual de expresión diferencial; una serina proteasa llamada *PLAU* conocida por convertir el plasminógeno en plasmina. *PLAU* estaba regulado a la baja en los pollos resistentes y al alza en los susceptibles (siempre en comparación con los pollos de control), lo que sugiere un papel de *PLAU* en el resultado de los pollos contra los virus de la gripe aviar altamente patógena. Nuestra hipótesis es que este papel podría estar relacionado con un aumento de la síntesis de plasmina en los pollos susceptibles, aumentando, a su vez, la activación de la hemaglutinina para infectar nuevas células, y causando coagulopatías y trastornos inflamatorios relacionados con la fibrinólisis inducida por la plasmina.

En el cuarto capítulo se analizó la persistencia de un virus de la gripe aviar de baja patogenicidad en el agua utilizando canales artificiales que simulaban el entorno en el que viven y transmiten la enfermedad los reservorios de la gripe aviar, en climas mediterráneos. La detección del ARN viral mediante PCR cuantitativa fue posible hasta el final del experimento (15 días); esto pone de manifiesto la validez del ARN ambiental para la vigilancia activa del virus de la influenza aviar en determinadas condiciones. Además, los resultados de la PCR cuantitativa revelaron que la combinación de sedimentos y agua fría era la condición con un efecto protector significativamente mayor sobre el ARN viral de la influenza aviar de baja patogenicidad. Aunque la detección del ARN viral fue alta, los resultados mostraron tiempos de persistencia infecciosa inferiores a los de otros procedimientos experimentales. Esto sugiere que los factores analizados por primera vez en nuestro estudio, como el efecto de los ciclos día/noche de la luz solar y el movimiento del agua, podrían estar relacionados con estas sorprendentes diferencias. Aunque se detectaron tiempos de persistencia bajos (un máximo de 2 días después de introducir el virus en el agua), planteamos la hipótesis de que la transmisión en

climas mediterráneos todavía puede ser posible, en aves silvestres que se agregan en humedales específicos durante períodos cortos. Además, estos resultados estaban relacionados con las condiciones ambientales reproducidas en los canales artificiales; si se imitaran otras condiciones, como las de las latitudes septentrionales, podrían haberse obtenido resultados diferentes, esperándose tiempos de persistencia más largos debido a las temperaturas más bajas y a la menor insolación durante la estación invernal.

En conjunto, la presente disertación mostró la importancia de utilizar metodologías multidisciplinarias para desentrañar las complejas interacciones que los virus de la gripe aviar tienen con el huésped y con el medio ambiente. Esto abre la puerta a futuras perspectivas, en las que el enfoque de "Una sola salud" no sea sólo una serie de buenas intenciones, sino una verdadera forma de trabajar.

La grip aviària és una malaltia amb un clar component d' "Una sola salut", ja que té elements rellevants relacionats amb el medi ambient, la salut animal i la salut humana. Aquí hem centrat la nostra atenció en la interacció del virus de la grip aviària amb l'hoste aviar i el medi ambient. Per desentranyar els complexos mecanismes implicats en aquestes interaccions hem analitzat de manera multidisciplinària diferents factors com la diversitat viral dins de l'hoste, la resposta de l'hospedador al virus i la persistència viral a l'aigua.

Al tercer capítol, hem analitzat les interaccions entre el virus i l'hospedador mitjançant una anàlisi d'RNA-Seq dual, d'hospedador i patogen, utilitzant mostres de pollastres inoculats experimentalment amb dos ceps diferents del virus de la grip aviària d'alta patogenicitat. En primer lloc, hem classificat els pollastres inoculats com a resistents o susceptibles. A continuació, hem realitzat una anàlisi de RNA-Seq des del cantó del virus i de l'hoste. Des del cantó del virus, es van detectar pocs canvis a nivell de cuasi-espècie , entre l'inòcul original i els hisops orals als tres dies post-inoculació a les aus susceptibles. Aquests pocs canvis apuntaven a un cert grau d'adaptació viral per guanyar aptitud viral en aquests pollastres, ja que els canvis es localitzaven a les ARN-polimerases dependents de l'ARN en els dos ceps, però no en els segments coneguts per interactuar amb la resposta immunitària del hoste. Tot i això, a més dels resultats obtinguts de l'anàlisi de compartimentació viral, suggerim que els esdeveniments estocàstics associats a múltiples colls d'ampolla durant la disseminació del virus de la grip aviària podrien perjudicar la diversitat viral i la selecció positiva. Encara que la comparació de l'evolució viral entre les aus resistents i les susceptibles no va ser possible a causa del baix nombre de partícules virals als pollastres resistents, la seva menor disseminació viral va fer improbables les diferències de nucleòtids a les cuasi-espècies d'aus resistents. En canvi, es van observar diferències enormes entre les aus susceptibles i les resistents a nivell transcriptòmic de l'hoste. Mentre que els pollastres susceptibles a la grip aviària altament patògena van mostrar enormes gens expressats de forma diferencial als 3 dpi, en comparació dels pollastres de control, els pollastres resistents no ho van fer. Tot i això, es van identificar prometedors gens expressats de forma diferencial en els pollastres resistents i susceptibles, mostrant regulacions oposades entre els dos resultats. Aquests gens expressats diferencialment estaven relacionats amb les cascades NF -κB i MAPK, mostrant un patró significatiu i distintiu. Van ser regulats

a la baixa en els pollastres resistents en comparació dels pollastres de control, mentre que van ser regulats a l'alça en els pollastres susceptibles en comparació dels pollastres de control, cosa que suggereix un paper perjudicial d'aquestes vies si no es regulen de forma òptima. Una anàlisi en profunditat d'aquestes vies hauria de ser un objectiu futur, per desentranyar quins elements són positius en la lluita contra el virus de la grip aviària, i quins altres són utilitzats pel virus de la grip aviària per replicar-se a l'hoste (per exemple , l'exportació nuclear de l'ARN viral). A més, ens centrem en un gen individual d'expressió diferencial; una serina proteasa anomenada *PLAU* coneguda per convertir el plasminogen en plasmina. *PLAU* estava regulat a la baixa als pollastres resistents i a l'alça en els susceptibles (sempre en comparació dels pollastres de control), cosa que suggereix un paper de *PLAU* en el resultat dels pollastres contra els virus de la grip aviària altament patògena. La nostra hipòtesi és que aquest paper podria estar relacionat amb un augment de la síntesi de plasmina als pollastres susceptibles, augmentant, alhora, l'activació de l'hemaglutinina per infectar noves cèl·lules, i causant coagulopaties i trastorns inflamatoris relacionats amb la fibrinòlisi induïda per la plasmina.

Al quart capítol es va analitzar la persistència d'un virus de la grip aviària de baixa patogenicitat a l'aigua utilitzant canals artificials que simulaven l'entorn on viuen i transmeten la malaltia els reservoris de la grip aviària en climes mediterranis. La detecció de l'ARN viral mitjançant PCR quantitativa fou possible fins al final de l'experiment (15 dies); això posa de manifest la validesa de l'ARN ambiental per a la vigilància activa del virus de la influença aviària en determinades condicions. A més, els resultats de la PCR quantitativa van revelar que la combinació de sediments i aigua freda era la condició amb un efecte protector significativament més gran sobre l'ARN viral de la influença aviària de baixa patogenicitat. Tot i que la detecció de l'ARN viral va ser alta, els resultats van mostrar temps de persistència infecciosa inferiors als altres procediments experimentals. Això suggereix que els factors analitzats per primera vegada al nostre estudi, com l'efecte dels cicles dia/nit de la llum solar i el moviment de l'aigua, podrien estar relacionats amb aquestes diferències sorprenents. Encara que es van detectar temps de persistència baixos (un màxim de 2 dies després d' introduir el virus a l'aigua), vam plantejar la hipòtesi que la transmissió en climes mediterranis encara pot ser possible, en aus silvestres que s'agreguen en aiguamolls específics durant períodes curts . A més, aquests

resultats estaven relacionats amb les condicions ambientals reproduïdes als canals artificials; si s'imitessin altres condicions, com les de les latituds septentrionals, podrien haver-se obtingut resultats diferents, esperant-se temps de persistència més llargs a causa de les temperatures més baixes i la menor insolació durant l'estació hivernal.

En conjunt, aquesta dissertació va mostrar la importància d'utilitzar metodologies multidisciplinàries per desentranyar les interaccions complexes que els virus de la grip aviària tenen amb l'hoste i amb el medi ambient. Això obre la porta a futures perspectives, en què l'enfocament d' “Una sola salut” no sigui només una sèrie de bones intencions, sinó una veritable manera de treballar.

La grip aviària és una malaltia amb un clar component d' "Una sola salut", ja que té elements rellevants relacionats amb el medi ambient, la salut animal i la salut humana. Aquí hem centrat la nostra atenció en la interacció del virus de la grip aviària amb l'hoste aviar i el medi ambient. Per desentranyar els complexos mecanismes implicats en aquestes interaccions hem analitzat de manera multidisciplinària diferents factors com la diversitat viral dins de l'hoste, la resposta de l'hospedador al virus i la persistència viral a l'aigua.

Al tercer capítol, hem analitzat les interaccions entre el virus i l'hospedador mitjançant una anàlisi d'RNA-Seq dual, d'hospedador i patogen, utilitzant mostres de pollastres inoculats experimentalment amb dos soques diferents del virus de la grip aviària d'alta patogenicitat. En primer lloc, hem classificat els pollastres inoculats com a resistents o susceptibles. A continuació, hem elaborat una anàlisi d'RNA-Seq des del cantó del virus i de l'hoste. Des del cantó del virus, es van detectar pocs canvis a escala de quasi-espècie, entre l'inòcul original i els hisops orals al cap de tres dies postinoculació a les aus susceptibles. Aquests pocs canvis apuntaven a un cert grau d'adaptació viral per guanyar aptitud viral en aquests pollastres, ja que els canvis es localitzaven als ARN-polimerases dependents de l'ARN en les dues soques, però no en els segments coneguts per interactuar amb la resposta immunitària de l'hoste. Tot i això, a més dels resultats obtinguts de l'anàlisi de compartimentació viral, suggerim que els esdeveniments estocàstics associats a múltiples colls d'ampolla durant la disseminació del virus de la grip aviària podrien perjudicar la diversitat viral i la selecció positiva. Encara que la comparació de l'evolució viral entre les aus resistents i les susceptibles no va ser possible a causa del baix nombre de partícules virals als pollastres resistents, la seva menor disseminació viral va fer improbables les diferències de nucleòtids a les quasi-espècies d'aus resistents. En canvi, es van observar diferències enormes entre les aus susceptibles i les resistents a nivell transcriptòmic de l'hoste. Mentre que els pollastres susceptibles a la grip aviària altament patògena van mostrar enormes gens expressats de forma diferencial als 3 dpi, en comparació dels pollastres de control, els pollastres resistents no ho van fer. Tot i això, es van identificar prometedors gens expressats de forma diferencial en els pollastres resistents i susceptibles, mostrant regulacions oposades entre els dos resultats. Aquests gens expressats diferencialment estaven relacionats amb les cascades NF -κB i MAPK,

mostrant un patró significatiu i distintiu. Van ser regulats a la baixa en els pollastres resistents en comparació dels pollastres de control, mentre que van ser regulats a l'alça en els pollastres susceptibles en comparació dels pollastres de control, cosa que suggereix un paper perjudicial d'aquestes vies si no es regulen de forma òptima. Una anàlisi en profunditat d'aquestes vies hauria de ser un objectiu futur, per desentranyar quins elements són positius en la lluita contra el virus de la grip aviària, i quins altres són utilitzats pel virus de la grip aviària per replicar-se a l'hoste (per exemple, l'exportació nuclear de l'ARN viral). A més, ens centrem en un gen individual d'expressió diferencial; una serina proteasa anomenada *PLAU* coneguda per convertir el plasminogen en plasmina. *PLAU* estava regulat a la baixa als pollastres resistents i a l'alça en els susceptibles (sempre en comparació dels pollastres de control), cosa que proposa un paper de *PLAU* en el resultat dels pollastres contra els virus de la grip aviària altament patògena. La nostra hipòtesi és que aquest paper podria estar relacionat amb un augment de la síntesi de plasmina als pollastres susceptibles, augmentant, alhora, l'activació de l'hemaglutinina per infectar noves cèl·lules, i causant coagulopaties i trastorns inflamatoris relacionats amb la fibrinòlisi induïda per la plasmina.

Al quart capítol es va analitzar la persistència d'un virus de la grip aviària de baixa patogenicitat a l'aigua utilitzant canals artificials que simulaven l'entorn on viuen i transmeten la malaltia els reservoris de la grip aviària en climes mediterranis. La detecció de l'ARN viral mitjançant PCR quantitativa fou possible fins al final de l'experiment (15 dies); això posa de manifest la validesa de l'ARN ambiental per a la vigilància activa del virus de la influença aviària en determinades condicions. A més, els resultats de la PCR quantitativa van revelar que la combinació de sediments i aigua freda era la condició amb un efecte protector significativament més gran sobre l'ARN viral de la influença aviària de baixa patogenicitat. Tot i que la detecció de l'ARN viral va ser alta, els resultats van mostrar temps de persistència infecciosa inferiors als altres procediments experimentals. Això suggereix que els factors analitzats per primera vegada al nostre estudi, com l'efecte dels cicles dia/nit de la llum solar i el moviment de l'aigua, podrien estar relacionats amb aquestes diferències sorprenents. Encara que es van detectar temps de persistència baixos (un màxim de dos dies després d'introduir el virus a l'aigua), vam plantejar la hipòtesi que la transmissió en climes mediterranis encara és possible, en aus

silvestres que s'agreguen en aiguamolls específics durant períodes curts. A més, aquests resultats estaven relacionats amb les condicions ambientals reproduïdes als canals artificials; si s'imitessin altres condicions, com les de les latituds septentrionals, podrien haver-se obtingut resultats diferents, esperant-se temps de persistència més llargs a causa de les temperatures més baixes i la menor insolació durant l'estació hivernal.

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Chapter I

General Introduction

1.1. The One Health approach

One Health is a multidisciplinary, integrated, and unifying approach that interconnects environmental, animal, and human health [1]. Historically, the investigation, study, and practice of human and animal health appeared together and were interconnected, but early public health standards divided the roles of physicians and veterinarians [2]. Nowadays, planetary changes are clearly showing not only the need to apply this interconnection again but to increase it, using the expertise from other fields, such as biologists, sociologists, economists etc., to improve human, animal, and environmental health.

Planetary changes are related to changes in human behaviour, trade globalisation, and deforestation, among others. The human population growth and the expansion to new geographical areas are increasing the close contact of humans with domestic and wild animals. The increase of intensive farming to feed this growing population, with areas of a massive density of animals, is also behind an increase in animal-human contact. Environmental changes, such as global warming and deforestation, can also increase the close contact between humans and wild animals, since some wild animals must leave their habitats for new ones, closer to human populations [3]. These close contacts between humans and animals increase the risk of pathogen transmission from animals to humans (zoonosis), as well as the transmission of pathogens from humans to animals (reverse zoonosis). In fact, 60% of known human infectious diseases and 75% of all reported emerging infectious diseases have an animal origin [4]. A clear example of the impact of those diseases is the current coronavirus pandemic (COVID-19), which has been responsible for millions of deaths. A zoonotic virus has been widely accepted as the cause of this pandemic event [5,6]. Furthermore, the rise in global trade and people's travels around the globe increase the risk of dissemination, when a new zoonotic and highly transmissible disease appears [7], as was the case with COVID-19.

Animal diseases not only directly impact human health, but they also negatively impact food security, as more than 20% of global animal production losses are linked to animal diseases [4]. This factor can negatively impact low-income

countries, as more than 75% of the billion people who live on less than $2 per day depend on subsistence farming and raising livestock to survive [8].

These facts illustrate the precise relationship between human, animal, and environmental health (Figure 1). Is in that context, when the One Health concept emerged. Recently, the Food and Agriculture Organization of the United Nations (FAO), the World Organisation for Animal Health (OIE), the United Nations Environment Programme (UNEP) and the World Health Organization (WHO) came together to define this approach [9]:

"*One Health is an integrated, unifying approach that aims to sustainably balance and optimize the health of people, animals, and ecosystems.*

It recognizes the health of humans, domestic and wild animals, plants, and the wider environment (including ecosystems) are closely linked and interdependent.

The approach mobilizes multiple sectors, disciplines, and communities at varying levels of society to work together to foster well-being and tackle threats to health and ecosystems, while addressing the collective need for clean water, energy and air, safe and nutritious food, taking action on climate change, and contributing to sustainable development."

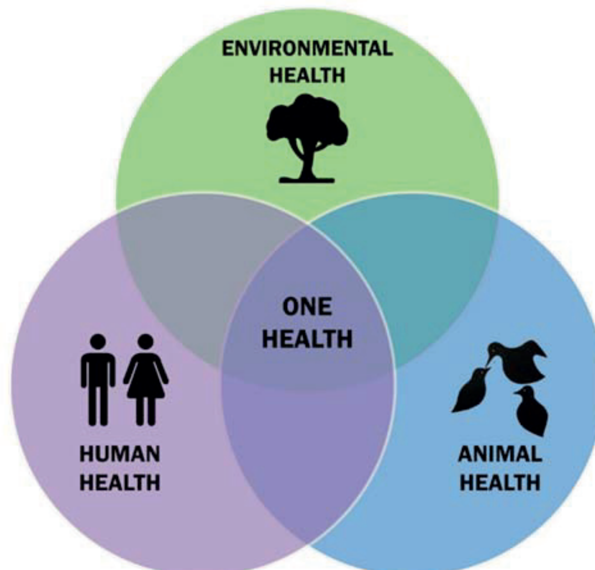

Figure 1. Schematic representation of the triad inherent of the One Health approach.

The One Health approach can be applied to control infectious diseases derived from different pathogens (e.g., bacterial, fungal, prion, viral, etc.). However, some of them can benefit more than others from global changes, such as RNA viruses. These viruses exhibit a large evolutionary plasticity, allowing them to adapt to new hosts faster than any other pathogen [10]. RNA viruses exist in the host as "mutant clouds", non-identical but related genomes subjected to variation, competition, and selection [11]. Therefore, RNA viruses were the first pathogens to seize the opportunities that global changes (density of livestock animals, global mobility, deforestation etc.) offer to them [12].

Avian influenza viruses (AIV) are RNA viruses known to spill over to human populations from birds; posing a risk to future pandemics, as was the case in the past [13]. AIV global outbreaks have a substantial economic impact on the poultry sector, causing increases in poultry product prices that in turn might became a risk to food security. Furthermore, recent outbreaks of AIV have caused massive mortalities in wild birds, and sporadic deaths in wild mammals, making AIV a conservation issue in several species [14]. These elements make AIV a perfect example of a pathogen that needs a One Health approach to be tackled (Figure 2); even more considering that AIV persistence in water environments plays a relevant role in the global transmission of this disease [15], since changes in wild bird's habitats can affect AIV spread [16].

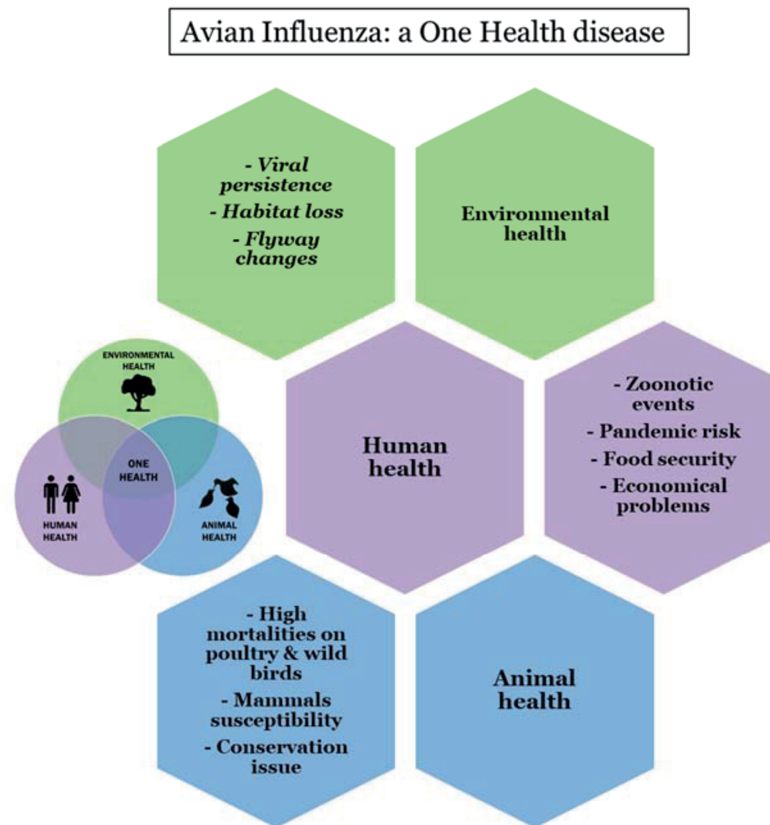

Figure 2. Schematic representation of the One Health implications in AIV outbreaks.

1.2. History of the avian influenza

The first records trace back 145 years ago in Italy, with historical reports of a highly lethal disease in chickens dated in 1878 [17]. This disease was named Fowl plague or Fowl pest and the first cases were identified by clinical signs and lesions. This was one of the first diseases described as being caused by a viral agent; in 1901, the causal agent was shown to be ultra-filterable [18]. By the mid-twentieth century, outbreaks of the Fowl plague were detected worldwide. In 1945, the first low pathogenic cases of Fowl plague were identified in domestic poultry species in Germany, showing a decrease in egg production and respiratory signs [19]. Its specific aetiology was unknown until 1955, when it was associated with the influenza A virus [20]. However, low and high pathogenic cases of fowl plague were not recognised as high pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI) since 1981 [21] and 2002 [22], respectively.

In 1961, the first cases of HPAI were detected in wild birds in South Africa, where common terns (*Sterna hirundo*) suffered high mortality rates [23]. The development of a serological test to identify these viruses in 1970 increased the cases reported (Beard, 1970a, 1970b), also helping to identify the first LPAI cases in wild birds by 1972. Since then, many asymptomatic cases have been detected in wild birds, mostly from the orders Anseriformes and Charadriiformes, which were recognised as reservoirs of the disease in the mid-1970s [26]. Till 1997, there were no references of HPAI cases in poultry transmitted to wild birds, neither maintained independently from poultry in wild bird populations. The emergence of H5 A/Goose/Guangdong/1/1996 (GsGD) lineage HPAI virus (HPAIV) in southeast Asia changed this paradigm [14].

Going back in time, in 1918, the most devastating influenza pandemic in the 20th century occurred, the misnamed "Spanish Flu", with millions of human deaths. It is now suspected that this pandemic was caused due to a zoonotic event from chickens infected with AIV [13]. Another zoonotic event from an AIV in 1957 caused another pandemic in humans, the "Asian Flu"[13], and a reassortment event from another AIV with the virus that caused the "Asian Flu" was behind a new pandemic named the "Hong Kong Flu" in 1968 [13]. These zoonotic events highlight the connections between human and bird health in this disease.

Since 2003, H5 GsGD HPAIV has become enzootic in Asia and panzootic worldwide, with massive outbreaks appearing each year around the globe, causing massive poultry mortalities and economic losses [27]. Diverse H5 GsGD HPAIV epizootics events affected Europe since then, but the worst wave in terms of severity, geographic spread, and number of wild bird species affected was the present season [28]. This worrisome tendency correlates with increased poultry production worldwide in recent years. In fact, since the early 1960s, the global supply of eggs *per capita* has doubled, while poultry meat supply has increased sixfold [29]. High-density poultry areas are more common nowadays, due to this increase in poultry production. This creates the ideal scenario for AIV to an increased number of transmission events and the generation of new variants of concern. This evolution process was behind the H5 GsGd HPAI lineage, which changed the role of wild birds in AIV epidemiology. Before that, wild birds were only known to transmit LPAI

variants to poultry and suffer mild or asymptomatic cases (except the 1961 common terns' outbreak), but since the GsGD lineage strains appeared, wild birds can transmit HPAIV directly to domestic birds and suffer severe disease with high mortalities [14]. Overall, the history of these viruses shows the importance of a One Health perspective to understand this disease, with significant effects on poultry, wild birds, and humans (Figure 3).

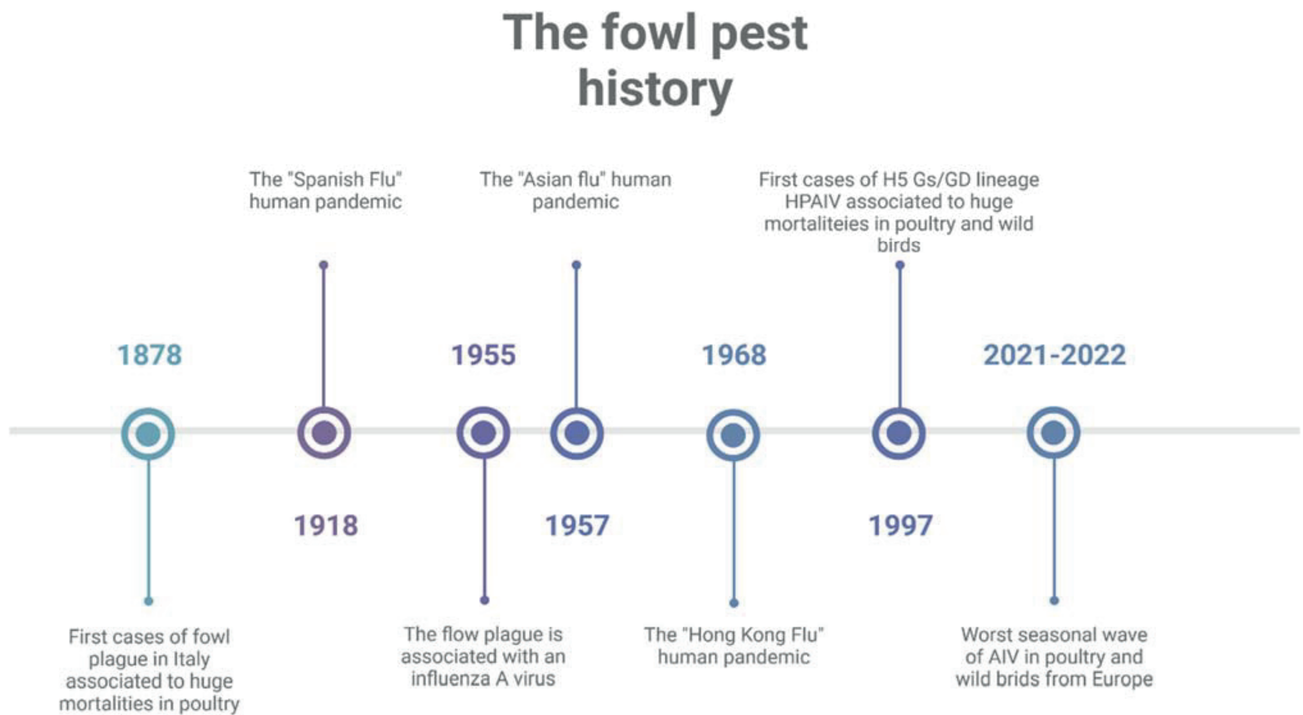

Figure 3. The relevant events of AIV history showing the importance of a One Health perspective.

1.3. The virus

1.3.1. Classification and nomenclature

Influenza viruses are RNA viruses from the Orthomyxoviridae family and are classified into four types (A, B, C and D). Avian influenza virus (AIV) is a type A influenza virus, isolated from and adapted to an avian host. Type A influenza strains have two major surface proteins, hemagglutinin (HA) and neuraminidase (NA), that are used to classify these viruses [30]. AIV has 16 HA subtypes (H1-H16) and nine NA subtypes (N1-N9). Two different subtypes from every protein (H17, H18, N10,

and N11) have been identified in bats [31], but, up to now, there is no evidence to support their presence in avian species. AIV is divided into two pathotypes, low pathogenic AIV (LPAIV) and HPAIV, based on its virulence in chickens. LPAIV cause asymptomatic disease or mild to moderate respiratory disease in chickens. In contrast, HPAIV cause severe systemic disease with high mortality rates in chickens [32]. HPAIV is restricted to H5 and H7 subtypes.

The standard nomenclature of influenza viruses consists of type (A/B/C/D); isolation species, if it is not human; the location where it was isolated; isolate number; the year when it was isolated, and if it is a type A influenza virus, HA and NA subtypes in brackets [33]. For example, if we have an H1N1 LPAIV strain isolated from a chicken in Spain, with 5093 as the isolate number, and it was isolated in 2021, we will name this virus like this: "A/Chicken/Spain/5093/2021 (H1N1)".

1.3.2. Viral structure and protein functions

AIVs are enveloped viruses, spherical to pleomorphic (100nm), that have a segmented genome comprised of eight segments of single-stranded negative-sense viral RNA (vRNA) that encode for at least ten essential proteins for virus replication [34,35]: the polymerase basic protein 2 (PB2), the polymerase basic protein 1 (PB1), the polymerase acidic protein (PA), the HA, the nucleoprotein (NP), the NA, the matrix proteins (M1 and M2), the non-structural proteins one (NS1) and two (NS2), also known as nuclear export protein (NEP). The envelope of AIVs contains a lipid membrane derived from the host cell, the HA protein, the NA protein, and the M2. The M1 and the NEP/NS2 underlie the inner surface of the envelope. Inside the envelope, the viral ribonucleoprotein (vRNP) complexes comprise the vRNA, the NP, and the RNA-dependent RNA-polymerase (RdRp) subunits (PB2, PB1, and PA). Overall, they constitute the viral structure of the AIV (Figure 4).

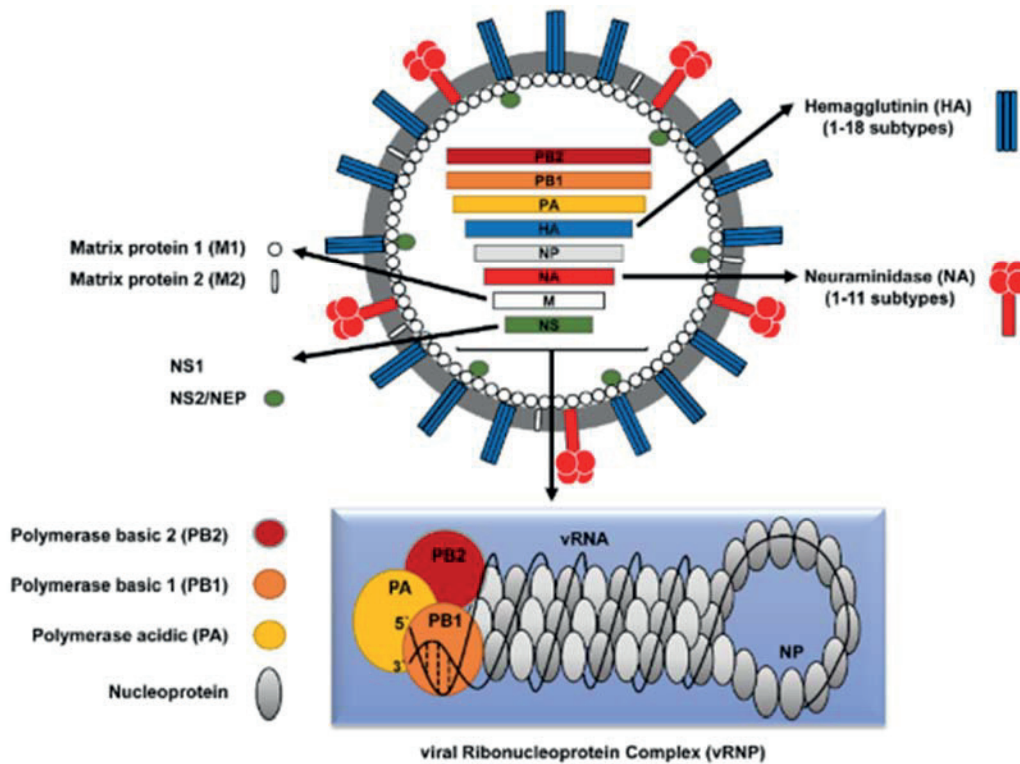

Figure 4. Structure of AIV obtained from reference [13].

The eight segments of vRNA range in size between 890-2341 nucleotides each, and the AIV genome contains, in total, approximately 13,600 nucleotides [36]. Due to this small genome size, some AIV strains have acquired different strategies to encode auxiliary proteins. These mechanisms are alternative splicing of viral mRNAs, non-canonical translation, non-AUG initiation, re-initiation, and ribosomal frameshifting [37]. The roles of the essential and auxiliary proteins of AIV are summarised in Table 1.

Table 1. AIV RNA segments, proteins, and their primary functions. Information obtained from [13,36]. nt: nucleotides size; aa: amino acids size.

<table>
<tr><td>Segment (nt)</td><td>Protein (aa)</td><td>Main Functions</td></tr>
<tr><td rowspan="2">1 (2341)</td><td>PB2 (759)</td><td>Host mRNA cap recognition and binding
Regulation of mitochondrial viability</td></tr>
<tr><td>PB2-S1 (508)</td><td>Inhibition of RIG-I-dependant interferon signalling pathway
Interference with RdRp</td></tr>
<tr><td rowspan="3">2 (2341)</td><td>PB1 (757)</td><td>Prime vRNA transcription
Transcribes vRNA into complementary RNA (cRNA)
Initiates vRNA synthesis</td></tr>
<tr><td>PB1-F2 (87-90)</td><td>Pro-apoptotic activity
Impairs host interferon response</td></tr>
<tr><td>PB1-N40 (718)</td><td>Balance between PB1 and PB1-F2 expression
Polymerase activity</td></tr>
<tr><td rowspan="4">3 (2233)</td><td>PA (716)</td><td>Cap-snatching by exonuclease activity</td></tr>
<tr><td>PA-X (252)</td><td>Modulation of host response and viral virulence
Promote viral replication</td></tr>
<tr><td>PA-N155 (561)</td><td rowspan="2">Promote viral replication and virulence</td></tr>
<tr><td>PA-N182 (534)</td></tr>
<tr><td>4 (1778)</td><td>HA (566)</td><td>Sialic-acid receptor binding
Membrane fusion
Major antigen</td></tr>
<tr><td>5 (1565)</td><td>NP (498)</td><td>vRNA encapsidation
Nuclear import of vRNP</td></tr>
<tr><td>6 (1413)</td><td>NA (454)</td><td>Cleavage of sialic acid receptors
Release of virion progeny
Penetrate the mucus barrier of the respiratory tract</td></tr>
<tr><td rowspan="5">7 (1027)</td><td>M1 (252)</td><td>Nuclear export of vRNP
Virion assembly and budding
Control of virion morphology</td></tr>
<tr><td>M2 (97)</td><td>Regulation of virion internal pH
Uncoatting process
Virion assembly and budding</td></tr>
<tr><td>M3 (9)</td><td>Unknown</td></tr>
<tr><td>M4 (54)</td><td>Unknown</td></tr>
<tr><td>M42 (99)</td><td>Supports viral replication</td></tr>
<tr><td rowspan="3">8 (890)</td><td>NS1 (230)</td><td>Support vRNA splicing, maturation and translation
Nuclear export of vRNP
Antagonize host antiviral responses
Inhibits host mRNA maturation and translation</td></tr>
<tr><td>NEP/NS2</td><td>Nuclear export of vRNP
vRNA transcription and replication</td></tr>
<tr><td>NS3</td><td>Host specificity</td></tr>
</table>

1.3.3. Virus replication cycle

The first step of the AIV replication cycle is the binding of HA to the host cell membrane. A previous step, essential for viral replication, is the cleavage of the HA into two subunits, HA1 and HA2, by host proteases in the cytoplasm of the previously infected cell, or by extracellular proteases [38]. The HA1 subunit of the virion binds with the host sialic acid (SA) receptor to enter the cell, mainly through clathrin-mediated endocytosis [39], or as an alternative pathway through macropinocytosis [40]. Once in the endosome, the lower pH environment activates the M2 ion channels at the AIV membrane. The protons and the K^+ ions enter the virion through the M2 ion channels and induce a conformational change in the HA2 subunit, resulting in the exposure of the fusion peptide motif. Several HA2 subunits form a pore between the AIV and the endosomal membranes, which release the vRNPs (uncoating) into the host cell cytoplasm [41]. Nuclear localisation signals are used by vRNPs to localise the pore and enter via the nuclear pore complex [42].

Once in the nucleus, vRNPs are transcribed using the RdRp (PB2, PB1, and PA), forming 5´cap structures with the PB2 binding to the 5´ cap of host mRNAs and the PA cleaving the cap structure 10-15 nucleotides downstream. The PB1 elongates the vRNA using the 5´cap structures as primers and adds a polyadenylated tail. The transcribed vRNA is transported then to the cytoplasm for translation with the help of NS1 and some translated proteins return to the nucleus via nuclear localisation signals to perform viral replication [43,44].

The virus' genome is also replicated in the nucleus by RdRp, using an intermediate RNA template of complementary RNA (cRNA) previously generated. The replicated vRNA is synthesised without a 5´cap structure or a polyadenylated tail and is encapsidated with the NP, the PB2, and the PB1 proteins to form a vRNP exported from the nucleus, with the help of the M1 and the NEP proteins. Outside of the nucleus, the M1 protein is associated with vRNP to avoid re-importing vRNP into the nucleus. Other translated proteins, such as the HA, the NA, and the M2, are transported to the host cell membrane through the Golgi network for post-translational modifications. Finally, due to the M1 and the M2 interaction, the HA

and the NA assemble with RNPs to form a mature AIV that is released with the NA cleavage of the host SA [36,41] (Figure 5).

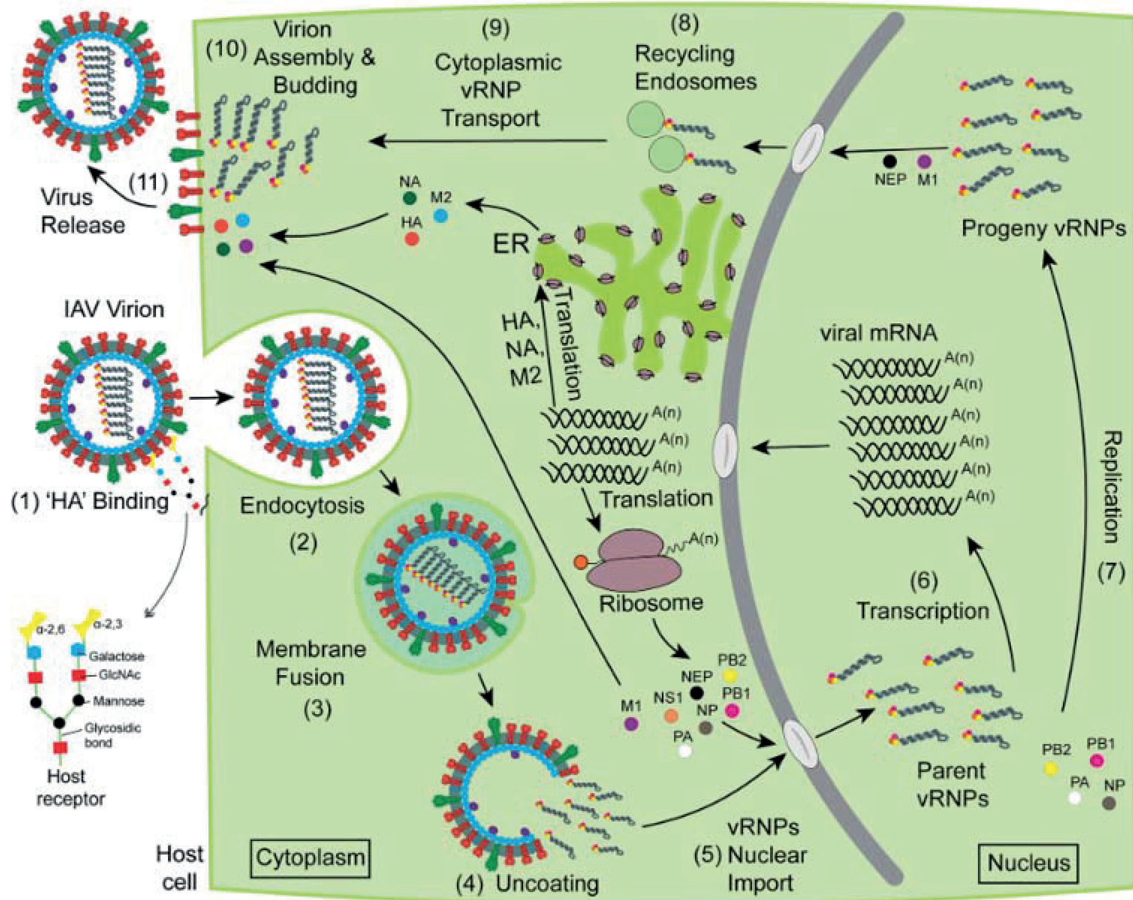

Figure 5. Replication of AIV modified from reference [36].

1.3.4. Viral evolution

The evolution of AIVs is triggered by two primary mechanisms that provide novel virus variants: a high mutation rate (antigenic drift) and a reassortment capability (antigenic shift) [45]. Both mechanisms allow the virus to rapidly adapt to a new host and imply an additional risk to public health.

The antigenic drift leads to the gradual accumulation of mutations in AIV. Among pathogens, RNA viruses are those with high mutation rates due to their error-prone polymerase [12]. AIVs are part of this group, and also have high mutation rates. This high mutation rate of the AIV RdRp is due to a lack of proofreading activity that

generates new mutations when it incorporates faulty nucleotides, with a rate of 10^{-3} to 10^{-4} [46]. This phenomenon is behind the antigenic drift that guides the virus to new variants in every replicated genome. One of the most relevant consequences of the antigenic drift is the evolution of H5 and H7 LPAIV subtypes from wild birds to HPAIV after its introduction in high-density poultry farms, where the virus finds a perfect environment to expand [47]. Therefore, the idea of viruses as identical genome copies is wrong. The term viral quasi-species describes viruses as mutant clouds of non-identical but related genomes that coexist in the host, including low-frequency variants [11]. Some of them acquire single nucleotide polymorphisms (SNPs) that allow the virus to escape the host barriers to replicate in new hosts [48], escape vaccine protection [49], or escape drug protection [50]. Those low-frequency variants that are beneficial for the AIV will be gradually positively selected to become predominant in the mutant cloud population (Figure 6).

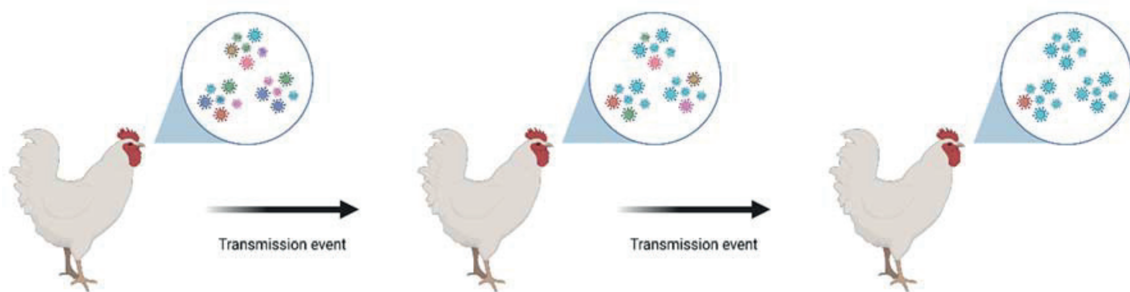

Figure 6. Viral quasi-species selection of a beneficial low-frequency variant (represented as a blue variant in the figure) from the mutant cloud population of the same viral strain.

The antigenic shift is another important mechanism that triggers AIV evolution. Occasionally, when two different AIV strains infect the same individual host, segments from the two strains can reassort into a new strain [45]. Even if it is not very common, the emergence of new influenza strains via the reassortment of human-adapted influenza strains with AIV is behind the worse influenza human pandemics [51]. This is due to the formation of novel antigenic proteins, for which the hosts are naïve, do not have pre-existing immunity and therefore may cause devastating outbreaks.

1.3.5. Viral determinants of pathogenicity

The HA protein is the primary determinant of the pathogenicity of AIV. It initiates viral infection by binding to the cellular membrane and induces membrane fusion. To be activated, the HA needs to be cleaved into the H1 and H2 subunit (Figure 7), this will allow the conformational changes required to fuse with the endosomal membrane when the pH drop (see 1.3.3. Viral replication cycle) [52].

Therefore, HA must be cleaved after its translation by proteases able to detect the HA cleavage site. HPAIVs have a multi-basic cleavage site that intracellular and ubiquitous proteases, such as furin proteases, can cleave inside the cell. In contrast, LPAIVs have a single basic cleavage site that does not allow cleavage inside the cell and needs the presence of proteases that are only present in intestinal and respiratory tissues (Figure 7). Thus, LPAIVs can only replicate in restricted tissues, in contrast, HPAIVs can replicate in almost all host tissues, increasing the pathogenicity of these strains [53]. However, occasionally some LPAIVs can spread systematically, thanks to a viral mechanism that converts host plasminogen into plasmin, a protease that allows an alternative cleavage of the HA [54]. Furthermore, the use of protease inhibitors as viral therapy highlights the importance of the HA activation-cleavage step for AIV to infect the cell [38].

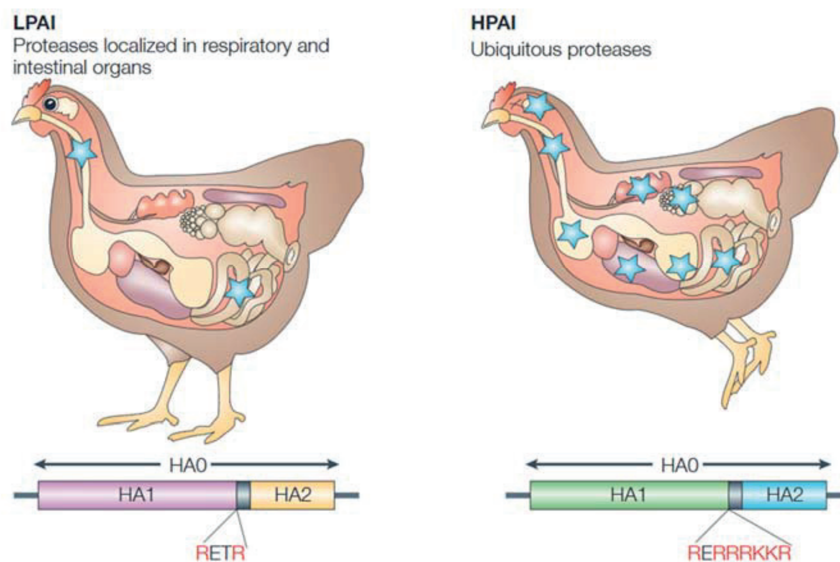

Figure 7. The cleavage site of the HA in LPAIV (basic cleavage site) and HPAIV (multi-basic cleavage site) explains the differential outcome after infection with the two pathotypes. Reproduced from reference [52].

Besides HA, other proteins have significant roles in viral pathogenicity. Protein PB2 regulates mitochondrial viability and uses the host cell resources to promote viral replication [55]. The PB1-F2 protein can upregulate polymerase activity, induce apoptosis, and impair host immunity [56–58]. Furthermore, the PB1-F2 protein is longer in HPAIVs than in LPAIVs, highlining its role in AIV pathogenicity [58]. The NS1 protein can suppress the host immune response by inhibiting interferon proteins and protecting the cell from early apoptosis to ensure viral replication [59,60]. In humans, the phosphoinositide-3-kinase (PI3K) pathway activation by the NS1 protein increased the virulence of the 1918 pandemic H1N1 strain [61]. Overall, AIVs use host mechanisms to increase viral fitness and virulence; therefore, expanding our knowledge of these interaction mechanisms is essential to discover new ways to stop viral replication [36].

1.4. The Host

1.4.1. Host Range of avian influenza viruses

Due to the evolutionary plasticity of RNA viruses, influenza A viruses can infect a broad range of hosts from different taxonomical groups (Figure 8). Specifically, AIVs have been isolated from at least 16 orders: Accipitrioformes, Anseriformes, Charadriiformes, Ciconiiformes, Columbiformes, Falconiformes, Galliformes, Gaviiformes, Gruiformes, Passeriformes, Pelecaniformes, Piciformes, Podicipediformes, Procellariiformes, Strigiformes and Suliformes [28,62]. Recently, unprecedented cases in sea birds from the order Suliformes have been related with an, also unprecedented, increase in H5 Gs/Gd lineage HPAIV outbreaks during summer [28]. However, the natural reservoirs of AIVs are aquatic birds from the orders Anseriformes (ducks and geese) and Charadriiformes (shorebirds, gulls, terns, and auks) [26]. Dabbling ducks (Sub-Family. Anatinae) are those with the highest rates of AIV detection [62]. As said, wild birds usually are asymptomatic to AIVs, except for AIVs from the H5 Gs/Gd lineage HPAIV, which can cause considerable mortalities in wild birds and is an emerging threat to these birds [14].

Even if AIV is usually detected on aquatic birds, some AIV strains can also infect terrestrial birds, such as predators, scavengers, and passerine species [63].

Wild birds can also transmit the virus to domestic birds, causing intermittent outbreaks in poultry farms (i.e., chicken, turkeys, ducks, geese, etc.) and acquiring, in some cases, a high degree of adaptation to poultry, resulting in an efficient and independent transmission within the poultry population [27,64].

Furthermore, some AIVs can cross the class barrier and infect mammal species causing sporadic cases, such as the cases that have been recently described in harbour porpoise, bottlenose dolphin, and American black bear infected with H5 Gs/Gd lineage HPAIV (European Food Safety Authority, 2022). These sporadic cases can occasionally become adapted lineages that can be maintained in the new host population (i.e., horse, pig, dog, and human)[13].

This phenomenon also highlights the risk of zoonotic events, as evidence the recent reports of human infections with H5N1, H5N6, H9N2, H10N3, and H3N8 strains that luckily are not showing an efficient human-to-human transmission [63]. Human cases from H10N3, H3N1, and current strains of H5N1 are fortunately and up to now asymptomatic, some fatalities have been related to H9N2, but all cases underlying pathological conditions. However, current cases of H5N6 and past human detections of H5N1 and H7N9 strains were linked to high mortality rates. Most previously reported human infections with avian influenza viruses were due to exposure to infected poultry or contaminated environments [65].

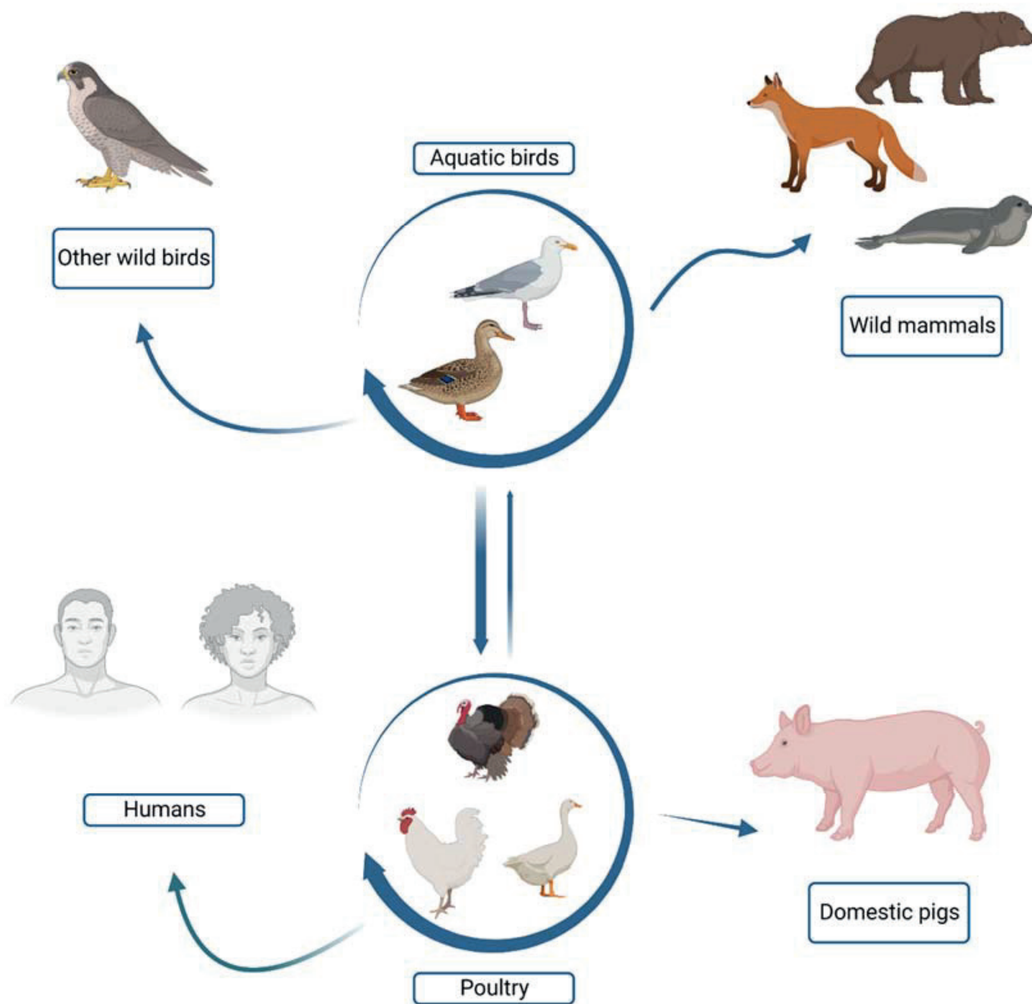

Figure 8. Host range of AIVs showing independent transmission in wild aquatic birds and poultry and sporadic cases in wild mammals, other wild birds, domestic pigs, and humans.

1.4.2. Disease in avian species

Even if only two pathotypes of AIV (LPAIV and HPAIV) are recognised, there is a wide range of clinical outcomes that are influenced by many factors, such as the species and age of the host, the subtype of virus and the presence or absence of other concomitants infections, and environmental factors [66]. However, a summary of all the possible clinical outcomes was suggested by Swayne et al. (2020): 1) highly virulent, 2) moderately virulent, 3) mildly virulent, and 4) avirulent. The highly virulent group is associated to strains from the HPAIV pathotype, which cause vast

mortalities, of almost 100%, in poultry and wild birds infected with H5 Gs/Gd HPAIV strains. In these cases, birds can be found dead without prior clinical cases, or showing nervous signs (e.g., tremors of head and neck, torticollis, repetitive movements, ataxia, paresis, and paralysis), diarrhoea, and loss of weight. The moderately virulent group results from an infection with a LPAIV, but with a concomitant secondary infection, or with the presence of stress factors. In these cases, the mortality can range from 5-97%, with the worst cases seen in young birds, reproductively active hens, or very stressed birds. The increase in the severity of the clinical course seen in LPAIVs with a concomitant infection can be related to bacterial pathogens that produce proteases, able to cleave the HA protein, allowing the virus to replicate widely, similarly to HPAIVs. The mildly virulent group is related to strains from the LPAIV pathotype without concomitant infection. It produces low mortality (less than 5%), mild respiratory signs (coughing, sneezing, and ocular discharge), mild intestinal signs (diarrhoea), and a reduction in egg production and weight. Finally, the last group is the avirulent group, generally associated with LPAIV strains that infect wild aquatic birds and do not cause any mortality or clinical signs. Overall, we can conclude that the outcome of the disease is related to the interaction of the AIV, the host, and its environment.

In more detail, the pathogenesis of the AIV begins with inhalation or ingestion of viral particles. Due to the presence of extracellular proteases in birds' respiratory and intestinal tissue, the HA of both pathotypes can be cleaved; therefore, multiple replications cycles occur in these tissues. In LPAIVs, the replication is restricted to these organs (see 1.3.5. Viral determinants of pathogenicity). In the case of HPAIVs, the first replication also does occur in the respiratory and intestinal epithelium. Still, within 24 hours, the virus is widespread in the host and is found in the capillary endothelium and some inflammatory cells. HPAIVs can replicate in more tissues than LPAIVs due to their ability to use furin-like proteases, which are ubiquitous and intracellular, to activate the HA (see Viral determinant of pathogenicity). After the infection of the capillary endothelium and inflammatory cells, the HPAIVs can spread into the host via the blood vessels (viremia) and the lymphatic system, reaching the peak of viral titers as soon as 48h [67].

The damage caused by AIV is due to three mechanisms: 1) the replication process, 2) inefficient regulation of the host's innate immune response, and 3) vascular dysfunction (Figure 9). The viral replication causes necrosis by the direct damage caused to the cells, and apoptosis by viral segments that induce this process [68] (see 1.3.5. Viral determinants of pathogenicity). Necrosis and apoptosis cause tissue damage and organ failure. However, in some cases, early induction of infected cells apoptosis by host immune signals can be beneficial to avoid a widespread infection [69]. Furthermore, the host immune mechanism to solve the infection can be, in some cases, detrimental. An optimal and early immune response can limit the viral spread, but an aberrant immune response can lead to immunopathologies [70], favouring viral replication [71]. HPAIV replication in chickens is associated with an exacerbated production of pro-inflammatory cytokines (cytokine storm) that cause capillary leakage and tissue damage [72]. In contrast, species such as ducks that are more tolerant to HPAIV can limit the duration of pro-inflammatory cytokine production [73]. Finally, vascular damage is induced due to the viral tropism of HPAIVs for endothelial cells [74]; this fact increases the vascular permeability, causes a thermoregulation impairment, increases the recruitment of pro-inflammatory cytokines, and causes coagulopathies. These alterations cause edema, haemorrhages, and micro-thrombosis, leading to ischemia and organ failures [75–78]. Moreover, this phenomenon is increased with the AIV conversion of plasminogen into plasmin which increases the inflammatory reaction via fibrinolysis and increases vascular permeability [79].

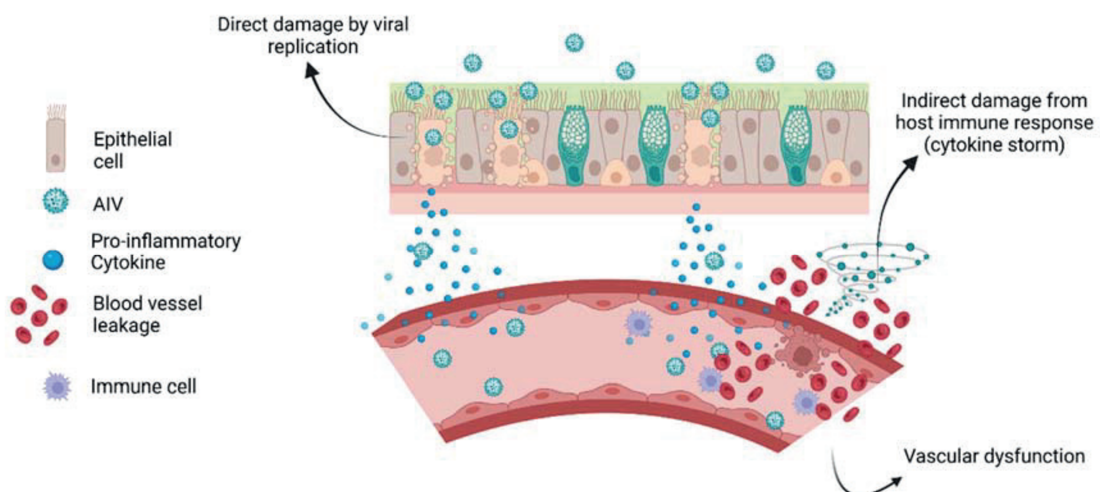

Figure 9. *Pathogenesis AIV is driven by three main mechanisms: direct viral replication, indirect damage due to host immune response, and vascular dysfunction.*

1.4.3. Immune response in avian species

Avian immune responses have unique features in contrast with mammals. The main difference is the maturation of B cells. In avian species, B cell maturation occurs at the bursa of Fabricius, a unique organ of birds. However, T cell maturation is produced, as in mammals, in the thymus. Both organs, together with the bone marrow, are the primary lymphoid organs. Birds do not have lymph nodes, that are replaced by secondary lymphoid organs such as the spleen, the Harderian gland, bronchial-associated lymphoid tissue (BALT), conjunctiva-associated lymphoid tissue (CALT), and gut-associated lymphoid tissue (GALT) that involves the caecal tonsils, the Peyer´s patches, and the Meckel´s diverticulum. Birds also lack neutrophils, but heterophils replace these last ones and are functionally equivalent. However, avian heterophils lacks myeloperoxidase and depends primarily on nonoxidative mechanisms for antimicrobial activity [80]. Furthermore, B cells from chickens only secrete IgM, IgA, and IgY (the functional equivalent of mammalian IgG) antibodies [81,82].

Once AIV infects a bird, the first hours are crucial to determine the disease outcome, highlighting the role of the innate immune response [83]. Innate immunity is the first line of defence of the host against viral agents such as AIV. After overcoming the host's physical barriers (e.g., mucociliary barrier), the viral pathogen-associated molecular patterns (PAMPs) will be recognised through specific pattern recognition receptors (PRRs) that are present in both immune and non-immune cells. Three PRR signalling pathways play essential roles after AIV infection: retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), toll-like receptors (TLRs), and nucleotide-binding oligomerisation domain (NOD)-like receptors (NLRs). The PRRs can be found on the cellular membrane or in the cell cytosol and recognise different compounds of AIV (i.e., double-stranded RNA, single-stranded RNA, and RNA with a 5´triphosphate overhang) [84,85]. RLRs are composed of three cytoplasmic receptors, the melanoma differentiation-associated protein 5 (MDA5), the laboratory of genetics and physiology 2 (LGP2), and the RIG-I; this last one is not present in chickens [86]. The recognition of AIV by RIG-I and MDA5 will activate the mitochondrial antiviral-signalling protein (MAVS) adaptor that will phosphorylate

the transcriptomic factors interferon regulatory factor (IRF) 7, IRF3, and the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which in turn will induce the production of proinflammatory cytokines and type I and III interferons (IFNs). TLRs are composed of TLR3 and TLR7; when they recognise AIV, they activate their adaptor molecules (TRIF and MYD88) that will also induce IRF7, IRF3, and NF-κB transcriptomic factors. The NLR family pyrin domain containing 3 (NLRP3) activates caspase-1 and induces pro-inflammatory cytokines after its activation [87] (Figure 10).

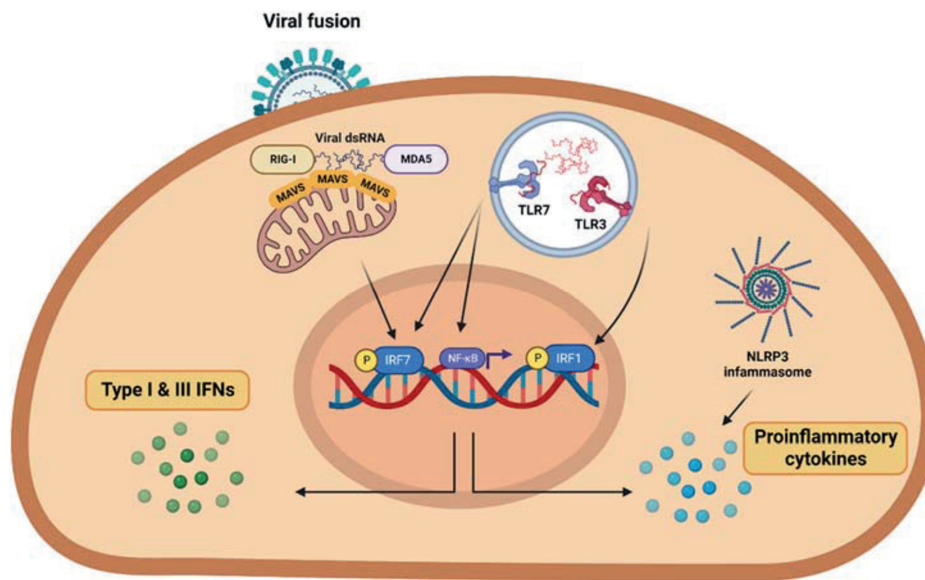

Figure 10. Innate immune response to AIV. PAMP and PRRs interaction led to the induction of proinflammatory cytokines and type I & III IFNs. Adapted from [87]

The production of IFN and proinflammatory cytokine leads to the induction of antiviral proteins and the recruitment of heterophils, macrophages, and dendritic cells at the site of infection. In the case of type I IFNs, they will activate an antiviral state through the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signalling, leading to the downstream expression of numerous interferon-stimulated genes (ISG). Among this ISG, there are Mx proteins that inhibit viral replication [88,89], the interferon-induced transmembrane (IFITM) that inhibits the fusion of virus-host cell membrane [90–92], the oligoadenylate synthase (OAS) family that degrades AIV RNA in the cytosol [93], the protein kinase R (PKR) that reduce the viral protein synthesis [94,95], and the interferon-induced protein with

tetratricopeptide repeat (IFIT) family proteins that sequester specific viral nucleic acids [96]. Type III IFNs can induce a similar antiviral state but using other receptors that are not expressed ubiquitously as type I IFN receptors, these receptors are expressed mainly in the airway epithelia and intestinal cells [97].

After a HPAIV infection, if an optimal and fast antiviral state is not induced, a dysregulation of the innate response can produce a cytokine storm that will increase the severity of the outcome disease. As explained before, the activation of PRRs will lead to the production of proinflammatory cytokines and ISGs. However, the virus has different mechanisms to inactivate the IFN-mediated effector functions (see 1.3.5. Viral determinants of pathogenicity). This phenomenon will increase viral replication, which in turn will induce an excessive pro-inflammatory cytokines expression (cytokine storm) without the production of ISGs, leading to tissue damage, multiple organ failure, and peracute death [83,98–101] (Figure 11). Furthermore, the activation of some immune pathways, such as NF-κB, will induce a host response necessary to combat the virus; however, if not correctly regulated, these pathways can become a double-edged arm that AIV can use to replicate (e.g., vRNP nuclear export) [71,102].

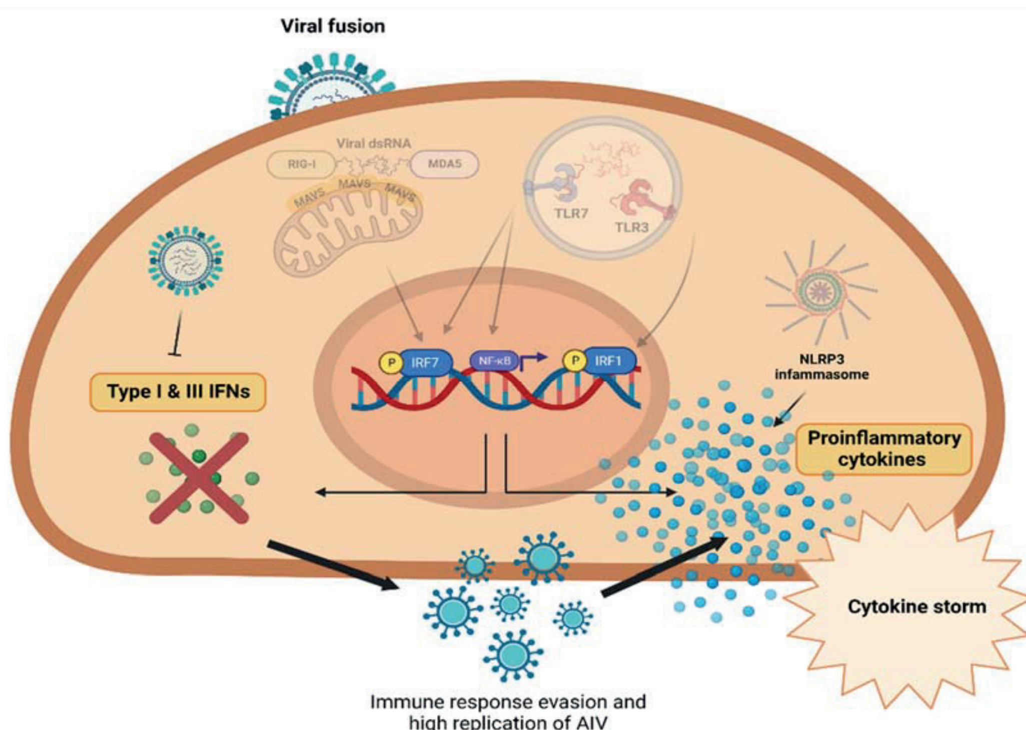

Figure 7. AIV inhibiting type I & III IFNs response enhances the production of proinflammatory cytokines that produce a deadly cytokine storm. Adapted from [87]

In naïve hosts infected by virulent AIV strains, adaptative immunity does not play an important role, as disease outcome is decided in the very first hours of infection. Moreover, considering that the systemic IgM response only appears after 5 days of infection, followed shortly by an IgY response. Unfortunately, little is known about the mucosal humoral immune response in birds against AIV [103,104]. Besides humoral response, cellular immunity can also protect against influenza A viruses in mammals, but little is known in birds [105–107]. Dendritic cells present the antigen to CD4 and CD8 cells, conferring protection in an antigen-specific manner. In AIV, it is thought that the effector response is primarily produced by cytotoxic T cells (CD8). However, this will be insufficient by itself to protect against HPAIV in naïve birds [103]. Nevertheless, the study of cellular response in poultry against AIV has long been neglected [108].

1.4.4. Host determinants of infection

The primary host determinant of infection with AIV is the species. Even if AIV has a widely distributed host range (Figure 8), disease susceptibility and outcome vary between species. It is commonly known that AIV infection is initiated by binding to host sialic acids (SAs) [109,110]. These host SAs are monosaccharides terminating the glycan chain of glycoproteins and glycolipids present at the cell membrane of epithelial cells. Original AIVs prefer SAs linked to galactose by α2.3 linkage [110,111]. In contrast, human-adapted strains of influenza A viruses prefer SAs linked to galactose by α2.6 linkage [112,113]. The distribution of SAs in different species is a clear determinant of infection (Figure 12). For example, humans have α2.3 SAs restricted to epithelial cells from the lower respiratory tract [114]; this restricts AIV infections in humans in the lower respiratory tract and difficult viral excretion. However, due to the flexibility of RNA viruses, some AIVs have been able to adapt to mammal species and transmit independently in mammal species (i.e., horse influenza, pig influenza, dog influenza, and human influenza lineages). Pigs express α2.3 SAs and α2.6 SAs in the respiratory tract, allowing this species to act as mixing vessels where AIV and human-adapted viruses could co-exist in a host and reassemble to become a new pandemic strain [13]. However, data on SA distribution in other species suggest that this role in pigs can be overestimated [115,116].

Furthermore, even if the dichotomy between α2.3 SAs and α2.6 SAs as host determinants has been broadly accepted, AIV can use low-affinity SAs to increase its binding affinity in tissues with low presence of high-affinity SAs, a process known as hetero-multivalent interactions [117]. Finally, apart from SA binding, AIV has alternative ways to enter the host cell that can explain viral infection of immune cells during HPAIV infections [118–120]. Other host factors, such as the presence/absence of RIG-I and different transcriptomic regulation of IFITM genes between different species, have been related to different outcomes between species [73,92,121].

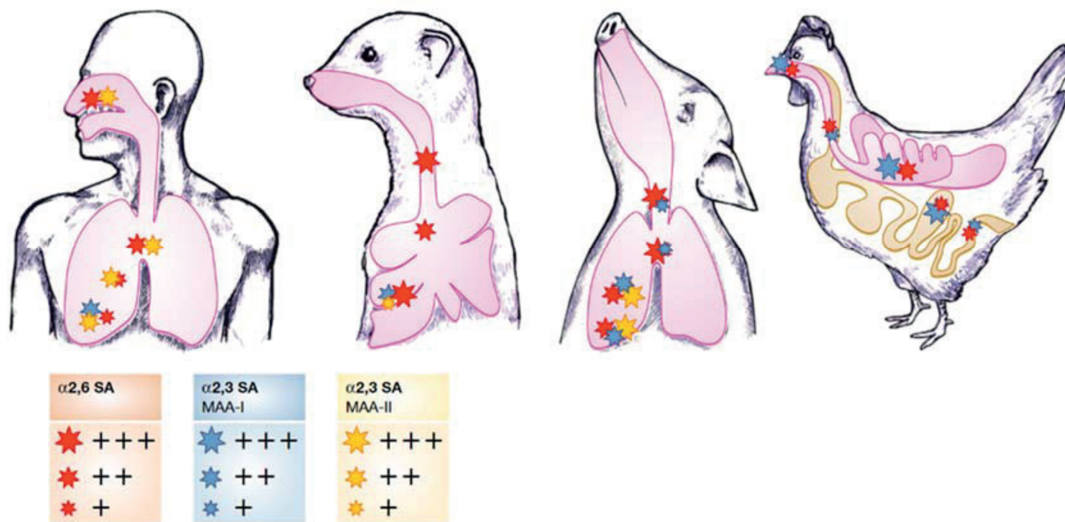

Figure 82. Sialic acids distribution in humans, ferrets, pigs, and chickens. Obtained from [109] .

Another important host factor is age. Even if differences have been reported between species, it seems a common fact that younger hosts are more susceptible to AIV than older ones [122–124]. Besides age, sex is another factor that plays a relevant role in other diseases. However, in LPAIV, the gender of hosts does not seem to play an important role, and in HPAIV, there is not enough data for potential correlations [62].

Within the same species, there are also differences in susceptibility related to different breeds, as in chickens [125]. It was suggested that local breeds, less selected for high production efficiency, were more resilient to viral infections than

commercial breeds [126]. However, a recent study showed that following experimental HPAIV infection, not all local breeds behave in the same way and only specific local breeds are more resistant to HPAIV than commercial breeds [67].

It seems that even if breed and species play a role, individual host factors are also important. Polymorphism of the chicken Mx gene was proposed to be related to different disease outcomes [127], but recent studies failed to demonstrate such a correlation [67,128,129]. A genome-wide association study analysed genomes of different birds that died or survived to AIVs and showed SNPs in genes related to the immune system, probably associated with the differential outcome [130,131]. Very recently, it was also suggested that there is a relationship between gut microbiota, chicken immunity, and AIV, opening the door to a new field that can explain individual outcome differences to AIV [132].

1.5. The environment

1.5.1. Viral transmission

Birds infected with LPAIV are known to excrete the virus from the digestive tract into the environment through the faeces. However, LPAIV can also replicate in the respiratory tract of infected birds. Therefore, oral excretion is also possible. In contrast, birds infected with a HPAIV excrete the virus mainly from the respiratory tract and in lower amounts from the digestive tract [62]. Nevertheless, as HPAIV can cause a systemic infection that affects many host tissues, the virus can also be present in the skin (i.e., feathers, feather follicles, and glands such as the uropygial gland) and contaminate the environment through these tissues [133,134].

Transmission of AIV can successfully occur in different ways and depending on the viral shedding route. LPAIV strains are usually transmitted indirectly through the faecal-oral route via contaminated water. This transmission is facilitated in aquatic environments, such as wetlands, where wild aquatic birds reside. Especially, dabbling ducks can be more susceptible to these viruses as they feed on surface waters, probably contaminated by faeces of other wild birds. Furthermore, a suggested alternative route could be through “cloacal drinking”, which will transmit

the virus by consuming contaminated water via the cloaca [62]. Transmission by direct contact cannot be discarded in LPAIV as oral excretion is possible. In the case of HPAIV, an increased oral excretion could favour a direct bird-to-bird transmission. However, indirect transmission of HPAIV could also be possible via air droplets or aerosols, faecal-oral route (such as in LPAIV), and the consumption of carcasses by predation [135]. This last indirect transmission is behind recent outbreaks in bald eagles and black vultures [136].

The first introduction of AIV into poultry flocks is commonly related to the transmission from other domestic and confined poultry, wild birds, pigs, or humans working closely with poultry. Furthermore, live poultry markets represent a risk for the introduction of AIV, as it allows live birds from different flocks to contact [137]. Nevertheless, the introduction of AIV in poultry farms with standard levels of biosecurity is also possible. It is suggested that airborne dissemination is possible between farms close to each other and near the ventilation points [138,139]. However, fomites (i.e., equipment, clothing, shoes, and transport vehicles) are probably the leading cause of dissemination between flocks [140,141]. Giving untreated water to poultry from ponds close to the farm is also a risk factor, as water can be contaminated with faeces from infected wild birds, and it is an entryway that was already reported in past outbreaks [142–144]. Indeed, wild birds can also transmit AIV through direct contact if domestic poultry and wild birds do not have any separation barriers [145,146]. Another entryway is the introduction of swine-origin influenza A viruses from fomites from pig farms or humans infected with these strains [145]. Once introduced, AIV can spread during an outbreak by secondary dissemination via fomite transmission, direct contact, and/or airborne dissemination. For secondary dissemination of conventional HPAIV, once LPAIV has evolved into HPAIV, wild birds do not play a relevant role [147,148]. Nevertheless, this concept changed entirely with the appearance of H5 Gs/GD lineage HPAIV that can be transmitted from poultry to wild birds and spread worldwide through international flyways [149–152] (Figure 13).

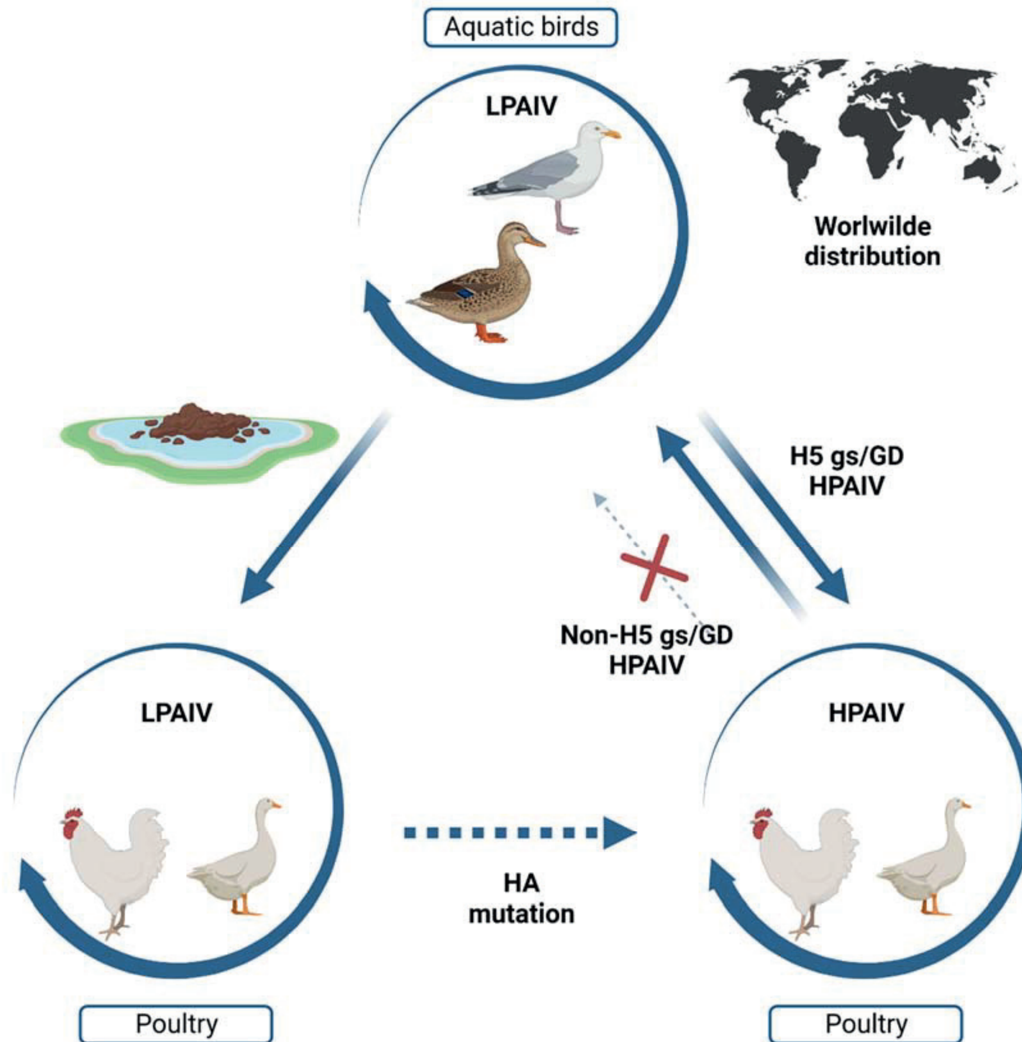

Figure 13. Poultry and wild birds in the AIV interface. Adapted from reference [45]

1.5.2. Environmental persistence

Overall, it seems clear that the environment plays a relevant role in AIV transmission. However, the question is if there is a bird-density independent transmission -without the need for the presence of infected wild birds-, or a bird-density dependent one, that requires the presence of infected wild birds. A density-independent transmission could be possible with AIV persistence in the environment after the infectious birds have left, making the infection of non-infectious birds possible. Mathematical transmission models seem to favour density-independent transmission, highlighting the role of environmental

persistence in AIV dynamics [153–155]. Furthermore, recent studies of AIV persistence in water under environmental conditions from northern breeding areas appear to support the interannual persistence of infectious AIV, a fact that could help the maintenance of AIV throughout the year in those regions when migratory birds left [15,156].

Different environmental factors can affect the viral persistence of AIV. If the virus is orally excreted in the air, relative humidity can play a role in AIV persistence [157]. If it is discharged into the water from the cloaca, persistence is influenced by many other factors. Previous experimental works showed that LPAIV remains infective for longer periods at cold temperatures (below 17^{0}C), neutral pH (7.2-8.4), low-moderate saline waters, filtered water, and without freezing-thaw cycles (see Table 2).

Although contrasting results have been found, some eye-visible organisms seem to filter and concentrate infectious viruses in their bodies [158,159], while others seem also to filter and concentrate viruses, but inactivate them [160,161]. If the virus is present in faeces, sediments, carcasses, or feathers, it can persist longer periods, suggesting a protective effect on these environments [162,163]. However, even if AIV is protected in sediments, transmission to new birds is not clear [164].

The strain of the virus is also an essential factor [165,166], as HPAIV strains had lower persistence times in water than LPAIV strains [167], even if the same factors (e.g., temperature and pH) affect them similarly [168].

It is worth mentioning that most of the experiments performed till now are far from reproducing the real conditions of aquatic habitats where wild birds live. Essential factors, such as diurnal temperature fluctuations, inactivation by sunlight, and the effects of an intact microbiome community in AIV water persistence have been pointed out as neglected factors that deserve to be analysed in experimental conditions [169,170].

Table 2. Environmental factors that increase the persistence time of LPAIV in water.

Environmental factor protecting infectious AIV	Reference
Low temperatures	[166,169,171–185]
Neutral pH	[172,174–176,179,180,182]
Low-moderate salinity	[167,172,174,176,181,184]
Filtered water	[174,177,182,184–186]
Absence of freeze-thaw cycles	[178,180,187]

1.6. Control

In order to control AIV outbreaks, different interventions are needed to: prevent the introduction of AIV, reduce transmission and virulence once introduced, and eradicate the virus. To accomplish these objectives, the current interventions are: biosecurity in poultry farms, active and passive surveillance, decreasing host susceptibility, eliminating infectious birds, and risk communication [66].

To avoid the entrance of new AIVs into a country, import control is needed, especially in live poultry. Applying the OIE Terrestrial Code (World Organisation for Animal Health - OIE -, 2010) is important. However, the transmission of AIV by migratory wild birds makes this effort every so often useless. Thus, the implementation of high biosecurity measures is highly recommended, to avoid contact with wild birds or with infectious fomites [66].

Active and passive surveillance is another important pillar for the early detection of infected wild birds, prior to their AIV transmission into poultry farms and as an early warning system. Active surveillance of wild birds requires the capture of animals, which sometimes is not easy or can be harmful to them. An alternative strategy is the use of environmental samples, the most common ones are faeces, but water, air, and sediments have also been used [189].

After poultry is infected, interventions are needed to avoid secondary transmissions to other farms, especially in the case of HPAIV. Following the OIE Terrestrial Code (World Organisation for Animal Health- OIE -, 2010), the infected farm will have to

cull all its birds (stamping out) and disinfect all farm facilities. A protection zone of 3 km radius will be implemented, where bird movement will be banned, plus a surveillance zone of 10 km, where reinforced eradication and control measures will be applied [190,191].

Once AIV is introduced in the farm, preventive measures to increase poultry resistance help to avoid, or reduce, viral infection and transmission. Vaccination of birds can be helpful to reduce host susceptibility; however, suboptimal vaccination can increase the mutation rate of some viral strains [192–194]. Furthermore, vaccination can allow the silent spread of HPAIVs due to vaccinated chickens that do not show evident clinical signs, but can transmit the disease; for that reason, some countries ban the exportation of vaccinated chickens [195,196]. Vaccination is a useful tool in AIV-endemic countries, but in non-endemic ones is still banned to avoid these negative aspects.

A future perspective is the genetic modification of poultry birds to generate resistant AIV breeds. Transgenic chickens expressing short-hairpin RNA that inhibits RdRp resulted in a reduction in AIV transmission; however, did not completely prevent infection and death [197]. Another study showed that cells genetically modified to inactivate a chicken protein (ANP32) - that supports AIV polymerase activity -, were resilient to the infection [198]. Therefore, even if resistant birds are a promising alternative, there is still a long way to go.

Finally, an efficient application of risk communication, and adequate economical compensations to poultry producers, are key to involve everyone in the fight against the disease.

Chapter II

Hypothesis and objectives

For a pathogen to successfully infect a host, needs to overcome its physical and immunological barriers and once excreted, survive enough time in the environment until reaches another host. Regarding AIVs, little is known about the host factors related to disease outcomes, and what we know is mainly related to age, species, and breed differences. However, individual variability has been observed among chickens of the same breed, suggesting the existence of individual factors related to AIV infection. We hypothesize that some chickens can stop viral replication, by optimally regulating the innate immune response, whereas others succumb to the infection due to an ineffective response. On the viral side, mutations in specific regions of the AIV genome have been associated with more virulent strains. However, chickens infected with the same strain showed different outcomes. In some cases, as AIV strains are not composed of identical copies but mutant clouds, they could defeat the host barriers by positive selection of low-frequency variants, able to overcome these barriers; in contrast, an efficient host response would prevent this process. The environment also plays a relevant role in the infection process of AIVs. It is known that the faecal-oral route is a relevant mode of viral transmission, especially, in waterfowl infected after the consumption of water contaminated with the faeces of AIV-infected birds. Thus, the study of AIV persistence in water is important to assess the risk of indirect transmission in this way, especially if we consider that waterfowl could transmit the disease worldwide by their flyways. Many previous studies have already evaluated this matter, but in laboratory-based conditions, that might be far from a realistic experimental set-up. We hypothesize that more realistic models will unravel more truthful persistence periods. Therefore, the general objective of this thesis will be to study **the AIV interaction with host internal factors and external factors that determine AIV dynamics using a multidisciplinary approach.** The specific objectives of this thesis are:

- To unravel the genetic basis of chicken resistance or susceptibility to HPAIV infection, as well as the impact of intra-host low-frequency viral quasi-species variants on the clinical outcome.
- To assess the external factors that determine LPAIV persistence in water by using artificial streams that mimic the environmental conditions of waterfowl habitats.

Chapter III

Study 1

Dual host and pathogen RNA-Seq analysis unravels chicken genes potentially involved in resistance to highly pathogenic avian influenza virus infection

Albert Perlas[1,2*], Jordi Argilaguet[1], Kateri Bertran[1], Raúl Sánchez-González[1,2], Miquel Nofrarías[1], Rosa M. Valle[1], Antonio Ramis[1,2], Martí Cortey[2 †], Natàlia Majó[1,2 †]

[1] IRTA, Centre de Recerca en Sanitat Animal (CReSA, IRTA-UAB), Campus de la Universitat Autònoma de Barcelona (UAB), Bellaterra, Spain

[2] Departament de Sanitat i Anatomia Animals, Universitat Autònoma de Barcelona, Campus de la Universitat Autònoma de Barcelona (UAB), Bellaterra, Spain

† These authors have contributed equally to this work and share last authorship

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3.1. Abstract

Highly pathogenic avian influenza viruses (HPAIVs) cause severe systemic disease and high mortality rates in chickens, leading to a huge economic impact in the poultry sector. However, some chickens are resistant to the disease. This study aimed at evaluating the mechanisms behind HPAIV disease resistance. Chickens of different breeds were challenged with H7N1 HPAIV or clade 2.3.4.4b H5N8 HPAIV, euthanized at 3 days post-inoculation (dpi), and classified as resistant or susceptible depending on the following criteria: chickens that presented i) clinical signs, ii) histopathological lesions, and iii) presence of HPAIV antigen in tissues were classified as susceptible, while chickens lacking all these criteria were classified as resistant. Once classified, we performed RNA-Seq from lung and spleen samples in order to compare the transcriptomic signatures between resistant and susceptible chickens. We identified minor transcriptomic changes in resistant chickens in contrast with huge alterations observed in susceptible chickens. Interestingly, six differentially expressed genes were downregulated in resistant birds and upregulated in susceptible birds. Some of these genes belong to the NF-kappa B and/or mitogen-activated protein kinase signaling pathways. Among these six genes, the serine protease-encoding gene *PLAU* was of particular interest, being the most significantly downregulated gene in resistant chickens. Expression levels of this protease were further validated by RT-qPCR in a larger number of experimentally infected chickens. Furthermore, HPAIV quasi-species populations were constructed using 3 dpi oral swabs. No substantial changes were found in the viral segments that interact with the innate immune response and with the host cell receptors, reinforcing the role of the immune system of the host in the clinical outcome. Altogether, our results suggest that an early inactivation of important host genes could prevent an exaggerated immune response and/or viral replication, conferring resistance to HPAIV in chickens.

3.2. Introduction

Avian influenza viruses (AIVs) are -ss RNA viruses from the family Orthomyxoviridae that can infect different avian species. They are divided into two pathotypes based on their virulence in chickens. Low pathogenic AIVs (LPAIVs) can be asymptomatic, but they typically cause mild to moderate respiratory disease, often accompanied by a decrease of water or feed consumption and drops in egg production. In contrast, highly pathogenic AIVs (HPAIVs) typically cause severe systemic disease with very high mortality in chickens [32]. Due to the high mortality rates, HPAIVs represent a big economic problem to the poultry sector. Besides, AIVs are also a threat to public health, since some viral strains, e.g., viruses of the Goose/Guangdong (Gs/GD) H5 lineage, can cause human infections with more than 50% case fatality rate [65,199,200]. The clade 2.3.4.4b H5N8 HPAIV of the Gs/GD lineage circulating in poultry and wild birds in Europe in recent years has also demonstrated its capacity to infect humans, albeit with mild clinical signs, which confirms that HPAIVs are a constant threat to public health [201,202]. The ability of an AIV to spill over to other species is linked to the evolutionary plasticity of RNA viruses that allows them to adapt to a new host faster than any other pathogen, and is often invoked to explain their zoonotic potential [10]. AIVs have a segmented genome comprised of eight segments that encode 10 to 17 proteins depending on the viral strain [37]. It is well known that these viruses exist in the host as quasi-species. The term viral quasi-species describes distributions of non-identical but related genomes in a cloud or swarm subjected to variation, competition and selection thought to be the target of evolutionary events [11]. This is one of the reasons why RNA viruses are currently one of the highest global public health concerns [203,204].

HPAIVs typically cause severe systemic disease, with the virus reaching different visceral organs within 24 h post-intranasal exposure and titers peaking at 48 h [70]. Thus, the first hours following HPAIV infection are critical to determine survival or death, indicating the key role of the innate immune response in determining clinical outcomes and resistant phenotypes [205]. Following AIV infection, host pattern recognition receptors (PRRs) recognize specific parts of the virus named pathogen-associated molecular patterns (PAMPs) to activate the innate defense. One

important group of PRRs are retinoic acid-inducible gene I (RIG-I) like receptors (RLRs), composed in chickens of two cytoplasmic receptors, melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2), as highlighted in chickens by a recent RNA-Seq analysis [86]. After viral recognition by the PRRs, some pathways are activated to induce the expression of type I interferon (IFN-I), which in turn enhance the expression of interferon stimulated genes (ISGs) and pro-inflammatory cytokines [73,87]. Several viral proteins are involved in the viral evasion of the innate immune response: the nucleoprotein (NP) and the matrix (M)-2 protein (M2) protein inhibit the activity of the IFN-I-dependent double-stranded RNA-activated protein kinase (PKR), which inhibits viral replication; while the non-structural (NS1) protein interferes with several innate immune signaling pathways [206]. Viral within-host compartmentalization has been largely analyzed in other RNA viruses, such as in the human immunodeficiency virus [207], but little is known about the within-host viral distribution in AIV infections. However, it has been suggested that stochastic bottlenecks associated with founder events and compartmentalization during AIV dissemination through the respiratory system, reduce viral diversity and limit the impact of positive selection on within-host evolution [208–210].

The severity of the clinical outcome after HPAIV infection varies based on the virus strain and the host genetic background [211]. Several authors have reported a different susceptibility to AIV infection depending on the chicken breed [125]. Moreover, some individuals may respond differently to the infection even within the same chicken breed [67]. Concerning the virus, certain AIV strains (or variants within quasi-species) are able to evade the host immune response more efficiently than others, with key amino acid mutations identified in the hemagglutinin (HA) protein [212–214]. Regarding the host, polymorphisms in the *MX* gene, an ISG gene that inhibits AIV transcription and replication in mammals, have been suggested to correlate with different infection outcomes [127]. However, several studies failed to demonstrate such correlation in vivo [67,128,129]. An RNA sequencing (RNA-Seq) analysis between resistant and susceptible chicken breeds suggested that genes related to hemoglobin, oxygen transportation and cell adhesion played a critical role in protecting against AIV infection [215]. Furthermore, genome wide association studies comparing birds that either survived or succumbed to AI outbreaks showed

single nucleotide polymorphisms (SNP) in genes associated with the immune response, such as *ZNF639*, *BCL6*, or *MAPK1* [130,131]. Lack of the viral sensor RIG-I in chickens, as opposed to ducks (typically much less susceptible to HPAIV), has also been proposed as an explanation for their increased susceptibility to HPAIVs compared to ducks [73,121]. Similarly, the IFN-induced transmembrane protein (*IFITM*) gene family has been shown to be strongly upregulated in response to HPAIV in ducks compared to chickens [92]. Taken together, the mechanism behind clinical outcome after HPAIV infection remains uncertain and calls for further investigation.

The outcome of HPAIV infection is a complex and multifactorial process where both the host innate immune response and the virus play an important role. The last years RNA-Seq have proved to be a powerful tool to characterize these complex processes in chickens against several pathogens [216–218]. The aim of the present study was to characterize the early host and viral factors that determine the individual clinical outcome of HPAIV infected chickens. With this goal, we investigated differences in gene expression in lung and spleen in chickens that are susceptible or resistant to a HPAIV-experimentally lethal infection. Furthermore, viral quasi-species distribution was also analyzed to further understand its early contribution to infection outcome and to the viral compartmentalization within the host.

3.3. Materials and methods

3.3.1. Experimental design

Bird samples were obtained from a previous experiment conducted by our group [67]. Briefly, 15-day-old chickens (*Gallus gallus*) of six different local breeds from Spain Empordanesa (EMP), Penedesenca (PENED), Catalana del Prat (C. PRAT), Flor d’Ametller (F. AMET), Castellana negra (C. NEGRA), and Euskal oiloa (E. OILOA), a commercial breed (Ross 308 Broiler), and a commercial-experimental line (specific pathogen free (SPF) White Leghorns) were used. The chickens included in this study did not receive any vaccination. Chickens were intranasally inoculated with 10^5 mean embryo lethal dose (ELD_{50}; reciprocal of that dilution of virus per unit volume

that results in the death of 50 percent of inoculated embryos) of either A/Chicken/Italy/5093/1999 (H7N1 HPAIV, Genbank accession numbers MK494920 to MK494927) or A/Goose/Spain/IA17CR02699/2017 (clade 2.3.4.4b H5N8 HPAIV, Genbank accession numbers DQ991325 to DQ991332), to assess the infection dynamics of each virus in the different breeds and to compare a recent H5N8 HPAIV isolated in Spain (Gs/GD lineage, clade 2.3.4.4b) with a classical H7N1 HPAIV. Based on our previous results, H7N1 HPAIV was more virulent to chickens than H5N8 HPAIV [67] . Three chickens from each breed, including uninfected control chickens, were euthanized at 3 days post-inoculation (dpi), and a complete necropsy and tissue sampling were performed. A full set of tissues in formalin were processed for routine histopathology and immunohistochemistry (IHC) targeting the AIV NP antigen. In the present study we used lung and spleen samples collected at 3 dpi and stored at -80 ºC, as well as 3 dpi oral swabs.

3.3.2. Classification of chickens as resistant and susceptible

In order to study expression differences relating to different host susceptibility, inoculated chickens were classified as susceptible or resistant. Chickens susceptible to infection were typified by presence of (i) evident clinical signs such as prostration, apathy and/or tremors; (ii) histopathological lesions such as hemorrhages, tissue necrosis and/or inflammation; and (iii) NP antigen in tissues associated to histopathological lesions. In contrast, chickens lacking clinical signs, histological lesions, and NP antigen in tissues at 3 dpi were classified as resistant. The breed factor was not considered for this classification since the objective was to characterize individual (not breed) factors to HPAIV resistance, as discussed later. Lung and spleen tissue samples collected at 3 dpi were used for the host transcriptome analysis and quantitative PCR validation, while 3 dpi oral swabs were used for viral quasi-species characterization (Sup. Table 1a, 1b, 1c, and 1d). Additionally, 3 dpi oral swabs from chickens that were not necropsied were also used for the viral quasi-species analysis. In these cases, instead of the previous criteria, chickens were classified as resistant if they survived until the end of the experiment (10 dpi) with no clinical signs. In contrast, chickens that died before the end of the experiment with HPAIV compatible signs were classified as susceptible.

3.3.3. Host transcriptome analysis (RNA-Seq)

RNA preparation

Lung and spleen samples collected at 3 dpi from chickens from each group (resistant, susceptible, and control) were used. The RNA was extracted using RNeasy RNA isolation kit (Qiagen, Madrid, Spain) following manufacturer's guidelines. The RNA was eluted in RNase free water and treated with a DNA-free kit (Ambion, Madrid, Spain). RNA quality and concentration were checked at Microomics Systems, S.L. (Barcelona, Spain) using a bioanalyzer (Agilent, Barcelona, Spain). At least three samples from lungs and spleens from control chickens and resistant and susceptible H5N8-inoculated chickens with an RNA integrity number (RIN) above eight were selected for subsequent transcriptomic analysis. However, samples from resistant H7N1-inoculated chickens showed low RNA quality and were discarded for further analysis.

Library preparation and sequencing

This study initially included 36 samples from 18 chickens. The library preparation and sequencing were performed by Microomics Systems, S.L. (Barcelona, Spain). Briefly, polyadenylated mRNA molecules were purified using poly-T oligo attached magnetic beads. Following purification, the mRNA was fragmented into small pieces. The protocol involved size selection and cDNA synthesis with random primers following the Illumina TruSeq Stranded mRNA Reference Guide [219]. After library preparation, sequencing of multiplexed samples was performed using Illumina Hiseq 2500 sequencer (Illumina, San Diego, CA) and 2x125 base-pair (bp) sequences were obtained with a sequencing depth by sample ranging from 20M to 37M (Sup. Figure 1) .

Bioinformatics analysis

Reads were filtered by quality control with FastQC v0.11.82 [220], then clean data were obtained by trimming adapters and nucleotides with a Phred score (a quality measure to assess the accuracy of a sequencing) of less than 30 at the beginning and end of the reads with Trimmomatic v0.39 [221]. Filtered reads were mapped to the reference genome with Hisat2 v2.1.0 [222] standard settings using the galGal6 chicken assembly, and the quality control alignment was done with qualimap v.2.2.2

[223]. FeatureCounts v1.6.4 [224] was used to generate table counts of each sample. Differentially expressed genes (DEGs) in samples from infected chickens compared to control chickens were normalized and obtained using the standard normalized methods performed by DESeq2 v1.24.0 [225]. A principal component analysis plot of each comparison was done to detect and eliminate outliers [226]. After this step, samples from one control chicken (lung and spleen), one H5N8 resistant chicken (lung and spleen), and one H7N1-susceptible chicken (lung) were discarded (Sup. Figure 2). Finally, a total of 31 samples were used for further analysis. The number of samples per group analyzed were as follows: three lung samples and three spleen samples from chickens resistant to H5N8 infection; four lung samples and three spleen samples from chickens susceptible to H5N8 infection; three lung samples and four spleen samples from chickens susceptible to H7N1 infection; and five lung samples and five spleen samples from control chickens. DEGs in samples from infected chickens compared to control chickens were obtained with a Wald test and the p-value was adjusted with a Benjamini and Hochberg method (BH-adjusted p values) using DESeq2 v1.24.0 [225]. DEGs with a BH-adjusted p value < 0.05 were considered significant. Heatmaps were constructed in R v4.0.5 [227] using the pheatmap package (RRID:SCR_016418). Venn diagrams were produced using the online tool Venny (https://bioinfogp.cnb.csic.es/tools/venny/index.html, accessed on 30 November 2021) To measure the viral transcript counts in each sample, the clean RNA sequencing reads were also mapped to the genome of the corresponding HPAIV strain with FeatureCounts v1.6.4 [224].

Enrichment analysis

Gene Ontology (GO) enrichment analysis of DEGs was performed with DAVID v6.8 [228]. Most representative GO terms with a Fischer's exact test $P<0.05$ after False Discovery Rate (FDR) correction were identified using REVIGO [229]. A stringent dispensability cutoff (<0.05) was used, as previously described [230]. KEGG pathways databases [231] were also used for a DAVID v6.8 enrichment analysis. To further elucidate if DEGs were potentially regulated by IFN-I, an enrichment analysis using Interferome v2.1 database [232] was performed.

Validation by quantitative PCR

To validate the results obtained by RNA-Seq, a quantitative polymerase chain reaction (qPCR) was performed for some genes of interest. RT-qPCR was performed in 18 lung samples: six samples from control chickens, and four and eight 3 dpi samples from chickens resistant and susceptible to HPAIV infection, respectively. In order to determine whether *PLAU* could be upregulated or downregulated in all the breeds used in the resistant group, at least one individual of each breed was present in the susceptible group in our qPCR validation. RNA was extracted as described above. All samples were adjusted with RNAse free water to 5 ng of RNA. A Power SYBR® Green RNA-to-CT™ 1-Step Kit (ThermoFischer) was used on 7500 Fast Real-Time PCR System (ThermoFisher) to quantify gene expression levels. Primers were validated and sent by BioRad (Madrid, Spain). The housekeeping genes *ACTB*, *RPL12* and *YWHAZ* were used to normalize the results as previously described [233,234] (Sup. Table 2).

3.3.4. Viral quasi-species comparison

Sample selection, egg passage validation and viral quasi-species construction

In our previous experiment, the viral titer present at 3 dpi in oral swabs was calculated by RT-qPCR [67]. The cycle threshold (Ct) values obtained (mean values around 30) suggested insufficient quantity of viral RNA to characterize the viral quasi-species using genome sequencing. Thus, in order to obtain an adequate viral titer, all 3 dpi oral swab samples were passaged in 10-day-old embryonated SPF chicken eggs by standard methods [235]. To establish whether the passage in eggs introduced any viral mutations and therefore a potential bias, two oral swab samples from each virus strain with high viral titers were directly deep-sequenced before and after a 48-72 h passage. Viral quasi-species from original viral stocks and from egg allantoic fluid were inferred as previously described [236]. Briefly, i) total RNA was extracted from allantoic fluid with TRIZOL LS (Ambion) and directly deep-sequenced without using primers; (ii) the genomic library was constructed for Illumina NGS using a commercial protocol and reagents (Thermo Fischer); (iii) MiSeq run of 2x250 bp was performed at Servei de Genòmica i Bioinformàtica (SGB, IBB, Barcelona); (iv) low quality reads (QC > 20) were trimmed using Trimmomatic

[221]; (v) quality reads were mapped against the corresponding HPAIV strain (H5N8 or H7N1) using the Burrows-Wheeler Aligner applying the BWA-MEM algorithm for long reads [237]; (vi) variants were annotated with SnpSift [238] to determine the frequency of each nucleotide at each position of the reference genome; and (vii) viral quasi-species were constructed in fasta format.

In addition to the egg passage validation, the quasi-species from the two original inocula and 12 oral swabs from susceptible birds (eight H5N8-inoculated birds and four H7N1-inoculated birds) were analyzed (Ct values after the passage in eggs were <23). However, even after the passage in eggs, viral titers from resistant birds were too low for quasi-species characterization (non-detectable virus).

Analysis of Molecular Variance (AMOVA)

To compare the viral quasi-species adaptation during the infection process, nucleotide changes between the original inocula and the 3 dpi oral swabs were identified. The average frequencies of each nucleotide per position and group (inoculum versus oral swabs for each HPAIV strain) were compared using AMOVA in Arlequin v.3.5.2.2 [239]. Positions presenting fixation index values between inoculum and oral swabs larger than Fct > 0.05 were considered differentially selected, and the nucleotide change was characterized as synonymous or non-synonymous at the codon level.

Within host viral compartmentalization

Furthermore, to analyze viral compartmentalization, next-generation sequencing (NGS) files from the host transcriptome analysis by RNA seq were also used for viral quasi-species construction following the above-mentioned protocol (2.4.1. section). Unfortunately, the amount of virus in H7N1 samples was not enough for viral quasi-species construction and the viral compartmentalization analysis had to be done using only H5N8 samples. The viral nucleotide diversity was then determined from all the samples (i.e., original inoculum, oral swab, lung, and spleen) with DnaSP 6 [240]. Additionally, the net nucleotide distance between samples from the same birds was also determined with DnaSP 6 from three susceptible birds infected with H5N8, since we had previously constructed the viral quasi-species from the oral swabs of these same birds (Sup. Table 1d).

3.4. Results

Minor transcriptomic changes in tissues from chickens that are resistant to HPAIV infection

Aiming to characterize the host determinants that confer resistance to HPAIV infection, we performed a transcriptomic analysis of lung and spleen tissues from resistant and susceptible chickens. Birds were infected with two different HPAIV strains (H7N1 and clade 2.3.4.4b H5N8) and samples were collected at 3 dpi, which is the earliest time point post-infection at which chickens can be categorized as resistant or susceptible by clinical signs, histopathological lesions, and IHC staining. Analysis of DEGs showed major transcriptomic changes in both lung and spleen samples from susceptible chickens compared to resistant ones (Figure 1). In particular, 5,887 and 5,436 DEGs from lungs, and 4,360 and 4,062 DEGs from spleens, were identified from H5N8 and H7N1 susceptible birds, respectively. More than 60% of DEGs were common in both susceptible groups in the two organs analyzed. However, more DEGs were seen in H5N8 inoculated birds than in H7N1 inoculated birds, and in lungs than in spleens. In clear contrast with susceptible chickens, only 39 and 20 DEGs were identified in lungs and spleens from resistant H5N8 inoculated birds, respectively (Figure 1). This marked differences validated our classification criteria, since resistant and susceptible samples clearly grouped differently in a PCA plot (Sup. Figure 3). Finally, as in susceptible birds, lungs of resistant birds showed more transcriptomic changes than spleens (Figure 1).

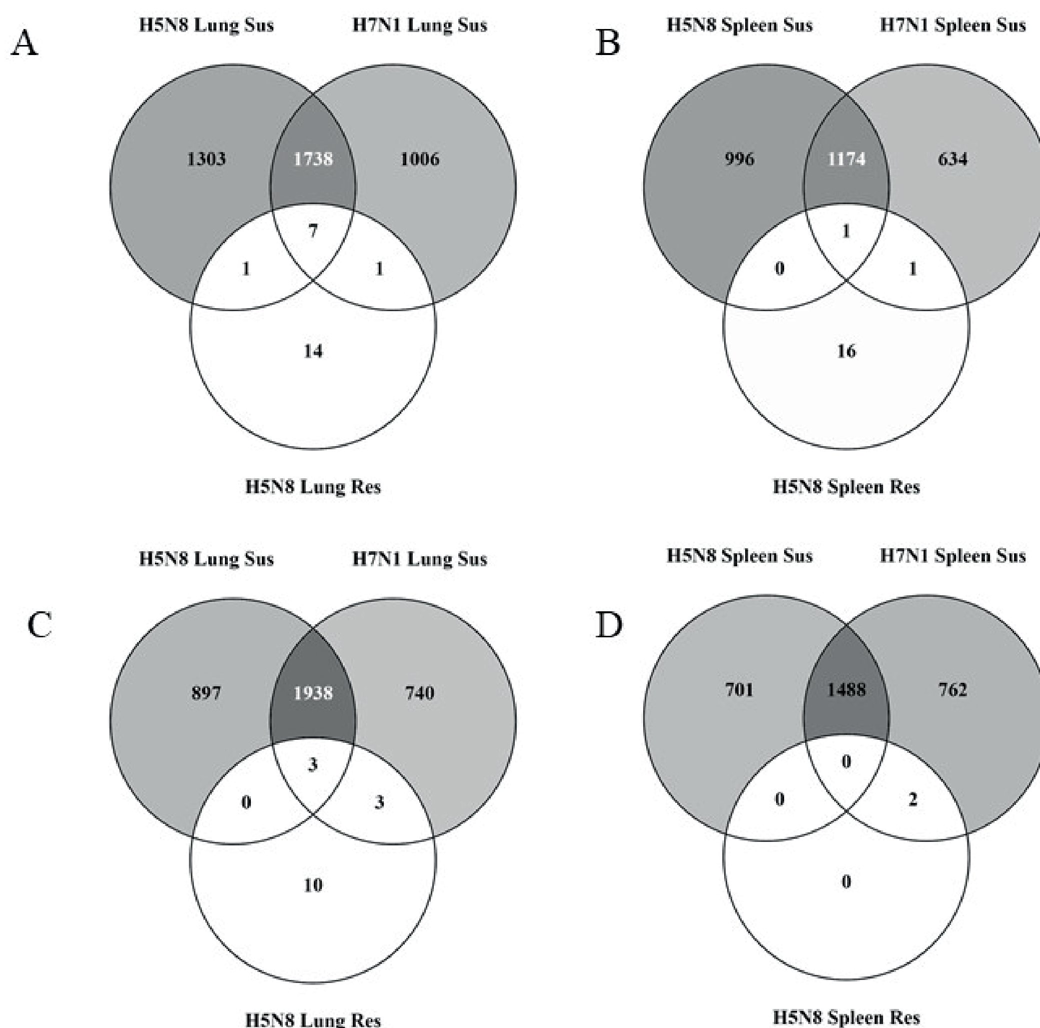


Figure 9. Venn diagrams illustrating overlapping of DEGs identified in lungs [upregulated (A) and downregulated (C)] and spleens [upregulated (B) and downregulated (D)] collected at 3 dpi from susceptible (Sus) and resistant (Res) H5N8 and H7N1 inoculated chickens.

Further characterization of transcriptomic changes from susceptible chickens by GO enrichment analysis showed an enrichment in processes related to immunological responses. Specifically, the most significantly upregulated GO biological processes were immune system process (GO:0002376) and defense response (GO:0006952) (Figure 2A). Downregulated GO biological processes were mainly related to homeostasis and growth (GO:0032502, GO:0071363) (Figure 2B). KEGG pathway analysis of susceptible chickens also showed enrichment in immune-related pathways such as cytokine-cytokine receptor interaction (Sup. Figure 4). In general, similar enriched processes were observed for both H5N8 and H7N1 susceptible

birds, but slight differences between lung and spleen were seen. More specifically, some pathways that were upregulated in lung, such as cell activation (GO:0001775), cytokine production (GO:0001816), immune system process (GO:0002376), and defense response (GO:0006952), were not differentially regulated or had a lower fold change in spleen. In contrast, enrichment analysis of DEGs from resistant birds did not generate any significant results.

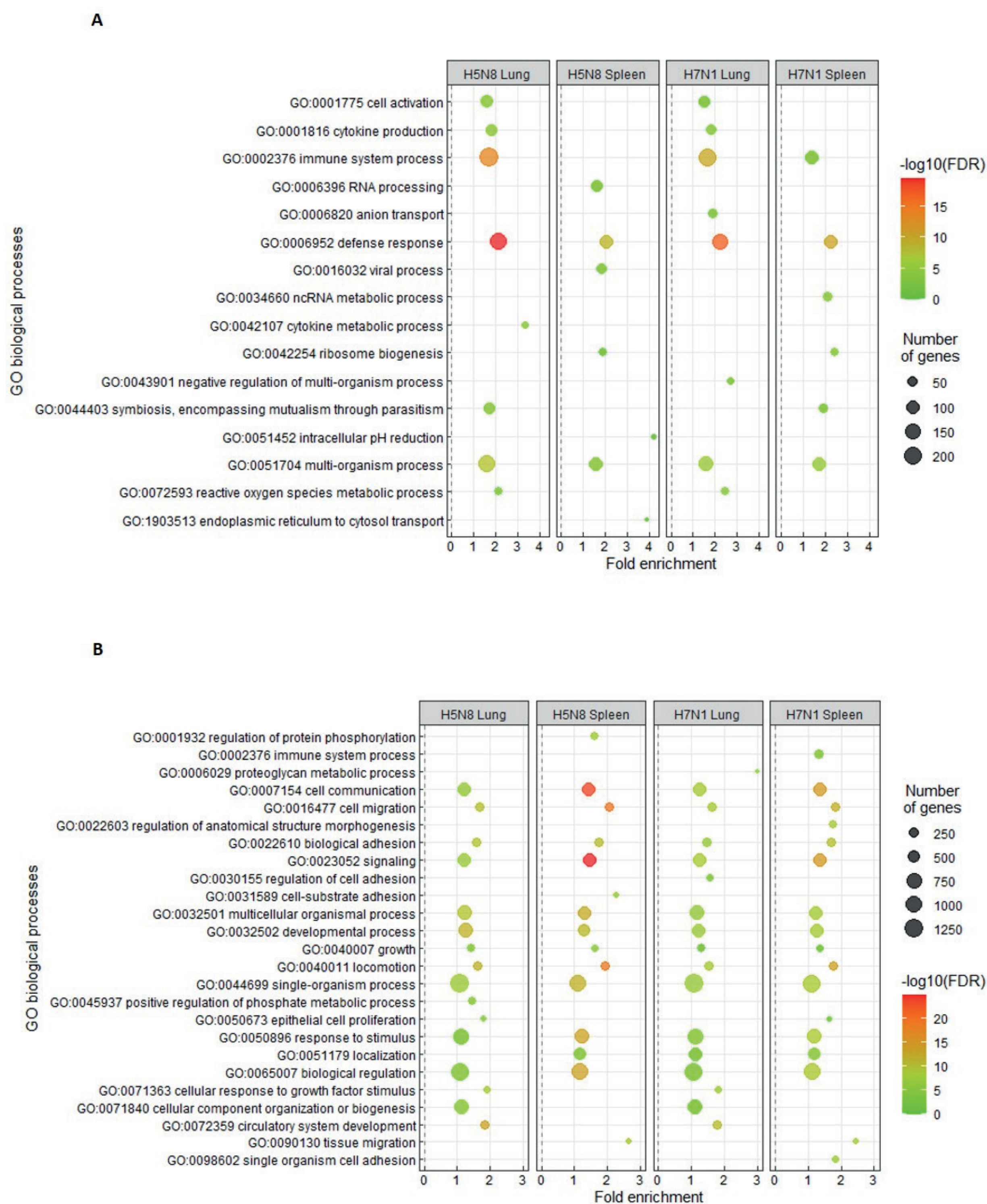


Figure 10. Enriched GO terms obtained from DAVID and REVIGO for upregulated (A) or downregulated (B) DEGs in lungs and spleens collected at 3 dpi from susceptible H5N8 and H7N1 inoculated chickens. The most representative and significant biological processes are represented and are sorted by fold enrichment. The dot size indicates the number of DEGs associated with the biological process. The dot color indicates the significance of the enrichment [-log10 (FDR-corrected P-values)].

To further define the transcriptional changes induced after infection, we compared the expression patterns of genes related to innate immune responses. PRR signaling as well as IFN-I and inflammatory responses were clearly upregulated in susceptible H5N8 and H7N1 inoculated birds (Figure 3). Besides, in line with the enrichment analysis results, susceptible H5N8 inoculated chickens had higher fold changes in these genes compared to susceptible H7N1 inoculated chickens. One exception was *IL8L1* and *IL8L2*, which showed higher expression levels in lungs and spleens of birds inoculated with the more virulent strain H7N1. Regarding differences between tissues, in line with the enrichment analysis results, lungs had higher fold changes in these genes than spleens. In contrast to susceptible birds, the expression pattern of important genes related to innate immune responses from resistant chickens lacked or presented very low differential regulation compared to the same genes from control chickens (Figure 3).

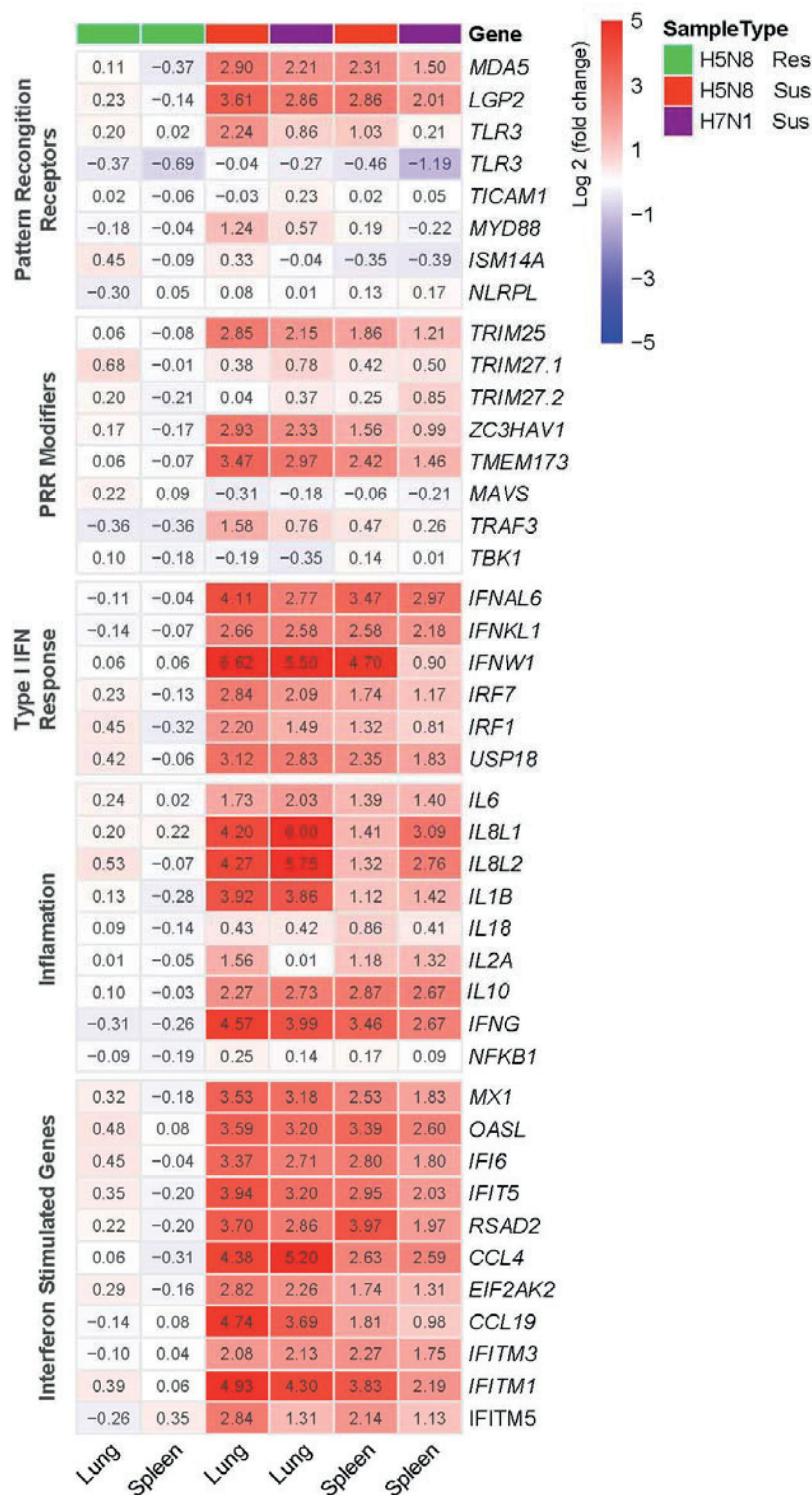
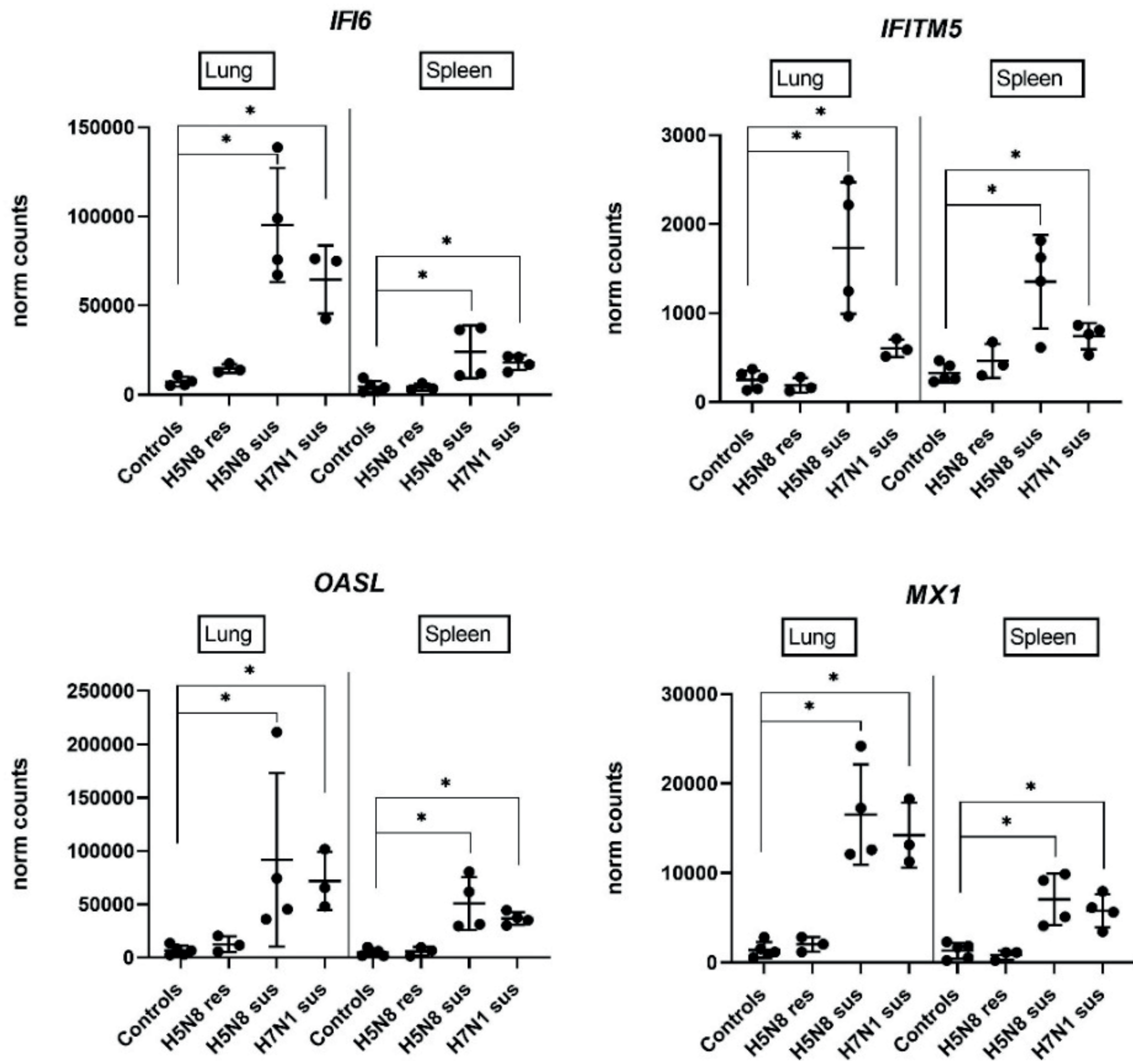


Figure 11. Heatmap illustrating the log2(fold change) in expression of genes representative of innate immune responses in lungs and spleens collected at 3 dpi from susceptible (Sus) and resistant (Res) H5N8 or H7N1 inoculated chickens. Columns were clustered using pheatmap (RRID : SCR_016418) in R.

A deeper statistical analysis of genes representative of IFN-I and inflammatory responses (*IFITM5, IFI6, MX1, OASL, IFNAL6, IFNW1, IL8L1, IL8L2*) confirmed significantly higher expression levels in H5N8 susceptible birds and in lungs, and very low differential regulation in resistant chickens compared to controls (Figure 4). Furthermore, we confirmed that *IL8L1* and *IL8L2* were the only genes showing higher expression levels in susceptible H7N1 inoculated birds (Figure 4).



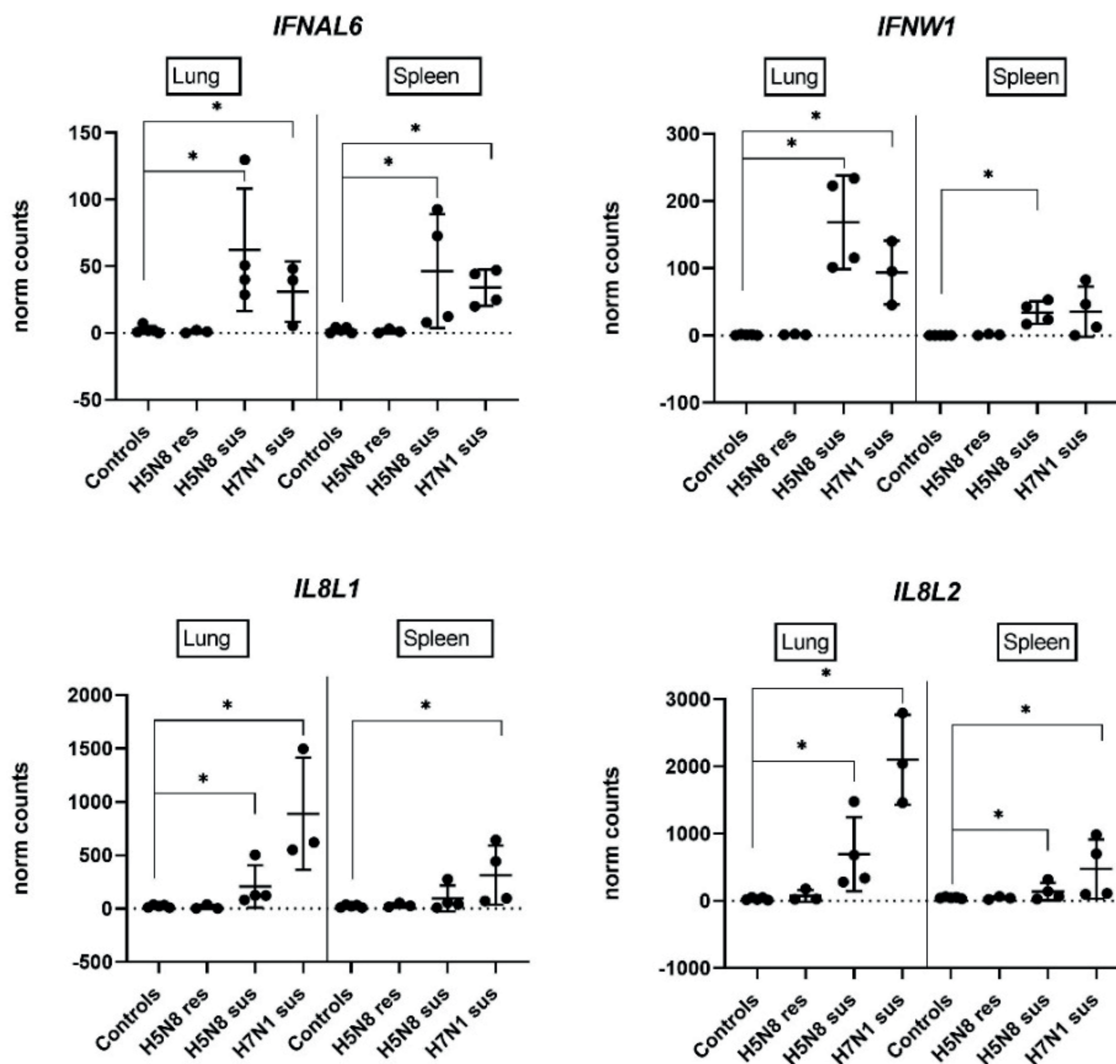


Figure 12. Expression levels of genes representative of IFN-I and inflammatory responses in lungs or spleens collected at 3 dpi from H5N8 or H7N1 inoculated chickens based on DEGs obtained by RNA-Seq. (∗) BH adjusted P ≤ 0.05 from Wald test p-value with DESeq2.

Low viral replication in chickens that are resistant to HPAIV infection

The viral transcripts from RNA-Seq data showed a clear association between the presence of virus and the number of DEGs. Higher levels of viral transcripts in lung tissues compared to spleen tissues were associated with more DEGs in lungs. Similarly, a positive correlation was also observed between viral transcripts and DEGs when comparing tissues from H5N8 and H7N1 inoculated chickens. Moreover, this analysis confirmed the lack of virus replication in lung and spleen tissues of

resistant birds, which was in line with the low number of DEGs in these organs (Sup. Figure 5). To further elucidate if the minor transcriptomic changes in resistant chickens were associated with lower viral replication and shedding by the oral route, we compared virus titers in 3 dpi oral swabs from resistant vs susceptible H5N8 inoculated chickens obtained in our previous experiment [67]. As expected, resistant chickens had significant higher viral Ct values (Ct mean value of 35.2) compared to susceptible chickens (Ct mean value of 22.5) (Sup. Figure 6).

To assess if an early innate immune response was induced in resistant chickens, an enrichment analysis using the Interferome v2.1 database [232] was performed. This analysis revealed that an important proportion of DEGs from lungs and spleens of H5N8 inoculated chickens are known to be regulated by IFN-I, in even higher percentages than the ones observed in susceptible birds (Figure 5).

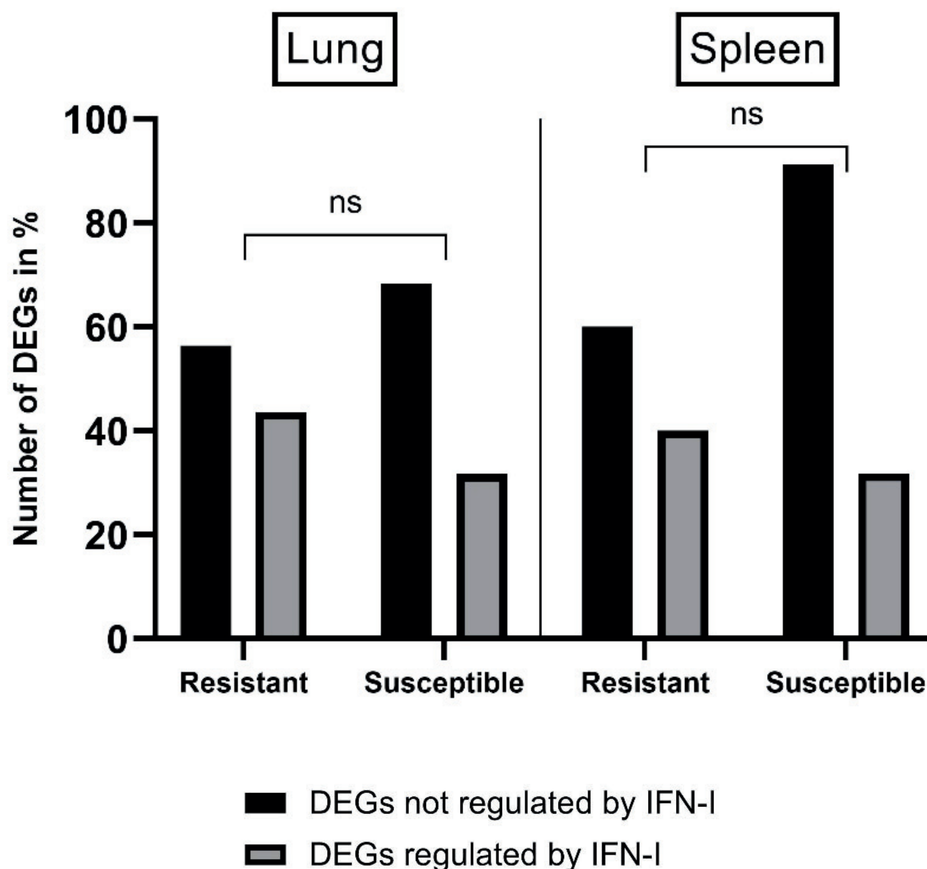


Figure 13. Percentages of DEGs identified in lungs and spleens from resistant and susceptible H5N8 inoculated chickens that are regulated or not by IFN-I based on results obtained from the database Interferome (v2.1). Non-significant differences in the

percentages of DEGs were seen between resistant and susceptible birds (ns) $P \geq 0.05$; 2-way ANOVA.

Downregulation of the serine protease encoding gene PLAU in lungs correlates with resistance to HPAIV infection

To further characterize the minor transcriptomic changes observed in resistant chickens, we grouped the 39 DEGs identified in lungs from resistant H5N8 inoculated birds, compared to control birds, by their expression patterns (Sup. Table 3). Most of the 39 genes were similarly upregulated or downregulated in both resistant and susceptible birds (Figure 6A). Among them, we found genes related to ion homeostasis (*SLC24A4, EPB42, RHAG*), immune system process (*BLOC1S6, FGF14, EPB42, ANKRD54, RHAG*), and response to stimulus (*DGKG, BLOC1S6, PRLHRL, FGF14, RRAD, ANKRD54*). Interestingly, six DEGs followed a different expression pattern, being downregulated only in resistant birds and upregulated in susceptible birds (Figures 6A and 7). Some of these genes belong to the NF-kappa B (NF-κB) (*PLAU, VCAM1, TNFRSF1A*) and/or mitogen-activated protein kinase (MAPK) (*TNFRSF1A* and *PGF*) signaling pathways [231], indicating a differential regulation of the inflammatory response in lungs for both infection outcomes. Among these six genes, the serine protease-encoding gene *PLAU* was of particular interest, being the most significantly downregulated in resistant chickens (Figure 6B and Sup. Table 4). Moreover, qPCR analysis of *PLAU* in a higher number of resistant chickens (Sup. Table 1c) showed a statistically significant downregulation when compared not only to susceptible chickens (which upregulated this gene), but also compared to controls, thus further validating a differential response between resistant and control chickens (Figure 6C). Overall, these results demonstrate a differential host response against HPAIV in successfully inoculated resistant and susceptible chickens, always in contrast with control chickens.

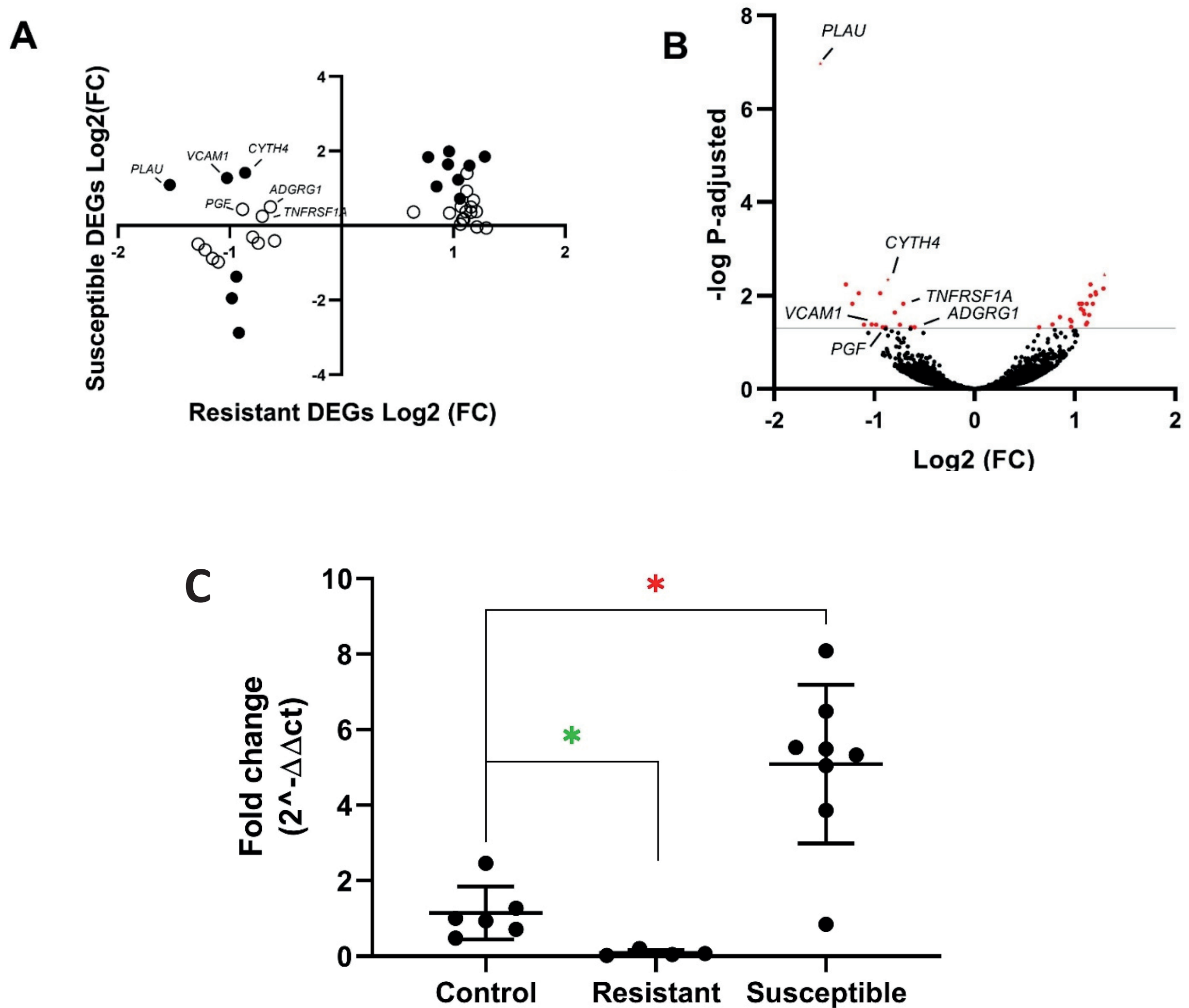


Figure 14. (A) Expression pattern of the 39 resistant-specific DEGs in lungs from susceptible (y-axis) and resistant (x-axis) chickens. Fold changes of the 39 genes in both groups are represented. Black dots indicate DEGs in both susceptible and resistant birds. White dots indicate DEGs only in resistant birds. (B) Volcano plot showing all genes identified by RNAseq in lungs from resistant chickens. In red are shown the 39 DEGs (BH adjusted $P < 0.05$). (C) Quantitative PCR of PLAU from lungs of resistant and susceptible H5N8 inoculated chickens collected at 3 dpi. Lungs from uninfected chickens were used as control. For each group the mean and standard deviation are shown. Green asterisk means significant downregulation and red asterisk means significant upregulation (*) BH adjusted $P \leq 0.05$ from Wald test p-value with DESeq2.

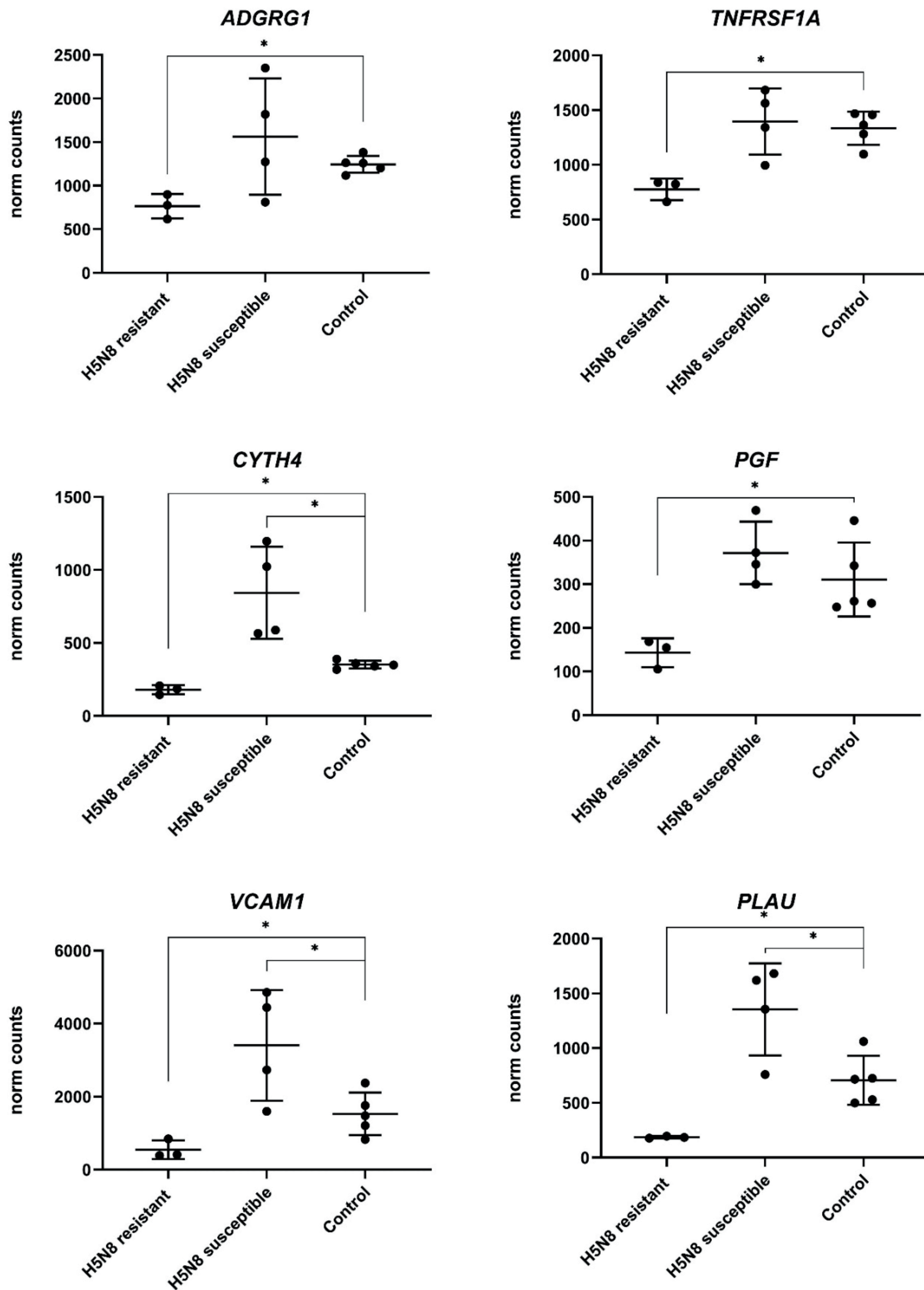


Figure 15. Expression levels of the six genes with opposite regulation between resistant and susceptible chickens inoculated with H5N8 virus from lungs collected at 3 dpi. For each group the mean and standard deviation are shown. (∗) BH adjusted $P \leq 0.05$ from Wald test p-value with DESeq2.

AMOVA results point to few quasi-species differences between inocula and 3 dpi oral swabs of susceptible H5N8 or H7N1 inoculated chickens

In order to validate the egg passage technique, the nucleotide frequencies per position were compared between four non-passaged samples (two H5N8 samples and two H7N1 samples) and the same samples after 48-72 h of egg-passage in SPF embryonated eggs. The comparison of the quasi-species obtained per segment with both treatments indicated a bias in the mean nucleotide frequencies per position that ranged between 0.5% in the neuraminidase (NA) of H7N1 to 1.2% in the polymerase basic 1 (PB1) of H5N8, with most values around 0.7%. All nucleotide frequencies were below the fixation index value (5%) selected for a nucleotide change to be considered significant.

The comparison of the nucleotide frequencies per segment between the original inocula and the 3 dpi oral swabs of susceptible birds offered contrasting results (Figure 8). Regarding H5N8 virus, no significant differences between the original H5N8 inoculum and the oral swabs of susceptible H5N8 inoculated chickens were detected in the HA, M, NP, polymerase acidic (PA), and PB2 segments, and only PB1 (n=7), NA (n=1), and non-structural protein (NS) (n=1) showed significant differences. The seven PB1 changes were located between nucleotide positions 246-346 (amino acid residues 82-115), a region known to have an alternative codification for PB1-F2 (nucleotides 227-385 of the PB1 A/goose/Spain/IA17CR02699/2017 (H5N8), MK494921). All nucleotide changes codified for non-synonymous mutations in the PB1 and PB1-F2 proteins. Regarding H7N1 virus, significant Fct values between the original H7N1 inoculum and oral swabs of susceptible H7N1 inoculated chickens were observed in 91 nucleotide positions, most of them in the PA (40 nucleotides, 37 non-synonymous) and PB2 (45 nucleotides, 43 non-synonymous) segments. These mutations were located between amino acid residues 572-656 of PA and 60-137 of PB2. Few nucleotide differences were detected in HA (n=2), M (n=1), and NP (n=1), while no differences were reported in NA and NS (Sup. Table 5).

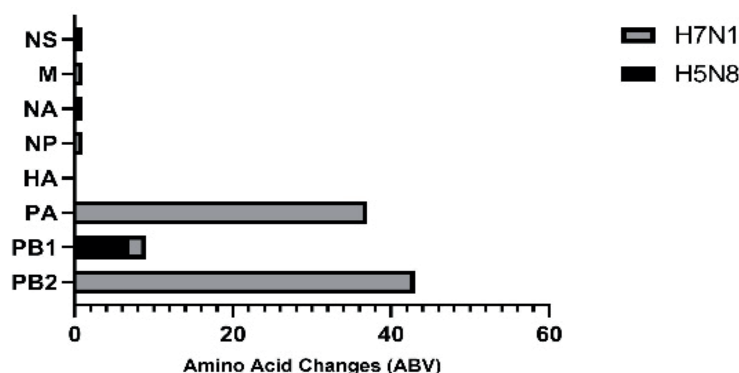


Figure 16. Stacked bars showing amino acid changes in absolute numbers in each segment. H7N1 viruses are represented by gray colour and H5N8 by black colour.

Compartmentalization analysis reveals low diversities in the inoculum and the oral swabs and high diversities in the lung

The quasi-species diversities obtained from the original inoculum, the oral swab, the lung, and the spleen at 3 dpi, from three birds infected with the H5N8 strain were analyzed. Significant differences were detected following an ordinary one-way ANOVA test. The lower nucleotide diversity was found in the inoculum, with a mean value for the combined viral segments of 1.9% (CI 95%: 1.147-2.74), followed by the oral swabs with 2.27% (CI 95%: 2.152-2.391), the spleens with 2.73% (CI 95%: 2.289-3.170). The highest diversity was found in the lungs with 3.06% (CI 95%: 2.744-3.369) (Figure 9). The nucleotide net distances between quasi-species were also analyzed. Significant differences were detected following an ordinary one-way ANOVA test. The lower nucleotide net distance was found between the lung and the spleen quasi-species, with an average net distance for the combined viral segments of 0.82% (CI 95%: 0.6738-0.9751), followed by the inoculum and the oral swab with 0.84% (CI 95%: 0.6008-1.088), the oral swab and the spleen with 0.98% (CI 95%: 0.7987-1.161), the inoculum and the spleen with 1.22% (CI 95%:0.986-1.463), and the oral swab and the lung with 1.32% (CI 95%: 1.091-1.545). The highest distance was found between the inoculum and the lung with 1.44% (CI 95%:1.19-1.682) (Figure 10).

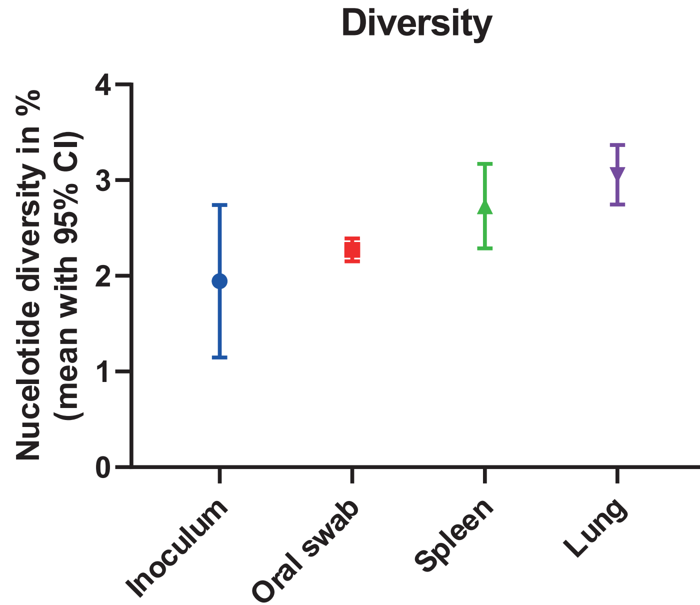


Figure 17. Inner nucleotide diversity in every type of sample analyzed expressed in percentage the mean of the nucleotide diversity with 95% confidence interval values of all the H5N8 segments and all the samples.

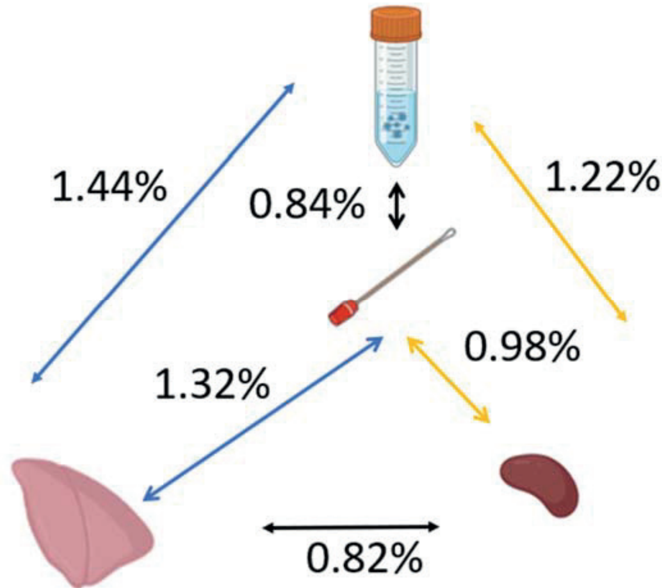


Figure 18. Schematic representation of the nucleotide distances between the type of samples analyzed expressed in percentage as the mean of the nucleotide distances values of all the H5N8 segments and all the samples.

3.5. Discussion

For an infection event to be successful, a pathogen must overcome the physical and immunological barriers of the host. Focusing on the pathogen of interest here, two HPAIV strains (H7N1 and clade 2.3.4.4b H5N8) were experimentally inoculated in a diverse group of chicken breeds [67]. Here, the transcriptome of resistant and susceptible birds (classified by clinical signs, lesions, and presence of viral antigen in tissues) and the viral quasi-species after infection were characterized. The results obtained showed the differences that are linked to host susceptibility to virus infection.

In our previous study, H7N1 HPAIV strain was more virulent than H5N8 HPAIV strain in different chicken breeds since it caused the highest frequency of severe clinical signs, highest mortality rates (70% versus 47% mortality rate), and shortest mean death time (3.3 dpi versus 4.9 dpi) [67]. Nonetheless, transcriptomic results in the present study indicated that both virus strains generally induced similar host transcriptional responses in susceptible birds. More specifically, a similar expression level was observed in IFN-I response, cytokine production, and ISG, even though susceptible H5N8 inoculated chickens presented more DEGs and slightly higher fold changes in immune-related DEGs, probably associated to a higher viral replication rate in lung and spleen of H5N8 inoculated chickens compared to H7N1 inoculated chickens [67]. However, a stronger *IL8L1* and *IL8L2* chemokine response was detected in lung and spleen of H7N1 inoculated chickens, perhaps a biomarker of an aberrant innate immune response [241] as seen before with this virus strain [72]. It is worth mentioning that differences in clinical outcome between the two HPAIV strains are mainly due to higher viral replication in the heart, central nervous system, and pancreas of H7N1 inoculated birds as previously discussed [67]. A similar gene expression was also observed between tissues (lung and spleen) of susceptible birds. Nevertheless, lung samples presented more DEGs, GO enriched pathways, and higher fold changes than spleen samples. These differences, as well as those observed when comparing the two virus strains, can be attributed to a different viral replication rate between tissues, since higher viral titers were detected in lungs compared to spleens. Furthermore, because trypsin-like enzymes in respiratory epithelial cells can cleave the viral HA and allow entry of the virus,

multiple replication cycles occur in the lung [211]. Altogether, these factors could explain why more virus and, consequently, more DEGs, were detected in lungs compared to spleens.

After considering the transcriptomic differences in susceptible birds between HPAIV strains and tissues, we focused on the differences between resistant and susceptible H5N8 inoculated chickens. The experimental design did not take into consideration the influence of the different chicken breed in the susceptibility of the infection, since we had previously shown that both susceptible and resistant chickens are present in different breeds [67]. We thus hypothesized that all chickens resistant to infection share a conserved transcriptomic signature in response to viral exposure regardless of their breed. This approach based on the comparison of individual chickens with different infection outcomes makes our analysis unique, in contrast with similar recent chicken RNA-Seq studies where resistant and susceptible chicken lines are compared instead [217,218,242]. A salient feature were the minor transcriptomic changes observed in resistant chickens versus controls, in contrast with the major transcriptomic changes in susceptible chickens. Based on these results, the pressing question was whether resistant chickens were actually infected, since they lacked direct evidence of IFN-I or inflammatory responses. Nevertheless, three main reasons support the fact that resistant chickens were infected; 1) the high viral dose used for the experimental infections [243] ; 2) the fact that within the 39 DEGs obtained in resistant chickens in contrast to control chickens, we found IFN-I regulated genes (Interferome v2.1 database) and genes related to defense response (Gene Ontology analysis); and 3) some DEGs downregulated in resistant chickens but upregulated in the susceptible ones were related to innate immune pathways such as Nf-kB and MAPK signaling. However, our study offers a static picture of the HPAIV infection at 3 dpi, which permits the comparison of resistant and susceptible birds based on clinical signs, lesions, and presence of antigen. It will be interesting to further characterize this resistant-specific responses analyzing other relevant tissues and time-points. Indeed, the few differentially expressed genes identified in these chickens might indicate that a more robust host response may occur in the upper respiratory tract or at earlier time-points post-infection. Given that AIV pathogenesis in chickens is determined by early immune responses [77,244–246], these minor transcriptomic changes in

lungs could be associated to a concomitant innate immune response in nasal mucosa where the virus first replicates and finds the first host barrier [247]. Finally, our study had a limitation in the number of resistant chickens analyzed, which probably resulted in the low number of DEGs observed. Despite this fact does not play down the results obtained, it is possible that analyzing a higher number of samples would unmask a broader transcriptomic response in lungs.

Despite most of the few DEGs from resistant chickens showed similar expression patterns to those in susceptible chickens, six of these DEGs showed an opposite sense of regulation. All six of these genes were found in lungs and were downregulated in resistant chickens and upregulated in susceptible ones. Three of the downregulated genes (*PLAU, VCAM1, TNFRSF1A*) belong to the NF-κB signaling pathway, suggesting that the activation of this inflammatory pathway might benefit virus replication. In fact, some drugs inhibit this pathway as a strategy against influenza viruses [248–250]. However, this host signaling pathway seems to play a controversial role in AIV infection. Even if low levels of NF-κB activity allow cells to avoid viral replication by retaining newly synthesized viral ribonucleoprotein complexes (vRNP) in the nucleus [251–253], complete inhibition of NF-κB functions can increase viral replication [254]. Apart from the NF-κB pathway, two genes (*TNFRSF1, PGF*) downregulated in resistant chickens and upregulated in susceptible ones are related to MAPK pathway. As in the case of NF-κB, the inactivation of the classical MAPK cascade leads to reduced viral titers by the retention of vRNP in the nucleus [255,256]. Altogether, these results suggest an early differential regulation of intracellular signaling pathways between resistant and susceptible chickens.

Moreover, the *TNFRSF1A* gene, which is involved in the influenza A infection pathway [231], is a tumor necrosis factor (TNF)-alpha receptor that recruits caspase-8; its activation initiates the subsequent cascade of caspases (aspartate-specific cysteine proteases) that mediates apoptosis [257,258]. Downregulation of *TNFRSF1A* could play a role in the retention of vRNP avoiding a caspase-mediated disruption of the nuclear pore complex and a subsequent reduction of viral replication, as discussed above. Interestingly, the gene *PGF*, a growth factor which is active in angiogenesis and endothelial cell growth [258], enhances the magnitude

and duration of TNF-alpha and toll-like receptors pathways, contributing to the subsequent release of inflammatory cytokines [259–261]. Thus, downregulation of *PGF* in resistant birds could avoid an exaggerated pathologic pro-inflammation in response to viral infection. Finally, the gene *VCAM1* has cell adhesion functions, more particularly in leukocyte-endothelial cell adhesion [262]. Its downregulation could suggest a resolution of a previous inflammatory process, reinforcing the hypothesis of an early immune response controlled during the very first hours.

Finally, the DEG found in lungs from resistant chickens showing the highest downregulation was *PLAU* (also known as *UPA*), which in contrast was clearly upregulated in susceptible birds, suggesting a key role in the final outcome of the disease. Its differential expression was further validated by qPCR from a larger number of samples of resistant, susceptible and control chickens from the same experiment. Additionally, in order to determine whether *PLAU* gene could be upregulated or downregulated in all the breeds used in the resistant group, at least one individual of each breed was present in the susceptible group in our qPCR validation. Our results indicate that different individuals of the same breed were able to regulate differentially this gene. Further studies are required in order to elucidate if *PLAU* expression is differentially regulated in different chicken breeds, through divergent epigenetic mechanisms or other transcriptional regulatory factors. *PLAU* encodes a serine protease that converts plasminogen to plasmin [262] and plays a relevant role in vascular degradation by increasing VE-cadherin degradation and in inflammation [263–265]. Our main hypothesis was related with the effect of *PLAU* in the plasminogen conversion to plasmin. This could be relevant in the context of HPAIV infection in two different ways; avoiding viral replication and/or avoiding fibrinolysis [79]. Specifically, plasmin is a serine-protease known to participate in the cleavage of the HA protein into HA1 and HA2, a necessary step for AIV infectivity [38,266] Thus, the downregulation of *PLAU* in resistant birds could lead to an inefficient AIV replication in the lung. Furthermore, the conversion of plasminogen to plasmin by *PLAU* is related to fibrinolysis-mediated inflammation [267,268], which has been suggested to play a role in a deleterious lung inflammation by increasing fibrin degradation products and vascular permeability in a mouse model [79]. We hypothesize that an optimized regulation of *PLAU* could reduce the coagulation dysfunction that HPAIV typically produce [78]. Besides,

PLAU is also known to play a relevant role in other viral infections, such as a biomarker of coronavirus disease (COVID-19) severity [269,270], associated with diffuse hemorrhages in classical swine fever virus [271], and with viral fusion to the cellular membrane in human immunodeficiency virus 1 infection [272]. Altogether, these results suggest that the downregulation of *PLAU* could have a relevant impact in the outcome of HPAIV disease in resistant chickens by avoiding vascular damage and permeability, deleterious lung inflammation and/or viral activation.

From the virus side, the AMOVA analyses identified several positions with significant changes along the viral quasi-species of 3 dpi oral swabs of susceptible H5N8 inoculated birds, mostly in PB1 (seven out of nine), whereas a large proportion of changes identified in H7N1 inoculated birds were located in PA and PB2. All three segments constitute the polymerase heterotrimer of AIV, which is involved both in transcription and replication of the viral RNA genome [273]. The PB1 protein is in the core of this complex, with its N and C terminal domains directly interacting with the C terminal domain of PA and the N terminal domain of PB2, respectively [274]. The mutations detected in PB1 are located just downstream of this interaction stretch, before the ATP binding site, in a region that encodes for PB1 and PB1-F2 proteins. Considering that all seven nucleotide mutations in PB1 and PB1-F2 of H5N8 inoculated birds were non-synonymous, those changes could imply a fitness gain for the virus, although further studies are needed to confirm this hypothesis. Most mutations observed in H7N1 inoculated birds were non-synonymous changes in the C terminal domain of PA and in the N-terminal domain of PB2, which is known to interact with PB1. Also, most mutations introduced amino acids with lower, more acidic, isoelectric points, potentially offering a fitness win. The H5N8 HPAIV strain used in this study is a goose-origin isolate and is less adapted to chickens than the H7N1 HPAIV strain that is a chicken-origin isolate [67]. This should have implied fewer changes along the viral quasi-species from H7N1 inoculated birds, as seen in more adapted strains [275], but the opposite was observed. This observation could be explained by the particularities of the randomly chosen samples analyzed, since oral swabs from H5N8 inoculated chickens had a higher replication rate than oral swabs from H7N1 inoculated chickens, offering a skewed picture of viral evolution dynamics. Interestingly, we found no or few substantial changes in the segments that encode for the proteins that interact with

the innate immune system (NP, NS, and M) or the cell receptors (HA and NA), indicating no adaptation in the segments that interact with the innate immune system of susceptible birds during the first 3 dpi. Overall, these results are in line with acutely infected hosts, where infections are dominated by purifying selection and low intra-host diversity [209]. Regrettably, the viral quasi-species of resistant birds could not be analyzed, as they quickly cleared the infection. However, because of their shorter viral shedding, nucleotide differences in quasi-species of resistant birds are unlikely. In susceptible birds, the lack of virus variation in the segments known to interact with the innate immune system of the host, coupled with the striking differences in gene regulation between susceptible and resistant birds, points towards a scenario where successful infection events seem not to be due to the changes in the viral quasi-species, but to the ability of the host's immune system to mount an early and efficient response against the pathogen.

Regarding viral compartmentalization, it is widely accepted that replication increases viral diversity, even more in RNA viruses, such as AIV [275]. Accordingly, our results suggest that viral replication at 3 dpi increased the diversity of the viral quasi-species within each bird (i.e., oral swab, spleen, and lung), in contrast with the original inoculum. Therefore, no founder effect (i.e., loss of genetic variation that occurs when a new population is established) related to the transmission process was detected. However, if diversity were analyzed at 1 dpi or at earlier time-pints we could have seen this effect, with its associated reduction of viral diversity in samples collected from birds. Among tissue samples, the highest viral diversity was found in the lungs, a result that correlates with the distance found between lungs and oral swabs, as was the largest distance detected between these samples. This may indicate that, after infection, viral particles firstly arrived to the lungs and via the hematogenous route spread to the spleen [276,277]. Rich and highly diverse viral populations in the lung could be stochastically selected due to founding effects during the viral transit to the spleen, impairing viral diversity found at the spleen in contrast with the lung. A similar reduction in viral diversity was suggested by others during the viral transit from the upper respiratory airways to the lower respiratory tract [278]. This hypothesis was highlighted by the fact that the nucleotide distance between the viral quasi-species detected in the lung and in the spleen was the lowest detected, suggesting a close relationship between these two viral populations.

Furthermore, due to trypsin-like enzymes in respiratory epithelial cells, multiple replication cycles occur in the lung [211], in contrast with the spleen, where H5N8 HPAIVs could have had fewer replication cycles, thus also avoiding a huge increase in viral diversity. Concerning oral swabs, it is worth mentioning that these samples contain mainly viable particles orally excreted in contrast with tissue samples (i.e., lung and spleen) where unviable intracellular particles are found. During AIV replication, around 70% are unviable viral particles [279], and some of them will not be able to exit the host cell. Therefore, not every viral quasi-species present in the oral tissue will be detected in oral swabs, and thus the nucleotide diversity found in oral swabs should be lower than in tissues, as was seen in our results. Indeed, it is known that the process of excretion is a bottleneck that viruses must overcome [210,236]. Overall, increasing the knowledge of within-host viral variability is an essential step to understanding the mechanisms behind viral distribution in the host, and deciphering how within-host evolution is related to evolution on a global scale. Till within-host viral evolution seems to be dominated by purifying processes and low intra-host diversity, evolution on a global scale seems dominated by positive selection processes with new high-prevalent strains being more adapted to new species and more transmissible. Our study was limited by a generally low within-host diversity that difficulted its analysis. To overcome this limitation a clearly defined viral population would be necessary, such as the use of AIV libraries bearing molecular barcodes implemented by others [210,278].

To sum up, some immune-related pathways and genes that could play a role in the outcome of the disease caused by HPAIV in chickens were identified. An early and optimal regulation of the NF-κB pathway and the MAPK cascades could be the reason why some chickens are resistant to AIV. This optimal host regulation could avoid an exaggerated immune response and/or viral replication by an early inactivation of important genes like *PLAU*. The relevance of the transcriptomic results is strengthened by the viral quasi-species analyses, where no evidence of viral adaptation to the host was reported among susceptible birds in the segments known to interact with the innate immune system and viral compartmentalization analysis, where viral diversity seems to be reduced by multiple founder events during viral tissue colonization. Although our results strongly suggest the implications of specific genes in HPAIV clinical outcome, further research is needed

evaluating upper respiratory tissues at earlier time-points after exposure in order to confirm their contribution.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

The project was conceived, and funding obtained by MN, AR, and NM. The experiment was designed by RSG, AP, RMV, and NM. The experiment was performed by RSG and samples collected and processed by AP and RMV. JA provided assistance with the analysis of RNA-Seq data. MC provided assistance with the quasi-species analysis. Figures were prepared by JA, AP, MC, and KB. The manuscript was drafted by AP with corrections from MC, JA, KB, and NM. All authors contributed to the article and approved the submitted version.

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Acknowledgments

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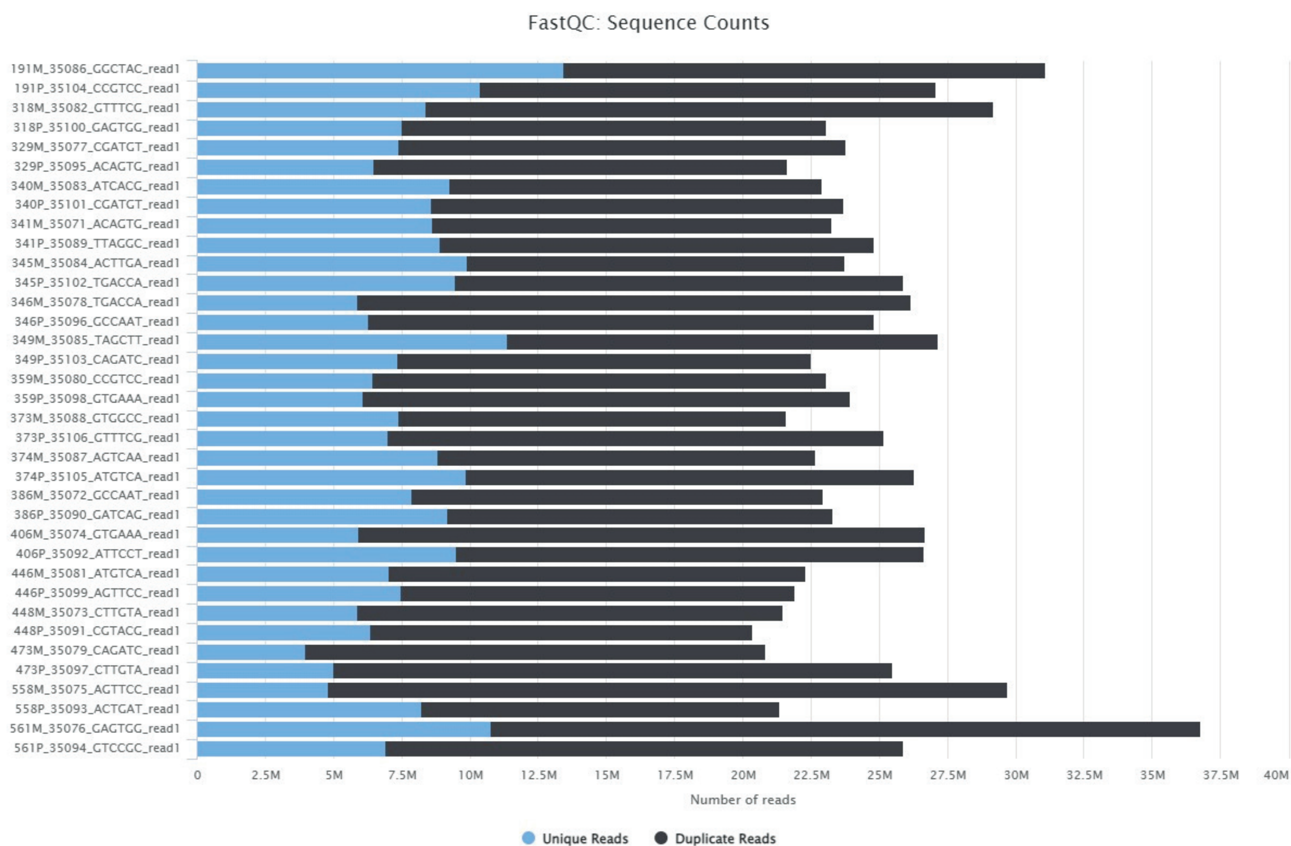
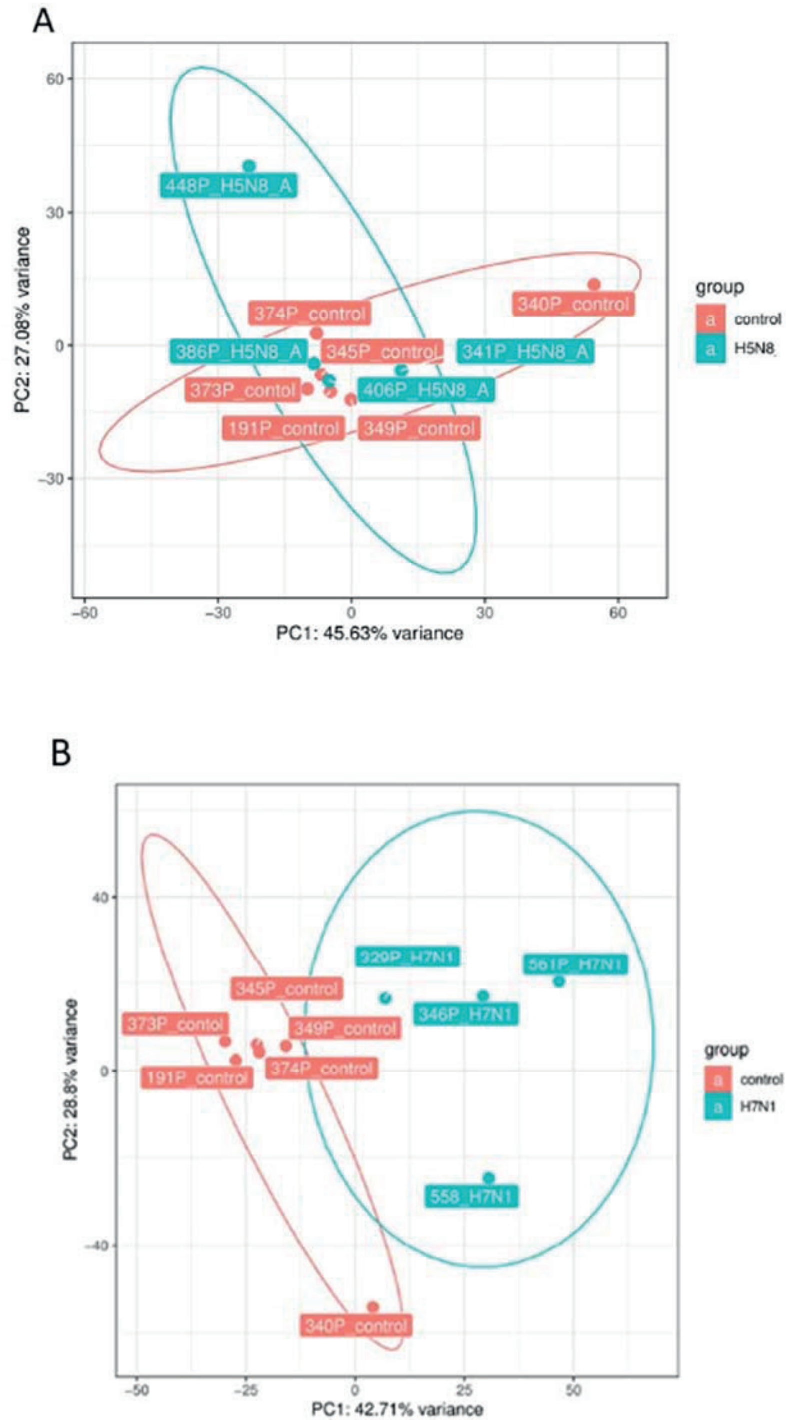
3.6. Supplementary Material

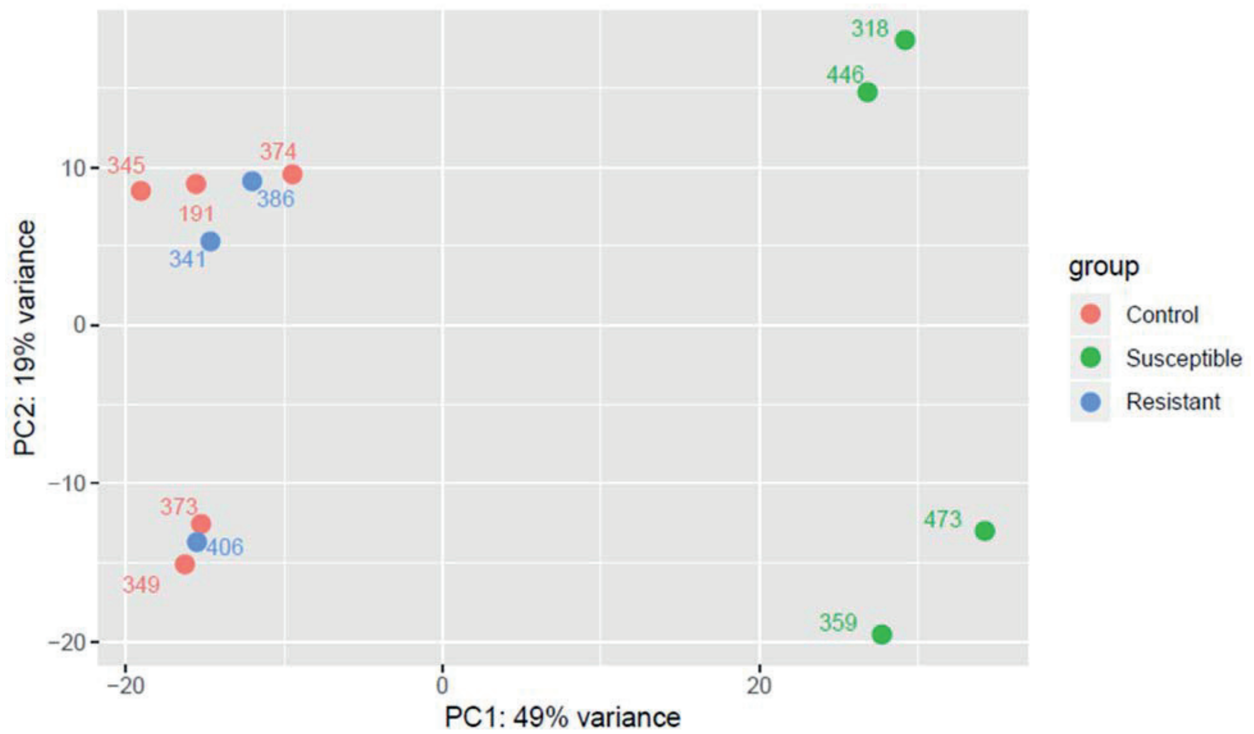
Supplementary Figures



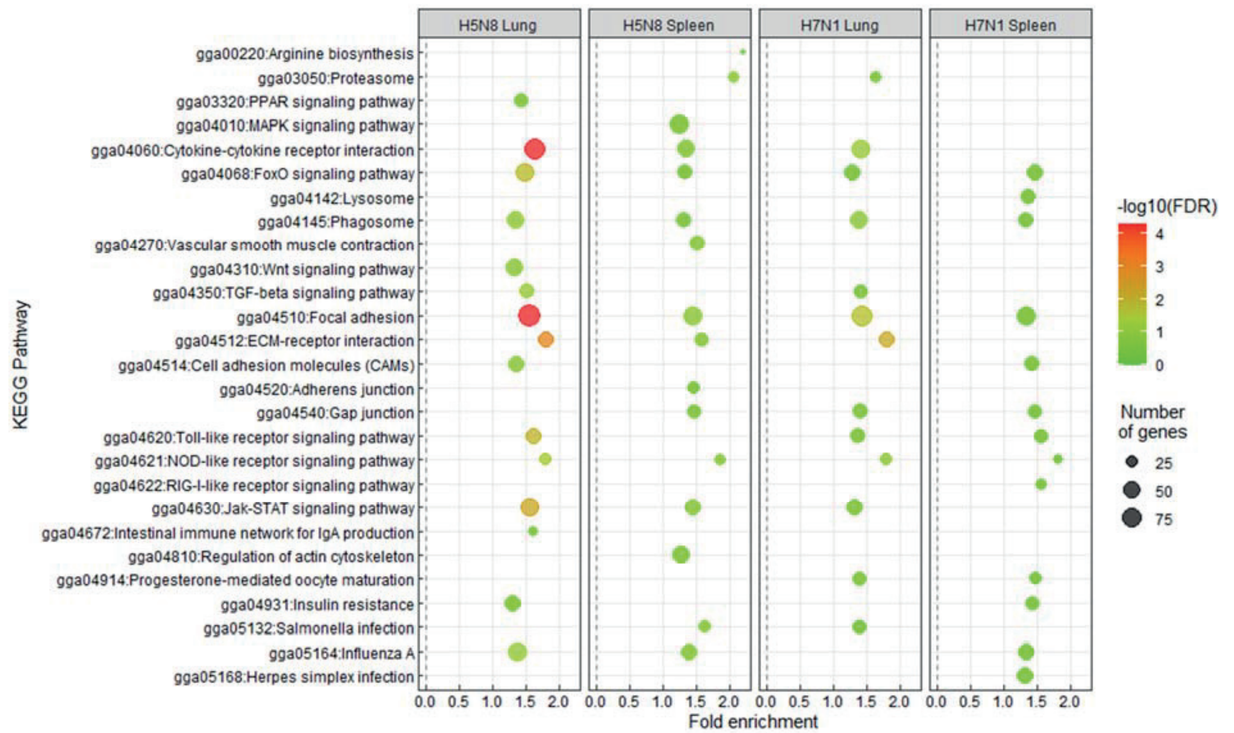
Suppl. Figure 1. Sequence counts for each sample showing unique (blue) and duplicate (black) reads. Duplicate read counts (in blue) are an estimate only.



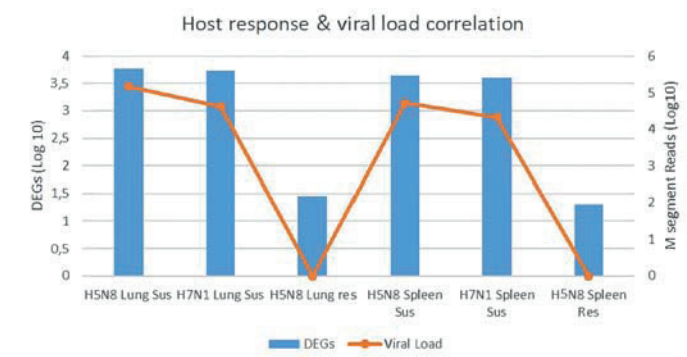
Suppl. Figure 2. Principal component analysis plot on variance stabilizing transformed (VST) counts of lung samples collected from resistant H5N8 inoculated chickens and control chickens (A), and from susceptible H7N1 inoculated chickens and control chickens (B). Used to discard 448, 340, and 558 (lung sample) chickens as outliers.



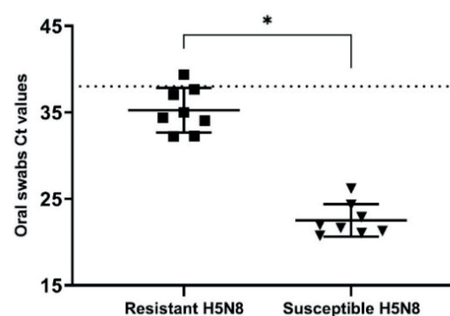
Suppl. Figure 3. Principal component analysis plot on variance stabilizing transformed (VST) counts of lung samples collected from susceptible and resistant H5N8 inoculated chickens and control chickens.



Suppl. Figure 4. Enriched GO terms obtained from KEGG for DEGs in lungs or spleens collected at 3 dpi from susceptible H5N8 or H7N1 inoculated chickens. The most representative and significant biological processes are represented and are sorted by fold enrichment. The dot size indicates the number of DEGs associated with the biological process. The dot color indicates the significance of the enrichment (-log10 (FDR-corrected P-values)).



Suppl. Figure 5. Number of DEGs (bars, left y-axis) and viral transcripts (line, right y-axis) identified by RNA-Seq in lungs and spleens collected at 3 dpi from susceptible (Sus) or resistant (Res) H5N8 or H7N1 inoculated chickens.



Suppl. Figure 6. Quantitative RT-PCR of M gene from 3 dpi oral swabs obtained from resistant and susceptible H5N8 inoculated chickens. For each group the mean and standard deviation are (*) P≤ 0.05; unpaired two-tailed t-test. The dotted line represents the limit of detection (Ct 38).

Supplementary Tables

Suppl. Table 1. Classification as resistant (RES), susceptible (SUS) or control (CON) of the samples (lung (LNG), spleen (SPL) or allantoic fluid) and breeds (Empordanesa (EMP), Penedesenca (PENED), Catalana del Prat (C. PRAT), Flor d'Ametller (F. AMET), Castellana negra (C. NEGRA), Euskal oiloa (E. OILOA), Broiler, and specific pathogen free (SPF)) used for this study. 1a. RNA-Seq sample selection and classification. 1b. Quasi-species sample selection and classification. 1c. *PLAU* validation sample selection and classification. 1d. Summary of all the samples.

1a.

RNA-Seq sample selection					
VIRUS	*CHICKEN ID*	*BREED*	*DPI*	*SAMPLE*	*GROUP*
H5N8	341	C.NEGRA	3	LNG, SPL	RES
	386	BROILER	3	LNG, SPL	RES
	406	EMP	3	LNG, SPL	RES
H7N1	558	E.OILOA	3	SPL	SUS
	561	E.OILOA	3	LNG, SPL	SUS
	329	BROILER	3	LNG, SPL	SUS
	346	BROILER	3	LNG, SPL	SUS
H5N8	473	F.AMET	3	LNG, SPL	SUS
	359	C.NEGRA	3	LNG, SPL	SUS
	446	SPF	3	LNG, SPL	SUS
	318	E.OILOA	3	LNG, SPL	SUS
CONTROL	345	BROILER	3	LNG, SPL	CON
	349	E.OILOA	3	LNG, SPL	CON
	191	EMP	3	LNG, SPL	CON
	374	SPF	3	LNG, SPL	CON
	373	C.PRAT	3	LNG, SPL	CON

1b.

Quasi-species sample selection					
VIRUS	*CHICKEN ID*	*BREED*	*DPI*	*SAMPLE*	*GROUP*
H7N1	558	E. OILOA	3	ALLANTOIC FLUID	SUS
	329	BROILER	3	ALLANTOIC FLUID	SUS
	346	BROILER	3	ALLANTOIC FLUID	SUS
	959	PENED	3	ALLANTOIC FLUID	SUS
H5N8	359	C.NEGRA	3	ALLANTOIC FLUID	SUS
	446	SPF	3	ALLANTOIC FLUID	SUS
	318	E. OILOA	3	ALLANTOIC FLUID	SUS
	319	E. OILOA	3	ALLANTOIC FLUID	SUS
	200	EMP	3	ALLANTOIC FLUID	SUS
	433	C.PRAT	3	ALLANTOIC FLUID	SUS
	467	F. AMET	3	ALLANTOIC FLUID	SUS
	471	F. AMET	3	ALLANTOIC FLUID	SUS

1c.

PLAU qPCR sample selection					
VIRUS	*CHICKEN ID*	*BREED*	*DPI*	*SAMPLE*	*GROUP*
H7N1	950	EMP	3	LNG	RES
H5N8	423	PENED	3	LNG	RES
	442	C.PRAT	3	LNG	RES
	387PN	BROILER	3	LNG	RES
H7N1	954	EMP	3	LNG	SUS
	946	PENED	3	LNG	SUS
	572	F.AMET	3	LNG	SUS
	558	E.OILOA	3	LNG	SUS
	561	E.OILOA	3	LNG	SUS
	346	BROILER	3	LNG	SUS
H5N8	433	C.PRAT	3	LNG	SUS
	367	C.NEGRA	3	LNG	SUS

CONTROL	349	E.OILOA	3	LNG	CON
	353	PENED	3	LNG	CON
	399	PENED	3	LNG	CON
	373	C.PRAT	3	LNG	CON
	378	F.AMET	3	LNG	CON
	379	F.AMET	3	LNG	CON

1d.

Chicken Id	Virus	Breed	Group	RNA seq	QSP	qPCR	Distance
341	H5N8	C.NEGRA	RES	X			
386	H5N8	BROILER	RES	X			
406	H5N8	EMP	RES	X			
558	H7N1	E.OILOA	SUS	X	X	X	
561	H7N1	E.OILOA	SUS	X		X	
329	H7N1	BROILER	SUS	X	X		
346	H7N1	BROILER	SUS	X	X	X	
473	H5N8	F.AMET	SUS	X			
359	H5N8	C.NEGRA	SUS	X	X		X
446	H5N8	SPF	SUS	X	X		X
318	H5N8	E.OILOA	SUS	X	X		X
345	Control	BROILER	CON	X			
349	Control	E.OILOA	CON	X		X	
191	Control	EMP	CON	X			
374	Control	SPF	CON	X			
373	Control	C.PRAT	CON	X		X	
959	H7N1	PENED	SUS		X		
319	H5N8	E. OILOA	SUS		X		
200	H5N8	EMP	SUS		X		
433	H5N8	C.PRAT	SUS		X		

467	H5N8	F. AMET	SUS	X	
471	H5N8	F. AMET	SUS	X	
950	H7N1	EMP	RES		X
423	H5N8	PENED	RES		X
442	H5N8	C.PRAT	RES		X
387	H5N8	BROILER	RES		X
954	H7N1	EMP	SUS		X
946	H7N1	PENED	SUS		X
572	H7N1	F.AMET	SUS		X
433	H5N8	C.PRAT	SUS		X
367	H5N8	C.NEGRA	RES		X
353	Control	PENED	CON		X
399	Control	PENED	CON		X
378	Control	F.AMET	CON		X
379	Control	F.AMET	CON		X

Suppl. Table 2. Primers used for quantitative real time PCR analysis of chicken mRNAs.

target	Primers 5´-3´
RPL12 Rev	GGCCCGTGTTATCTCAGAGG
RPL12 Rev	GGATCCCAAAGAGACGAGCG
ACTB Fwd	AGCGAACGCCCCCAAAGTTCT
ACTB Rev	AGCTGGGCTGTTGCCTTCACA
YWHAZ Fwd	AGGAGCCGAGCTGTCCAATG
YWHAZF Rev	CTCCAAGATGACCTACGGGCTC
PLAU	qGgaCED0031197 Bio RAD
Il1B Fwd	GGGCATCAAGGGCTACAA
Il1B Rev	CTGTCCAGGCGGTAGAAGAT
VCAM1 Fwd	GAGATTGTTTGTGTGGCCAGATT
VCAM1 Rev	TGGAGATGCATTAATGGAAGTATTTT

Supplementary Table 3. List of DEGs identified from lungs in resistant birds.

Upregulated DEGs	Downregulated DEGs
ANAPC10	*LOC107051977*
LOC112529948	*ADGRG1*
HEMGN	*TNFRSF1A*
HBM	*LOC112532108*
R3HDM4	*DGKG*
HBA1	*CYTH4*
EPB42	*PGF*
RRAD	*ITGBL1*
LOC112531211	*SLC24A4*
RHCE	*FGF14*
C8ORF88	*VCAM1*
HBBA	*LOC107056992*
BLOC1S6	*ADRB3*
MFSD2B	*LOC107055113*
RHAG	*PRLHRL*
NT5C3A	*PLAU*
SLC25A37	
NARF	
MIR147-1	
LOC107054431	
C4H4ORF54	
SAT1	
ANKRD54	

Suppl. Table 4. Number of *PLAU* reads by lung sample in the resistant (RES), susceptible (SUS) and control groups from the RNA-Seq analysis.

CHICKEN ID	BREED	GROUP	*PLAU* READS
386	BROILER	RES	184
406	EMP	RES	196
341	C.NEGRA	RES	177
318	E.OILOA	SUS	1355
359	C.NEGRA	SUS	1680
446	SPF	SUS	1620
473	F.AMET	SUS	759
374	SPF	CONTROL	1061
373	C.PRAT	CONTROL	716
191	EMP	CONTROL	529
345	BROILER	CONTROL	499
349	E.OLIOA	CONTROL	724

Supplementary Table 5: Amino acid changes at the quasi-species level in each segment and virus.

H7N1		H5N8	
Segment	Amino acid changes	Segment	Amino acid changes
M	T181A	NA	Y113F
NP	K98R	NS	S266I
PA	Y59E P65S F71L R73K R74H L75R Y76F T78I A80E L86M L90V C93S V94I R572K F573I T575M H578G M582R R584S Q593E V598A C601S V612F I618T F625P V627G I628V R631G T632S P634G T638R V649L F652S Y656E	PB1	K82D R86D *88A *89A D105H D110N L115A
PB1	M571R S770C		
PB2	G60D R61K M67I F68P D69E G70R I71N V77L R79S C81T K82N K84A *85G P86S G87D L89V A91V A92S T93P V94L K95A K96V Q104P D120E A122V T132P Q134H *138Q A635S H654P K657N N658Y I662T I663K A673G S675L T690V R702K		

Chapter IV

Study 2

Persistence of low pathogenic avian influenza virus in artificial streams mimicking natural conditions of waterfowl habitats in the Mediterranean climate

Albert Perlas[a,b]; Kateri Bertran[a,c]; Francesc Xavier Abad[a,c]; Carles M. Borrego[d,e]; Miquel Nofrarías[a,c]; Rosa Valle[a,c]; Lola Pailler-García[a,c]; Antonio Ramis[a,b]; Martí Cortey[b]; Vicenç Acuña[d,f]; Natàlia Majó[a,b]

[a] Unitat mixta d'Investigació IRTA-UAB en Sanitat Animal. Centre de Recerca en Sanitat Animal (CReSA). Campus de la Universitat Autònoma de Barcelona (UAB), Bellaterra, 08193, Catalonia. Spain.

[b] Departament de Sanitat i Anatomia Animals, Universitat Autònoma de Barcelona, Campus de la Universitat Autònoma de Barcelona (UAB), Bellaterra, Spain

[c] IRTA. Programa de Sanitat Animal. Centre de Recerca en Sanitat Animal (CReSA). Campus de la Universitat Autònoma de Barcelona (UAB), Bellaterra, 08193, Catalonia. Spain.

[d] Catalan Institute for Water Research (ICRA), C. Emili Grahit 101, 17003, Girona, Spain.

[e] Grup d'Ecologia Microbiana Molecular, Institut d'Ecologia Aquàtica, Universitat de Girona (UdG), Plaça Sant Domènec 3, 17004 Girona, Spain.

[f] Universitat de Girona (UdG), Plaça Sant Domènec 3, 17004 Girona, Spain.

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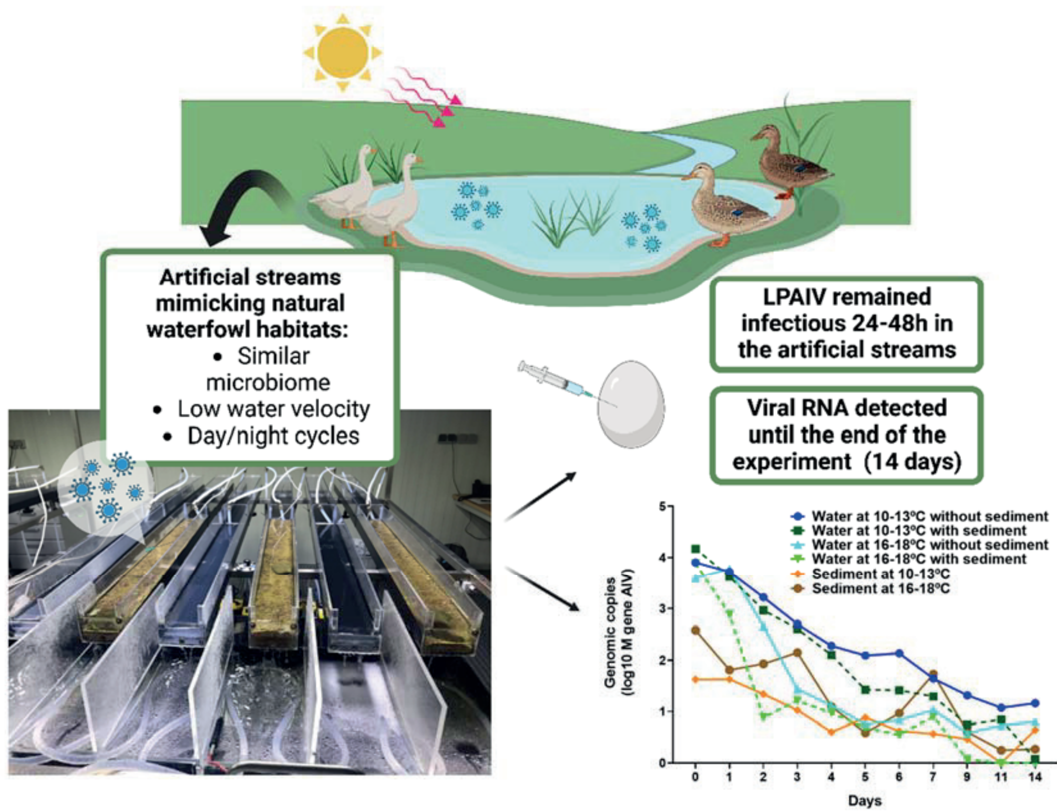
4.1. Abstract

Avian influenza viruses (AIVs) can affect wildlife, poultry, and humans, so a One Health perspective is needed to optimize mitigation strategies. Migratory waterfowl globally spread AIVs over long distances. Therefore, the study of AIV persistence in waterfowl staging and breeding areas is key to understanding their transmission dynamics and optimizing management strategies. Here, we used artificial streams mimicking natural conditions of waterfowl habitats in the Mediterranean climate (day/night cycles of photosynthetic active radiation and temperature, low water velocity, and similar microbiome to lowland rivers and stagnant water bodies) and then manipulated temperature and sediment presence (i.e., 10-13 ºC vs. 16-18 ºC, and presence vs. absence of sediments). An H1N1 low pathogenic AIV (LPAIV) strain was spiked in the streams, and water and sediment samples were collected at different time points until 14 days post-spike to quantify viral RNA and detect infectious particles. Viral RNA was detected until the end of the experiment in both water and sediment samples. In water samples, we observed a significant combined effect of temperature and sediments in viral decay, with higher viral genome loads in colder streams without sediments. In sediment samples, we didn´t observe any significant effect of temperature. In contrast to prior laboratory-controlled studies that detect longer persistence times, infectious H1N1 LPAIV was isolated in water samples till 2 days post-spike, and none beyond. Infectious H1N1 LPAIV wasn´t isolated from any sediment sample. Our results suggest that slow flowing freshwater surface waters may provide conditions facilitating bird-to-bird transmission for a short period when water temperature are between 10-18 ºC, though persistence for extended periods (e.g., weeks or months) may be less likely. We hypothesize that experiments simulating real environments, like the one described here, provide a more realistic approach for assessing environmental persistence of AIVs.

Graphical abstract:



4.2. Introduction

Avian influenza viruses (AIVs) belong to the *Orthomyxoviridae* family and have a segmented, single-stranded RNA genome. AIVs have an evolutionary plasticity that allows them to replicate in different species like waterfowl, poultry, and mammals, including humans. Based on the ability to cause disease and death in chickens, AIVs can be classified into two different pathotypes: low pathogenic (LP) and highly pathogenic (HP). LPAIVs can be asymptomatic, but they typically cause mild to moderate respiratory disease in chickens, often accompanied by a decrease in water or feed consumption and drops in egg production. In contrast, HPAIVs cause severe systemic disease with very high mortality in chickens and other Galliformes [62]. Outbreaks of AIV currently represent a big challenge for the poultry sector, wildlife, and public health, as exemplified by the 2021-2022 HPAIV epizootic in Europe [63]. Even if there is a low zoonotic risk of the current HPAIV strains circulating in Europe, the emergence of strains with zoonotic potential is an obvious concern, as some HPAIVs have been able to replicate in humans [63].

Wild waterfowl are the main reservoir of AIVs [280]; they can disseminate these viruses globally through their migration routes and occasionally spread them to poultry, sometimes with devastating effects [149,281,282]. Furthermore, migratory waterfowl can also suffer from high mortality rates by HPAIV infections, threatening wildlife conservation [14]. Therefore, the study of waterfowl habitats that intersect different avian migration routes is key to understanding transmission dynamics and, ultimately, designing optimal management strategies. Since transmission of AIV among wild birds occurs mainly via the faecal-oral route through water containing viral particles [169,283,284], environmental transmission through this vehicle may facilitate spillovers to poultry.

Decades of laboratory-based investigations provide evidence that AIV may remain infectious in water and sediments for extended periods (weeks or months) under experimentally controlled conditions. The role of some abiotic factors is well studied: low water temperatures, neutral pH, and low-salinity water conditions substantially increase AIV persistence [170,285,286]. The effect of biotic factors has also been studied, showing that in filtered and sterilized water AIV can persist for

longer periods than in untreated water collected in natural sources with the presence of microorganisms [174,177,182,184–186]. Furthermore, wild bird-origin AIVs have repeatedly been isolated [180,284,287–289] or detected by RT-PCR [290,291] from surface water, and isolated [292] or detected by RT-PCR [293,294] from sediments collected from freshwater and estuarine wetlands. Regarding the amount of AIV needed to initiate an infection in waterfowl via the faecal-oral route, a recent study suggests that even very low titers, around 2 $\log_{10}$ 50% tissue culture infection dose ($TCID_{50}$) may be sufficient [295]. These findings are in line with the hypothesis that environmental transmission plays a crucial role in AIV epidemiology. However, field experiments carried out under environmental conditions to validate results from laboratory investigations are rare [156]. Only recently, a combination of field- and laboratory-based approaches suggested that waterfowl AIVs could remain viable for months in surface water of northern wetlands in North America, supporting the tenet that surface waters may act as an important vehicle in which AIV may be both transmitted and maintained, potentially serving as an environmental reservoir for infectious AIVs [15]. Nevertheless, the effect of UV light and/or water movement were not examined in that study.

Most information about AIV persistence in water has been obtained from studies where AIV was diluted in water and maintained inside a tube (virus-in-a-tube experiments), under controlled conditions lacking the realism of natural ecosystems. Here, we used artificial streams that can mimic biotic and abiotic natural conditions of waterfowl habitats, providing a more refined inference on the duration of AIV infectivity in wetland systems. More specifically, we assessed the effect of temperature (10–13°C *vs.* 16–18°C), the effect of sediments (with the associated microbiota), and the interaction of these two variables on LPAIV water persistence in a more realistic model. Based on previous works, we hypothesized that: 1) colder water temperature (10–13°C) might have a greater protective effect on infectious viral particles than warmer water temperature (16–18°C); 2) the presence of sediments could have a sorption effect on AIVs protecting them from abiotic effects; and 3) correspondingly, the interaction of colder water temperature with the presence of sediments could have the main protective effect on AIVs. Given recent HPAIV detections in wetlands from the Mediterranean coast of Catalonia

[296] and throughout Southern Europe, we also used our results to speculate on AIV persistence in Mediterranean waterfowl habitats during the winter-spring period.

4.3. Materials and methods

4.3.1. Virus stock

The LPAIV isolate A/Duck/Italy/281904/06 H1N1 LPAIV was used (kindly provided by Dr. Ana Moreno from the Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Italy). The working stock was prepared in embryonated chicken eggs (ECE) [297], filtered (0.2 μm), and titrated in Madin–Darby Canine Kidney (MDCK, ATCC CCL-34) cells using the $TCID_{50}$, calculated following the Reed and Muench method [298].

4.3.2. Experimental design

The experiment was performed at the artificial streams facility of the Catalan Institute for Water Research (ICRA, Girona, Catalonia, Spain). This indoor experimental system simulates the natural water environment under controlled conditions [299]. Here, four different treatments were evaluated (three replicates per treatment), with two sediment conditions (presence [S1] *vs.* absence [S0]) and two temperature conditions (10–13°C [T0] *vs.* 16–18°C [T1]) akin to those found in a Mediterranean river during winter-spring (Figure 1). Water temperature varied over time following day/night cycles of light, a constant parameter from the experimental set-up, as explained below. Each artificial stream was 2 m long, had a rectangular cross-section of 50 cm^2 and was set up as an open system with constant slope and steady, uniform flow. Water and sediments were obtained from a non-polluted lower section of the Ter River (Jafre, Catalonia, Spain) close to a wetland area where many species of waterfowl are present (Aiguamolls del Baix Ter, Catalonia, Spain). Sediments were mostly sandy and median sediment grain size was 0.02 mm. Controlled parameters constant to all treatments were daily cycles of photosynthetic active radiation (PAR) (12 h daylight + 12 h darkness) that emulated

the solar radiation, reproducing all the wavelengths at the same intensity modifying water temperature, and 5.5 l of water in a recirculating flow of 0.01 l/sec. These cycles of photosynthetic active radiation were chosen to mimic autumn day/night cycles in the Mediterranean region, the season when some waterfowl species arrive from their summer breeding areas. The recirculating flow of 0.01 l/sec was the lowest flow possible in the artificial streams facility and was chosen to mimic very low flow in wetland areas and to avoid a fast eutrophication process due to still water in the streams. All streams were in acclimation for 3 weeks prior to virus spike, a necessary step for the sediment microbiota to colonize the water. The acclimation process was verified as explained below (Section 2.3). Following acclimation, water and sediment samples from all streams were collected and used as negative controls. Subsequently, all streams were spiked with 53.5 ml of H1N1 LPAIV at a titer of 7.3 log_{10} $TCID_{50}$ directly into each 5.5 l stream, resulting in a spike dose of approximately 5.3 log_{10} $TCID_{50}$/ml per stream. The dilution was carried out by gently spiking the virus longitudinally on each stream water surface. Following the virus spike, all streams were sampled and monitored for 14 days as detailed below. Water evaporation was compensated for by adding 400 ml of Ter river water per stream daily. As a positive control, viral persistence was also assessed under virus-in-a-tube conditions at Centre de Recerca en Sanitat Animal (IRTA-CReSA, Bellaterra, Catalonia, Spain). Briefly, 24 Eppendorf tubes (12 tubes at 10°C and 12 tubes at 16°C) containing each 1.5 ml of water from Ter River without sediment were spiked with H1N1 LPAIV at a final titer of 5.3 log_{10} $TCID_{50}$ per ml and maintained at their corresponding temperature for 14 days.

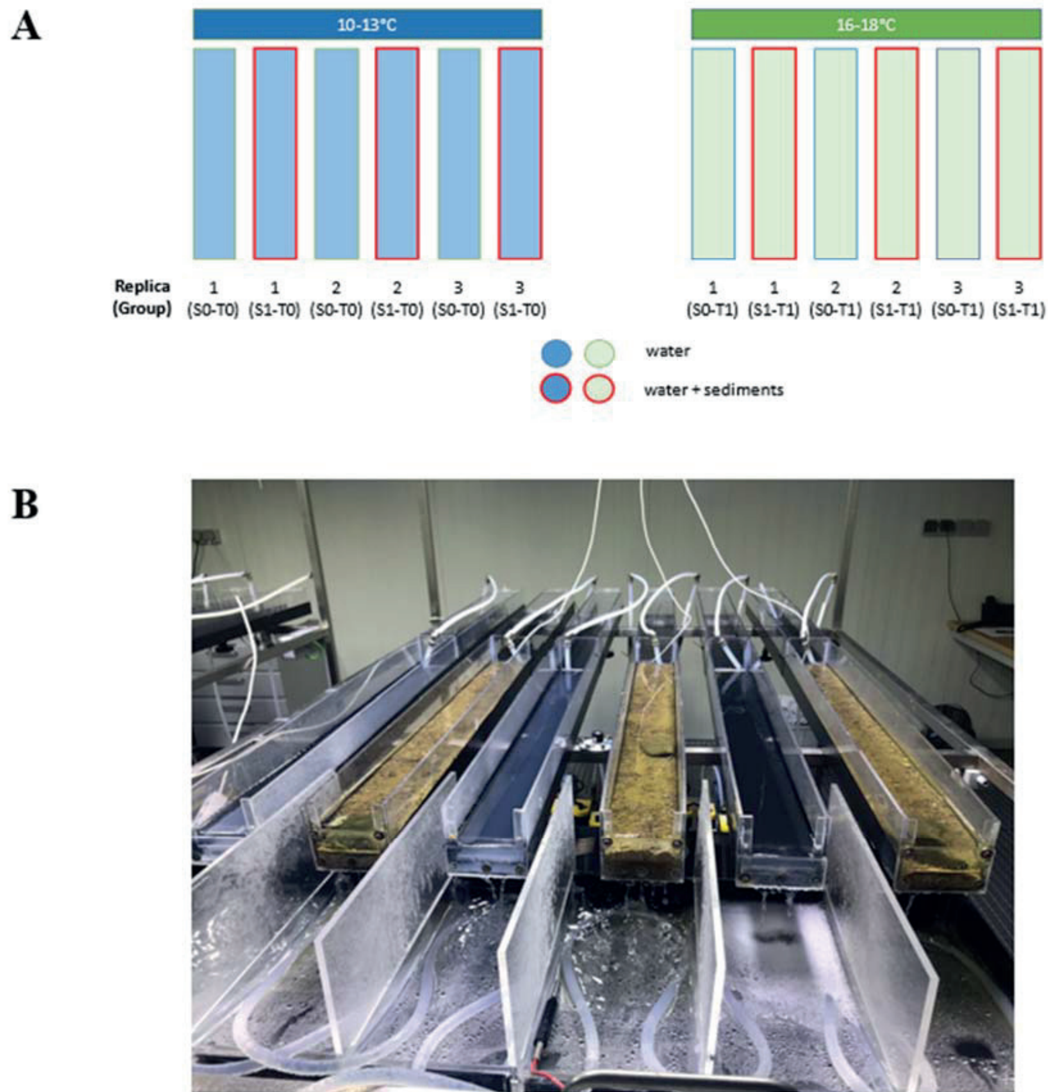


Figure 1. A. Schematic representation of the artificial streams. B. Artificial streams at ICRA, showing six of the 12 streams used in the experiment.

4.3.3. Characterization of water and sediments

Water and sediment samples were collected throughout the experiment for characterization purposes. Dissolved oxygen, pH, and specific conductivity were measured every 48h by noon at each artificial stream using Wissenschaftlich-Technische Werkstätten (WTW) hand-held probes (Weilheim, Germany). Water temperature in each artificial stream was recorded every 10 min during all the experiment by means of VEMCO minilog temperature data loggers (TR model; AMIRIX Systems Inc, Halifax Canada) (5–35 °C, +/-0.2 °C). Photosynthetically active

radiation was also recorded every 10 min using 4 quantum sensors (LI-192SA; LiCOR Inc, Lincoln, NE, USA) located across the whole array of streams.

Biofilm colonization was allowed in the artificial streams for 3 weeks before virus spike. Briefly, superficial sediments (1-10 cm depth) were brought from a nearby lowland river (Ter River near the mouth, Girona), then spread, with no further treatment, in the artificial streams forming a layer of 3-4 cm depth. A colonization period was then allowed so that the biofilms could adapt and grow on the artificial streams. During this acclimation period, biofilm status was monitored twice per week for their maximum photosynthetic efficiency. These measurements provided information on the physiological status of the biofilm [300] in the artificial streams and were made to assess the physiological similarity of the biofilms in all the artificial streams before exposure to the different temperatures. Biofilm samples were collected in streams with sediments after the acclimation period (pre-spike) and at 14 days post-spike at both temperature ranges (see Section 2.6).

4.3.4. Sampling of water and sediment

Water and sediment samples were collected from all streams at 12 different time-points: pre-spike (as a negative control), 0 (10 min post-spike), 1, 2, 3, 4, 5, 6, 7, 9, 11, and 14 days post-spike. Briefly, 9 ml of water per stream and time-point were collected with a micropipette and stored in 1.5-ml aliquots (Suppl. Figure S1) at –75 ºC. Approximately 2 ml of sediments per stream and time-point were collected following the "core" method (Suppl. Video 1), with a 15-ml falcon tube and stored at –75 ºC. Similarly, one tube per temperature condition and time-point from the virus-in-a-tube experiment was collected and stored at –75 ºC.

4.3.5. Viral detection and quantification in water and sediment samples

Water and sediment samples were used for virus isolation and RNA extraction. While water samples were processed without any additional manipulation, the procedure for extracting virus from sediments was optimized for virus recovery prior to processing samples. To that end, two different methods were tested and compared using sediment samples collected as negative controls from our experiment that were subsequently spiked with 5.3 $\log_{10}$ $TCID_{50}$/ml of sediment with H1N1 LPAIV. For the first method, a sterile gauze was wet with 5 ml of brain

heart infusion broth supplemented with 2% Penicillin/Streptomycin (Invitrogen, Spain). Then the sediment pebbles were manually rubbed with the wet gauze, and the gauze was placed in a sealable plastic bag. A masticator homogenizer (IULmicro, Spain) was used for 1.5 min to punch and squeeze the supernatant out of the gauze and inside the plastic bag. Finally, the supernatant was collected into sterile 50-ml falcon tubes using a micropipette and squeezing the remaining supernatant out of the gauze with a 20-ml syringe. This sediment supernatant was used for virus isolation and RNA extraction alongside water samples. For the second method, the same steps were followed, but a pestle instead of a masticator was used, as previously described (Stephens & Spackman, 2017). The first method using the masticator was able to recover 4.75 log_{10} $TCID_{50}$, in contrast with the method using the pestle which only recovered 4.2 log_{10} $TCID_{50}$, validating the use of the method using the masticator.

Water and processed sediment samples were used for quantification of M gene copies. Viral RNA was extracted using Nucleospin RNA virus kit (Macherey–Nagel, Düren, Germany) following manufacturer's instructions. A highly conserved region of 99 bp present in AIV M1 gene was amplified and detected by one-step Taqman real-time RT-PCR (rRT-PCR) in Fast7500 equipment (Applied Biosystems, USA) [302]. A standard curve from the same region of the AIV M1 gene was obtained as previously described by our group [303] and used to obtain the gene equivalent copies from the rRT-PCR results. Furthermore, virus isolation from water and sediment samples was conducted in ECEs by standard methods [297]. Briefly, 3 ECEs were inoculated with 0.2 ml of undiluted water and sediment samples by the chorioallantoic sac route. Standard hemagglutination assay was used to test the allantoic fluid from each ECE for virus replication [304]. Hemagglutinating allantoic fluids were assumed to represent the presence of infectious AIV in water and sediment samples.

4.3.6. Characterization of the sediment microbiota

DNA was extracted from biofilms developed on sediments using the FastDNA Spin for Soil kit (MP Biomedical) according to the manufacturer's instructions. High-

throughput multiplexed *16S rRNA* gene sequencing with the Illumina MiSeq System (2×250 PE) was carried out using primer pair 515f/806r (Caporaso et al., 2011) targeting the V4 region of the *16S rRNA* gene complemented with Illumina adapters and sample specific barcodes. Details on the analysis of sequence datasets including comparison of alpha and beta diversity of samples across treatments are described in Supplementary Material.

4.3.7. Statistical analysis

Results obtained from AIV rRT-PCR were used to evaluate the effects of the temperature and the presence or absence of sediments by two methods. First, a Kruskal-Wallis Test [305] was used to assess differences between groups. If any differences were found, a Dunn's Test [306] for multiple comparisons was applied to test which treatments were significantly different. Second, to compare the treatments for each time point, a mixed-effects model with the stream as a fixed factor and treatment and time point as random factors was adjusted, along with a contrast of the estimated marginal means. Results obtained from virus isolation were used to determine differences between treatments in their survival curves with a Gehan-Breslow-Wilcoxon test. All the statistical analyses were performed with the R software [307] and the subsequent packages: car, FSA, lmerTest, emmeans and tidyverse.

4.4 .Results

Physical and chemical characterization of water and sediment samples

The temperature of the streams under colder conditions was maintained between 10°C and 13°C (mean of 11.6°C and a standard deviation of 0.95), while the temperature of the streams under warmer conditions was maintained between 16°C and 18°C (mean of 17.5°C and a standard deviation of 0.89) throughout the experiment, temperature variation was due to day/night PAR cycles. The pH in all the streams and time points was around 8, and the specific conductivity was

moderate (≈ 500 µs/cm), without significant differences among streams at any time. The physical and chemical characterization of the water did not show significant differences among treatments throughout the experiment (Suppl. File 1).

Characterization of the sediment microbiota

Characterization of microbial communities in the streams containing sediments was performed to assess potential variations across treatments (i.e., water temperature) that could affect viral persistence during the experimental period. Bacterial communities in sediments did not show significant differences in richness and diversity among streams differing in water temperature (10–13 ºC *vs.* 16–18 ºC) (Suppl. Figure S2). However, a Principal Coordinates Analysis (PCoA) ordination of samples using the Bray-Curtis dissimilarity distance segregated samples according to both the water temperature (horizontal axis, 26% sample variance, Suppl. Figure S3) and the experimental time (time 0 *vs.* time 14 days, vertical axis, Suppl. Figure S3) (PERMANOVA test, F-value= 2.4207; $p < 0.016$).

Viral RNA quantification in water and sediment samples

Water and sediment samples from the artificial streams experiment were used for RNA extraction and quantification of H1N1 LPAIV M gene copies by rRT-PCR at 12 different time points. All negative control samples (samples collected pre-spike) were negative, confirming the absence of AIV from water and sediments from Ter River. Overall, water and sediment rRT-PCR results showed presence of viral RNA throughout the 14-day experiment, although a steady decline over time was observed (Figures 2 and 3).

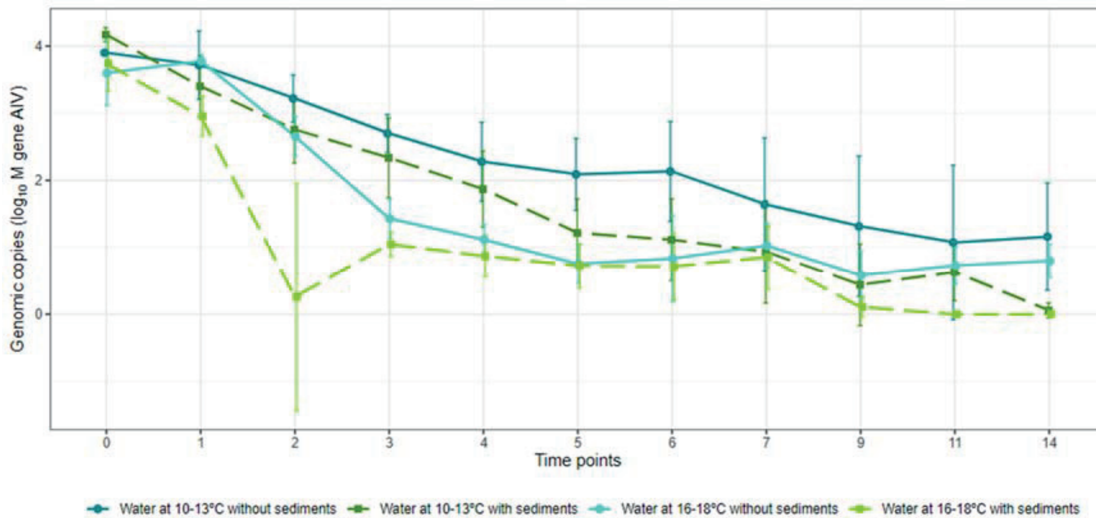


Figure 2. M gene copies in **water** samples in the four analysed treatments in the artificial streams experiment. Results are expressed as mean and standard deviation (three replicates per treatment) expressed in log_{10} values.

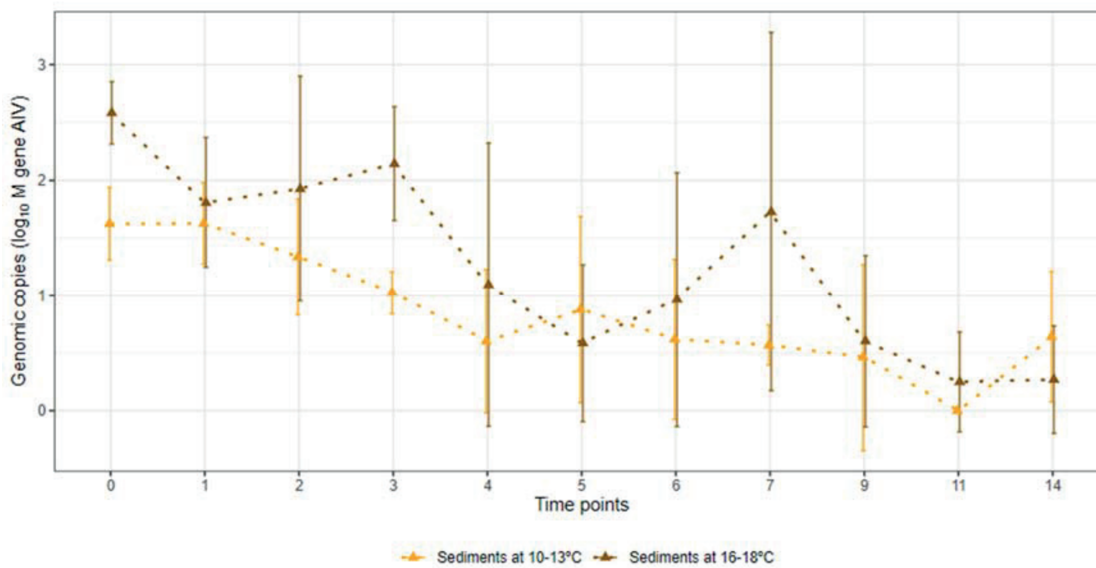


Figure 3. M gene copies in sediment samples in the two analysed treatments in the artificial streams experiment. Results are expressed as mean and standard deviation (three replicates per treatment) expressed in log_{10} values.

In water samples, significant differences in viral RNA decay were only observed between streams at different temperatures (10–13 ºC *vs.* 16–18 ºC) regardless of sediment condition (multiple comparison Dunn's test). However, the interaction between 10–13 ºC temperatures and the absence of sediment compared with the interaction of 16–18 ºC temperatures and the presence of sediment showed the most significant differences in LPAIV RNA persistence (Suppl. Table S1). In sediment samples, no significant differences in viral RNA decay were observed between streams at different temperatures (Kruskal-Wallis test) (Suppl. Table S2). Significant lower numbers of genomic copies were detected in sediment samples compared to water samples on the first days (taking together both temperature conditions), but similar numbers were found in both types of samples on subsequent days (Figure 4 and Suppl. Table S2).

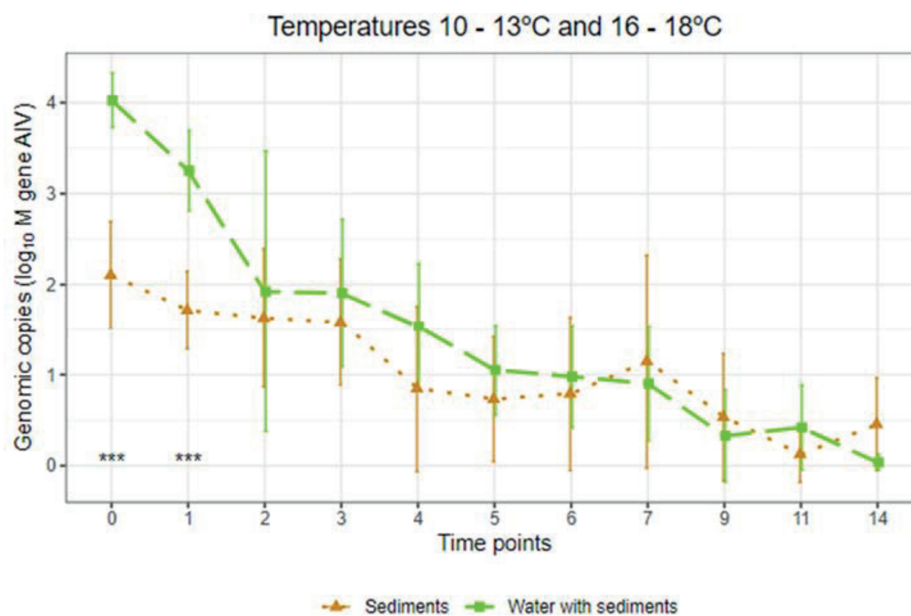


Figure 4. M gene copies in sediment samples and water samples from streams with sediments. Results are expressed as mean and standard deviation (three replicates per treatment) expressed in $\log_{10}$ values.

Viral isolation from water and sediment samples

To detect infectious H1N1 LPAIV, viral isolation in ECE from water and sediment samples was carried out. Viral isolation from water samples was successful from all the streams at 0 days post-spike, from six of 12 streams at 1 day post-spike, from one stream at 2 days post-spike, and no infectious viruses were detected beyond that (Table 1). No significant differences in viral survival among treatments were detected (Table 1). No infectious virus was recovered from any of the sediment samples. In contrast to the artificial streams, viral persistence in water under virus-in-a-tube conditions yielded infectious viruses from all samples at all time points, both at 10 ºC and 16 ºC temperature conditions (Table 1).

Table 3. Results of virus isolation from water samples for each replicate by treatment in each of the different experiment setups (i.e., artificial streams and virus-in-a-tube conditions). Results are shown as the number of positive replicates/total replicates. NA = not apply.

Treatment	Sediment	Temperature	Experiment setup	Day 0	Day 1	Day 2	Day 3 to14
S1-T0	Present	10–13 ºC	Artificial streams	3/3	3/3	0/3	0/3
S0-T0	Absent	10–13 ºC	Artificial streams	3/3	1/3	1/3	0/3
S1-T1	Present	16–18 ºC	Artificial streams	3/3	1/3	0/3	0/3
S0-T1	Absent	16–18 ºC	Artificial streams	3/3	1/3	0/3	0/3
NA	NA	10 ºC	Virus-in-a-tube	3/3	3/3	3/3	3/3

4.5.Discussion

Most of the data regarding environmental AIV persistence comes from virus-in-a-tube experiments, which do not completely reproduce real environmental conditions [170,285,286]. Even if some studies have attempted to reproduce more realistic environmental conditions [15,308], the persistence of AIV in water has not been yet fully characterized in simulated environments. In this study, we mimicked biotic and abiotic natural conditions of waterfowl habitats using artificial streams to analyse the effect of temperature and sediments on the persistence of LPAIV. Even if we still didn´t fully characterize the simulated environment, we tried to go one step further mimicking factors such as UV light cycles and streamflow.

First, we characterized the streams after the acclimation period. Similar physical and chemical properties were found among the streams at the same temperature and sediment conditions. Water had a neutral-to-basic pH and moderate specific conductivity throughout all the experiment; these characteristics were previously associated with the longest AIV persistence periods [170,297,309]. Furthermore, the characterization of bacterial communities in the sediments was also analysed to assess the potential effect of the sediment microbiota on viral persistency. No great differences in richness and diversity of sediment microbial communities were observed across treatments, although beta diversity analyses revealed differences between streams according to water temperature and incubation time. This observation suggests that maturation of biofilm communities was clearly influenced by ambient conditions (i.e., temperature). Recently, Romero and co-workers reported a 92% similarity between bacterial communities from the artificial streams and those from the source location [299]. Here, since the conditions of the artificial streams were very similar to those of the source river (non-polluted reach of the river Ter, see Materials and Methods), we can assume that the studied sediment microbiota was analogous to that in the real, streambed biofilms. Overall, our results indicate that the conditions, both abiotic and biotic, in the artificial streams were comparable to those existing in surface water from the area of Spain from which water and sediment was collected and where AIV may persist.

Viral RNA genomic copies throughout the experiment were determined in water and sediment samples by rRT-PCR. It is worth mentioning that we did not concentrate

water samples since our goal was to obtain the most realistic results. Genomic copies of the spiked LPAIV were detected both in water and sediment samples until the end of the experiment (14 days), suggesting that the use of environmental samples for viral RNA detection could be a good surveillance method for the early detection of viral RNA from AIVs, as was previously suggested but not to reveal actual infectious AIV virus [189,286,310]. However, fewer genomic copies were found at day 0 than expected, a phenomenon probably related to viral aggregation after the addition of LPAIV to water at different pH and conductance levels [311–314]. Significant differences in genomic copies in water were observed between streams at different temperature conditions regardless of the sediment condition, but not between streams with or without sediments at the same temperature condition. This observation suggests that, in our study, the presence of sediments —and therefore of associated biotic factors— played a less important role in the degradation or retention of viral RNA than the temperature. These results could be associated with the absence of organisms capable of filtering or feeding LPAIV in our artificial streams [159–161]. Also, water movement (even if minimal) could be the reason for the low sediment retention. However, a significant combined effect of temperature and sediments in viral decay was observed, with higher viral molecular signal in colder streams without sediments. Furthermore, there was a reduction in the difference of RNA amplification signal between water samples and sediment samples over time, with almost no differences in the last days. These results suggest some degree of viral sorption in sediments. In contrast, and as expected, the temperature had a clear significant effect on viral detection in water samples, with significantly higher amounts of gen amplification signal detected in the colder streams. The favouring effect of cold-water temperature in LPAIV persistence is widely known [170,172,173,176]. The lack of significant differences in viral molecular signals in sediment samples between streams at different temperatures could be related to a protective effect of the sediments against abiotic factors, such as temperature.

The ultimate objective of our study was to analyse viral infectivity over time to determine the infectious capacity of LPAIV in Mediterranean waterfowl habitats. For that purpose, water and sediment samples were inoculated in ECE to determine their infectivity. The detection of viral RNA by rRT-PCR did not associate with the

recovery of infectious particles in water samples. Even though viral isolation was successful from all water samples at 0 days post-spike (10 minutes after spike) and from six streams on day 1, isolation in ECE was only successful in one stream at 2 days post-spike. No significant differences in viral isolation results were observed between streams differing in water temperature and presence of sediment, probably due to the rapid decrease in viral infectivity observed in all streams. Surprisingly, we found relatively short viral persistence compared to virus-in-a-tube studies, even though our initial spiking dose was close to other studies [176]. Consistently, we also found relatively short viral persistence in our artificial streams compared to our virus-in-a-tube experiment, performed as a positive control. Our artificial streams results are in line with a simulated environment study that reproduced real conditions of Cambodian lakes in experimental aquatic biotopes, where LPAIV isolation from water was possible during a maximum of 4 days post-spike [308]. Nonetheless, another realistic study performed in Alaska showed longer persistence results, with evidence for interannual LPAIV persistence [15]. The differences observed between these simulated environments studies are probably related to differences in water temperature, since higher temperatures were used to reproduce Cambodia's temperatures (25 ^{o}C) than Alaska's (0 ^{o}C – 16 ^{o}C), and to the different methodologies used, even if initial spiking doses were like ours. We suggest that abiotic factors such as UV light with the associated variation in water temperature and/or the water movement, which were not incorporated in previous studies [15,308] but were analysed in ours, could be related to our short viral persistence. In fact, UV light, at a specific wavelength, is used as a biosafety technique to inactivate AIV in routine laboratory work [315]. Furthermore, infectious viruses were not protected inside a tube as in virus-in-a-tube experiments. For these reasons, PAR cycles could have greatly influenced the results. We suggest that northern breeding areas, with very few hours of light during winter and an incidence angle that can make the radiation less penetrating, could reduce the effect of this factor. We also hypothesize that faeces or feathers could protect viral particles against UV light. In any case, further studies need to be done to confirm these hypotheses.

No infectious LPAIV was recovered from any of the sediment samples, suggesting that sediments did not play a major role in viral persistence in our study. However, several studies have described that sediments can act as AIV reservoirs, although viral infectivity was not evaluated [293,294,316] or a gem carrier was used [163]. In fact, one study tried to isolate infectious virus from sediment samples and was successful only at high infectious doses (≥ 100 plaque-forming units per 200 µl), linking such low recovery rate from sediment samples to inhibition, disruption of viral particles, or viral attachment to the sediment [164].

Our study was performed in artificial streams mimicking the environmental conditions of the waterfowl habitat in a Southwestern European region with a Mediterranean climate (Catalonia, Spain) during the winter-spring period. In this specific region and conditions, we can find AIV susceptible species, such as the grey heron (*Ardea cinerea*), the yellow-legged gull (*Larus michahellis*), and the mallard duck (*Anas platyrhynchos*) among others [317]. Indeed, outbreaks of HPAIV in waterfowl have already been detected in recent years. For example, in a wetland area from Catalonia (Parc Natural dels Aiguamolls de l'Empordà, Girona, Spain), which is close to the Ter River, HPAIV has been detected in a white stork (*Ciconia ciconia*) during the 2016-2017 season, and in a white stork and a greylag goose (*Anser anser*) during the 2020-2021 season. More recently, seven positive wild birds were detected in wetlands in Catalonia and 38 wild birds all around Spain during the 2021-2022 winter migration period [296]. Our experimental results corroborate that waterfowl habitats in these mild-temperature latitudes can still provide the right conditions for bird-to-bird (faecal-oral) transmission of AIVs among waterfowl, both migratory and resident, that normally aggregate in a specific area for a short period, as seen in the recent outbreaks with numerous AIV-positive wild birds from the same location. Even if more experiments need to be done, with different water and sediments sources or with different AIV strains, our results seem to suggest that interannual persistence of LPAIV in water ecosystems is unlikely in such Mediterranean climate. This underscores direct or almost direct faecal-oral transmission in contrast to environmental transmission (i.e., without the presence of infected birds), due to the low persistence times found. However, these

results could differ significantly in other latitudes with environmental conditions more favourable for AIV persistence. In fact, studies reproducing Alaska's conditions suggest that its water ecosystems play a crucial role in LPAIV transmission among migratory waterfowl, as they are a breeding place for these birds [15,318]. This highlights a paradox of our study, while it better approximates real conditions in the field, it also narrows the parameters to which the results can be applied, here restricted to some relevant conditions mimicking a specific region and season. Furthermore, our study did not consider the protective effect of faeces and feathers on LPAIV persistence that some authors suggested [162,163] so longer persistence times could be found if this protective effect would have been incorporated. Nevertheless, artificial streams that reproduce waterfowl breeding ecosystems, such as Alaska's, could be used to study the effect of abiotic factors such as UV light, and to confirm the role of these ecosystems in the interannual persistence of LPAIV.

Declaration of competing interest

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

CRediT authorship contribution statement

The project was conceived, and funding was obtained by MN, AR, and NM. The experiment was designed by AP, KB, VA, MC, FXA, and NM. The experiment was performed by AP, VA, and KB. Samples were collected and processed by AP, KB, VA, and RV. LPG has done the statistical analysis. CMB has done the microbiome analysis of sediments. Figures were prepared by LPG, AP, and KB. The manuscript was drafted by AP with corrections from MC, KB, VA, FXA, MN, AR, LPG, and NM. All authors contributed to the article and approved the submitted version.

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4.6. Supplementary Material

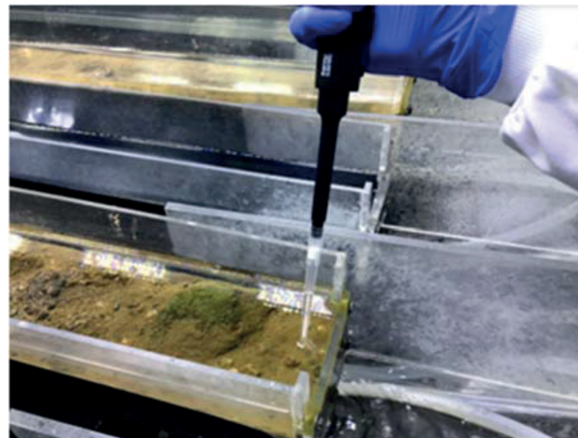
DNA extraction, High-throughput sequencing, and analyses of sequence datasets

DNA was extracted from biofilm samples using the FastDNA Spin for Soil kit (MP Biomedical) according to the manufacturer's instructions. Quality of the final DNA was measured with a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific; Wilmington, DE, USA) by measuring A260/A230 and A260/A280 absorbance ratios. The concentration of DNA in final extracts was measured using a Qubit 2.0 fluorometer (Life Technologies; Carlsbad, CA, USA).

High-throughput multiplexed 16S rRNA gene sequencing with the Illumina MiSeq System (2×250 PE) was carried out using primer pair 515f/806r [319] targeting the V4 region of the 16S rRNA gene complemented with Illumina adapters and sample specific barcodes. Sequencing was performed at the Sequencing and Genotyping Unit of the Genomic Facility/SGIker of the University of the Basque Country. Demultiplexing, quality filtering, clustering into amplicon-sequence variants (ASV) and construction of the ASV table were performed in QIIME2 [320]. The analysis yielded 2,561,844 total reads of high quality (80% of reads averaged ≥ Q35 scores) distributed across 12 samples with a minimum and maximum number of reads per sample of 93,569 and 328,230, respectively. Deblur algorithm was used to build the ASV table (18,284 ASV in total). Representative ASV sequences were aligned against Greengenes v12_08 [321]. The feature-classifier script implemented in QIIME2 was employed for taxonomic assignment using the SILVA reference database v. 138 [322]. Before analysis of alpha and beta diversity, the ASV table was filtered to remove spurious ASVs having less than 100 counts across samples. 2,623 ASVs were retained after filtration (14% of original ASV), comprising up to 88% of the unfiltered reads and indicating that filtered ASVs were extremely rare across samples.

For community analysis (alpha and beta diversity), the filtered ASV table was rarefied by randomly selecting a subset of 84,000 sequences per sample to minimize bias due to different sequencing depth across samples. Alpha and Beta diversity analyses were performed in the Microbiome Analyst (MA) platform [323] after transforming the data using the Centered Log Ratio algorithm. Alpha diversity was assessed using richness (Chao1) and diversity (Shannon) indices and compared through a Kruskal-Wallis pairwise test. Beta diversity was assessed by computing Bray–Curtis distance between samples and then differences between conditions (high *vs.* low temperature) was assessed by permutational multivariate analysis of variance (PERMANOVA).

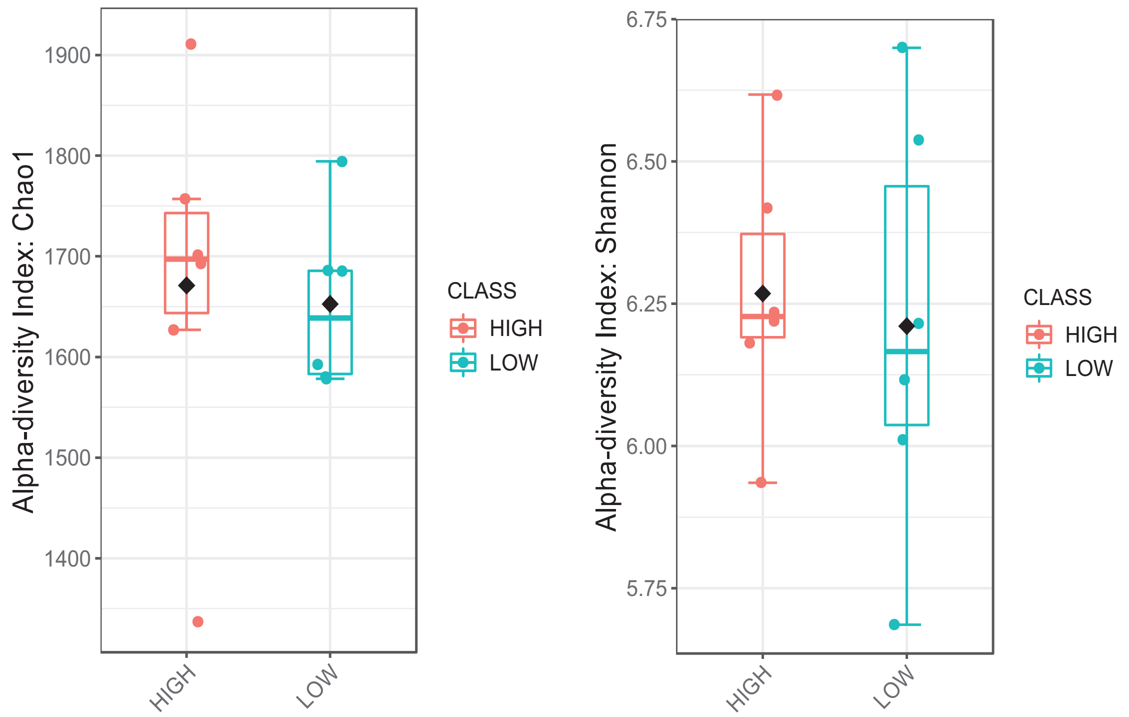
Supplementary Figures and Tables



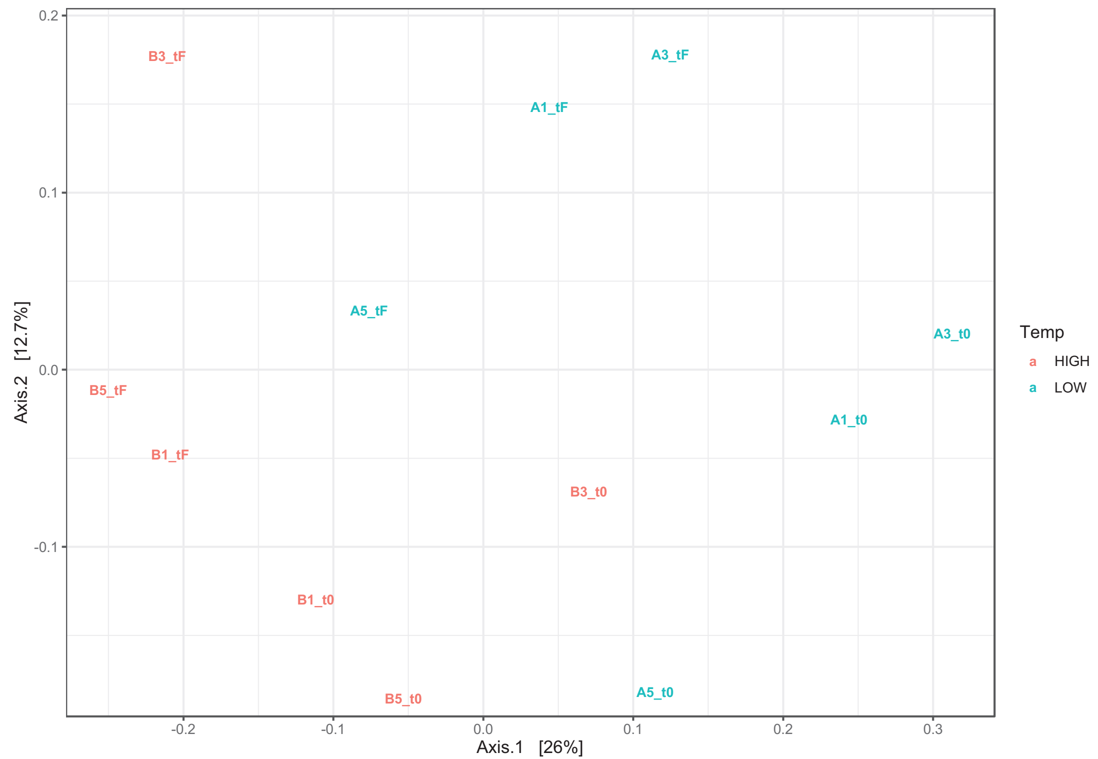
Suppl. Figure S1. Water sample collected with a micropipette.

Suppl. Video. Sediment sample collected with the "core" method. This supplementary material was uploaded to Zenodo online repository: https://zenodo.org/record/7389054#.Y4m4WHaZOUk

Suppl. File. Excel file with data of the physical and chemical characterization of the water (anions, cations, total organic carbon). This supplementary material was uploaded to Zenodo online repository: https://zenodo.org/record/7389109#.Y4m5cHaZOUk



Suppl. Figure S2. Comparison of richness (Chao, left) and diversity (Shannon, right) indices across conditions (High vs. Low temperature).



Suppl. Figure S3. PCoA ordination of samples according to the Bray-Curtis dissimilarity distance. Samples rarefied at the minimum library size (84,000 seqs/sample). Samples are colored by temperature condition and labelled by sample name as follow: A and B refer to channels, 1-3-5 refer to different replica channels; t0: samples collected at time 0; tF: samples collected at the end of the experiment. PERMANOVA test resulted in a significant difference across samples (F-value= 2.4207; R2 = 0.19489; $p < 0.016$).

Suppl. Table S1. *Summary of the p-values of the multiple tests. The first column represents the results from the multiple comparison Dunn's Test and the other ones shows the results from the contrast of the estimated marginals means in each time point.*

Comparison	Dunn's	0	1	2	3	4	5	6	7	9	11	14
S0.T0 - S0.T1	0.0438	1.0000	1.0000	0.5541	0.0158	0.0398	0.0126	0.0161	0.5983	0.3505	1.0000	0.7826
S0.T0 - S1.T0	0.2781	1.0000	1.0000	0.9274	1.0000	1.0000	0.3519	0.2714	0.8260	0.3600	1.0000	0.0627
S0.T0 - S1.T1	0.0002	1.0000	0.2608	0.0000	0.0052	0.0185	0.0094	0.0026	0.0611	0.0098	0.0821	0.0477
S0.T1 - S1.T0	0.2729	1.0000	1.0000	0.9274	0.0218	0.0690	0.3519	0.3504	0.8260	0.7032	1.0000	0.2703
S0.T1 - S1.T1	0.1006	1.0000	0.2268	0.0004	1.0000	1.0000	0.8680	0.5025	0.6945	0.3508	0.3547	0.2509
S1.T0 - S1.T1	0.0071	1.0000	0.3372	0.0000	0.0083	0.0398	0.3378	0.1829	0.3521	0.2675	0.2520	0.8611

Suppl. Table S2. Summary of the p-values of the multiple tests. The first column represents the results from the multiple comparison Dunn's Test and the other ones shows the results from the contrast of the estimated marginals means in each time point. S: sediment samples, W: water samples.

Comparison	Dunn's	0	1	2	3	4	5	6	7	9	11	14
S (10–13 ºC) – S (16–18 ºC)	0.3160	0.1056	0.7124	0.4562	0.0960	0.9554	1.0000	1.0000	0.0980	1.0000	1.0000	1.0000
S (10–13 ºC) - W (10–13 ºC)	0.0055	0.0000	0.0005	0.0059	0.0097	0.0163	1.0000	0.5357	0.4622	1.0000	0.5198	1.0000
S (16–18 ºC) - W (16–18 ºC)	0.3017	0.0261	0.0872	0.1365	0.1705	0.9554	1.0000	1.0000	0.0872	0.7937	1.0000	1.0000

Chapter V

General Discussion

The interconnection between environmental, animal, and human health is now more evident than ever after the COVID-19 global pandemic most probably occasioned by a zoonotic coronavirus that jumped from a wild animal [5]. Furthermore, global and fast changes, such as deforestation, climate change and the increase in worldwide mobility will imply new dynamics in infectious diseases and new challenges to global health. A One Health approach will be essential to tackle these problems. The disease caused by AIVs is the perfect example of this, with clear environmental (e.g., viral persistence in the environment, waterfowl distribution throughout flyways, etc.), human (e.g., zoonotic strains, economic problems, food security, etc.), and animal (e.g., huge mortalities in poultry and wild birds, mammals' susceptibility, conservation issues, etc.) factors involved. In this dissertation, we have applied a One Health approach to disentangle the bird-AIV-environment interconnections. For that, we have used innovative methods with a multidisciplinary approach to analyse AIV holistically.

Because the results have been already extensively discussed in the specific chapters, here we will focus on the future perspectives of this work .

In the third chapter, a dual host and pathogen RNA Seq analysis was performed using bioinformatic tools to unravel genes related to disease resistance at the viral and host level. This analysis was performed in full compliance with the three R principles, which promote the ethical use of animals in research, taking advantage of a previous experiment and demonstrating that it is possible to obtain striking results by reusing samples [324]. However, even if very relevant results have been obtained, such as the downplaying of viral within-host diversity and the potential role of *PLAU* in the chicken outcome against HPAIV, the complex interaction between the virus and the host would need extended analysis to be completely unravelled. The use of other omics technics in addition to transcriptomics will be suitable to complete the characterization of the AIV-host interactions. For example, the bird´s microbiota can play a relevant role in the protection against AIV by modulating the innate immune response [132,325]. Therefore, the characterization of bird microbiota by means of metagenomics from resistant and susceptible birds could serve to detect beneficial microorganisms against AIV, as previously shown in avian malaria [326]. The use of genome-wide association analysis could also shed

light on the individual factors that lead to differential AIV outcomes at the DNA level. Some analyses have already been done, but the requirement of huge samples could be a limiting factor [130,327]. Finally, an epigenetic analysis to understand more deeply the factors that regulate the host transcriptomic response to AIV [328], a single cell RNAseq analysis to understand host response at a cellular level [329], and a proteomic analysis to finally determine the proteins translated after an AIV infection [330,331] will complete a multi-omics study necessary to resolve the convoluted mechanisms behind host-viral interactions that lead to disease resistance or susceptibility.

From the virus side, a fully comprehensive analysis of viral compartmentalization and within-host diversity would need to be further complemented by studying other tissues (e.g., cloacal samples, brain, etc.). In addition, the use of accurate long-read sequencing technologies could provide a lower error rate to detect with more precision low-frequency variants and it will eliminate biases due to the different methodologies used in our study. Another engaging approach would be the use of innovative bioinformatics tools, such as machine learning algorithms to predict resistant and susceptible birds before the occurrence of the first clinical signs or deaths. This would solve one of the main problems we had to confront when we designed our study, which was how to determine as resistant or susceptible, chickens sacrificed before showing any clinical signs. That problem limited our study to 3 dpi. The use of trained models with data from susceptible and resistant chickens from 3dpi could be able to classify as susceptible or resistant chickens sacrificed at earlier time points, as previous machine learning algorithms have done to predict disease outcomes [332].

For further validation of the role of DEGs with opposite regulations between resistant and susceptible chickens (i.e., *PLAU, VCAM1, TNFRSF1A,* and *PGF)* and to keep working in full compliance with the three R principles, we could set up lung organoids from chicken tissues as a new *in vitro* platform to use as a chicken model. Organoids are self-organizing and self-renewing three-dimensional cellular structures, similar to organs in structure and function [333]. Lung organoids can be produced from human adult stem cells (i.e., basal cells) obtained from human lung tissue that in the appropriate conditions differentiate into different cell lines, such

as multi-ciliated cells, mucus-producing secretory cells, club cells, and basal cells [334]. Furthermore, organoids have also been obtained from different tissues of domestic animals [333]. This organoid model could be useful to validate the relevant DEGs detected in chapter three, silencing those genes with small interfering RNA [335] and analysing the effect on AIV replication with qPCR and a cell viability test.

If in the third chapter the viral-host interaction was analysed, in the fourth chapter we focused our work on the viral-environment interaction. A unique facility, able to mimic the environmental conditions of waterfowl habitats, was leveraged to determine the persistence time of LPAIV in water from these habitats. Here we showed that a more realistic experimental set-up produced more realistic LPAIV persistence data. As with the host, the interactions with the environment are also complex and deeper analysis is recommended to further understand them. We suggested that day and night cycles of sunlight and water movement could have had a huge impact on viral persistence. To clear up the role of these factors new experiments could be performed selecting these variables. Another interesting experiment would be to mimic the real conditions from other regions, not only the Mediterranean area but also the northern breeding areas where waterfowl stay in summer, transmitting the disease to wild birds from all around the world. Furthermore, we have only used one LPAIV strain, and it is known that some differences exist between strains [165,166], therefore, the use of different strains would be desirable in the future to better understand the role of viral diversity on persistence. In line with this, to unravel viral nucleotide locations linked with viral persistence, a quasi-species analysis could be performed contrasting the viral mutant clouds from the original spiked virus and the mutant clouds from the last infectious virus detected, to determine the low-frequency variants selected in this process. Finally, to complete in the future the analysis of this work, the confluence of the two chapters would be possible by inoculating waterfowl species with water samples obtained from the artificial stream at different time points, thus validating the role of environmental persistence in these birds.

Overall, the results from this thesis evidence that using a multidisciplinary approach is valuable to unravelling the interactions between the host, the virus, and the environment. Additionally, the results obtained from the viral-host interaction

study could be profitable in the future, to determine new therapeutical targets to reduce AIV replication, or even to select AIV-resistant birds. Besides, the results obtained from the viral-environment interaction study helped to understand the persistence times of AIV related to wetlands, a fact that can help in the prevention strategies against global challenges, such as climate change, which can potentially modify the migratory flyways of waterfowl to new wetlands locations. The aforementioned future perspectives will guide our work in the next projects to enter the open doors of this thesis.

Chapter VI

Conclusions

1. Transcriptomic signatures of resistant and susceptible chickens at early stages after H5N8 HPAIV infection reveal that early and optimal regulation of immune pathways (NF-κB or MAPK) could be related to vRNP retention and therefore to HPAIV resistance in chickens.

2. Downregulation of *PLAU (*Plasminogen Activator, Urokinase*)* in H5N8 HPAIV resistant chickens could play an important role in the HPAI outcome by avoiding viral activation and/or fibrinolysis deleterious effects.

3. Within-host viral compartmentalization after H5N8 HPAIV infection points towards multiple stochastic founder events, associated with viral transit and high global diversity in lungs and spleen. In contrast with tissue samples, in oral swabs the lower levels of diversity observed were associated with a minor presence of non-viable viral particles and the effects of innate host immunity.

4. The detection of viral RNA from water and sediment samples obtained from H1N1 LPAIV in spiked artificial streams suggests that eRNA could be a useful target for AIV surveillance under certain conditions, although might not reflect the environmental infectiousness.

5. The use of artificial streams mimicking waterfowl conditions showed that the effect of water temperature had a greater impact on AIV persistence than the presence of sediments.

6. Experiments performed closer to real conditions generate more realistic LPAIV persistence data. The use of sunlight cycles and water movement in our artificial streams experiment could be related to the short persistence times detected in the present work.

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