



Genetic Protection against Avian Influenza

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A report for Australian Eggs Limited
by K.R. Short

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Foreword

This project was conducted to investigate methods to understand and ultimately reduce the mutations that arise in avian influenza in chickens. Specifically, we wished to focus on the role that the chicken proteins Sem3A and neuropilin (NRP1) played in the evolution of avian influenza. This is based on the evidence that Sem3A is a known immune modulator that binds to NRP1 on endothelial cells. Independent of its interaction with Sem3A, NRP1 preferentially binds avian influenza viruses with a multibasic cleavage site (MBS) and therefore may play a role in selecting for the evolution of these viruses.

This project was funded from industry revenue, which is matched by funds provided by the Australian Government.

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Prof. Kirsty Short is a virologist at the University of Queensland who specialises in the research of respiratory viruses, namely influenza virus and SARS-CoV-2.

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Abbreviations

HA	Haemagglutinin
HPAIV	Highly pathogenic avian influenza
LPAIV	Low pathogenic avian influenza
MBSC	Multibasic cleavage site
NRP1	Neuropilin 1
Sema3A	Semaphorin 3A
VEGF	Vascular endothelial growth factors

Executive Summary

Understanding the evolution of avian influenza is essential for the poultry industry. Specifically, through evolution avian influenza can acquire mutations that increase its severity, spread, or ability to infect humans, putting both poultry and workers at risk. Early detection of evolutionary changes allows for more effective control measures, reducing economic losses from mass culling and trade restrictions.

These data present the first evidence that exogenous Sema3A reduces the number of mutations that arise *in vitro* in chicken cells in the influenza HA. These data raise the intriguing possibility that the addition of exogenous Sema3A (e.g. in feed) may reduce the number of mutations that emerge in avian influenza viruses upon infection of poultry. This could include mutations associated with increased transmission from poultry-to-poultry workers.

There is a current theory, based on the emergence of synonymous mutations over time, that mutation rates of avian influenza virus are higher in chickens than ducks, suggesting that surveillance for high fitness mutations is best targeted at poultry flocks. However, these observations remain to be proven experimentally. Our results indicate that higher fitness HA mutations are more likely to occur in duck cells, even though chicken cells allow for the faster emergence of mutations. This finding has important implications for avian influenza virus surveillance. Namely, these data suggest that whilst increased mutations emerge in chickens, surveillance for mutations of increased fitness may be best targeted at wild bird populations. This is highly pertinent at the current point in time where surveillance is recognised as a key tool in the protection of Australian from avian influenza.

Overall Conclusions

- Increased mutations were recorded in the influenza virus HA after passaging on chicken, rather than duck, endothelial cells.
- The addition of exogenous Sema3A reduces the number of mutations recorded in the influenza virus HA and species-dependent differences in viral mutations may reflect different levels of Sema3A expression.
- Mutations of higher fitness (defined by increased replication) emerged in the influenza virus HA following passaging in duck, rather than chicken, endothelial cells.

1 Introduction

Avian influenza represents a real and looming threat to Australia's poultry industry. The recent outbreaks of H7 influenza viruses on farms in Victoria, NSW and the ACT highlight the capacity of this virus to adversely affect our poultry industry, with over 1.8 million birds already euthanised¹. This may be further magnified should the H5N1 avian influenza virus, which has now spread to every other continent in the world, arrive in Australia this spring¹.

Avian influenza viruses originate in wild birds, where they typically cause minimal, if any, disease¹. From here, the viruses can spill over into poultry where they once again are typically associated with minimal disease (although a reduction in egg laying has been reported in infected birds)¹. However, the spread of this so called 'low pathogenic' avian influenza amongst poultry can facilitate the evolution of the virus¹. Some mutations acquired by the virus may be neutral, i.e. they do not affect the severity or transmissibility of the virus. Other mutations may facilitate the more effective transmission of the virus to poultry workers². Disease caused by low pathogenic avian influenza in humans can range in severity, although it is important to note that spillovers of the low pathogenic H7N9 caused a 40% mortality in humans². Perhaps the most notable viral mutation that can occur in poultry is the insertion of a specific amino acid sequence in the surface protein of the virus (the 'multibasic cleavage site' or MBCS)². This changes the virus from being 'low pathogenic' to being 'highly pathogenic'. Highly pathogenic avian influenza virus (HPAIVs) can cause up to 100% mortality in infected poultry and is typically associated with widespread haemorrhaging and oedema. Following its emergence in poultry, highly pathogenic avian influenza viruses can spread back to wild birds and poultry workers where it may cause significant mortality². Understanding the processes that drive the evolution of avian influenza in chickens represents a novel opportunity to identify new druggable targets to intervene in the process.

2 Chapter 1: Virus evolution in chicken and duck cells

2.1 Introduction

To begin to explore avian influenza evolution in chickens, we first sought to explore the *in vitro* emergence of mutations after passaging in chicken vs duck cells. There is a current theory, based on the emergence of synonymous mutations over time, that mutation rates of avian influenza virus are higher in chickens than ducks, suggesting that surveillance for high fitness mutations is best targeted at poultry flocks³. However, these observations remain to be proven experimentally.

2.2 Methods

2.2.1 Cell culture

Chicken (ChAEC) and duck (DuAEC) aortic endothelial cells were previously isolated and characterised⁴. Briefly, 17-day-old embryonated chicken eggs (*Gallus gallus domesticus*) and 21-day-old Pekin duck embryonated eggs (*Anas platyrhynchos domesticus*) were purchased from Darling Downs Hatchery (Queensland, Australia). Primary aortic endothelial cells were cultured from the aortic arches of chicken and duck embryos as described previously using EGM-2MV medium (Lonza, Basel, Switzerland) with 10% FBS (Gibco, Waltham, MA, USA). Collection of avian cells was approved by the University of Queensland's Animal Research Ethics Committee (002/18). All avian cells were cultured at 40°C 5% CO₂.

2.2.2 H7N6 stock generation

LPAIV H7N6 was isolated from an infected emu in Victoria in 2020. Viral stocks were amplified in embryonated chicken eggs, approved by the University of Queensland Animal Ethics Committee (AE000089).

2.2.3 Infection of avian endothelial cells with LPAIV

All infectious work with LPAIV was conducted in a PC3 certified facility at the University of Queensland (UQ) and approved by the UQ Institutional Biosafety Committee (UQ IBC, approval IBC/525Bv2/SCMB/IMB/2022). Briefly, 24-well plates were coated with 0.2% gelatin in PBS (Sigma-Aldrich) for 5 mins at 4°C. Plates were washed and avian cells were seeded at a density of $\sim 1.25 \times 10^5$ cells/well and left to adhere overnight. Once 80% confluent, cells were taken into PC3, and 300 mL of virus or EGM was added to triplicate wells and left to incubate for 2 days. This constitutes as passage 1 (P1). Once CPE was observed (data not shown), inoculum was pooled and clarified by centrifugation at 1,000 g for 5 mins at 4°C. 300 mL of supernatant was added to new plates, constituting the next passage. After 48 hours of infection, 100 mL of the cell culture supernatant was added to 300 mL of RLT buffer and b-mercaptoethanol, and prepared for PCR.

2.2.4 PCR, sequencing and analysis

RNA was isolated with QIAamp viral RNA mini kit (QIAGEN GMBH) and incubated according to the instructions from the manufacturer, with minor modifications. A 100 mL sample of supernatant containing virus was lysed with 350 mL of lysis-binding buffer. After binding to the column and washing, the RNA was eluted in 20 mL of nuclease-free double-distilled water.

Complementary DNA (cDNA) was reverse transcribed using Invitrogen Superscript iii (Invitrogen) as per the manufacturer's protocol with minor modifications. A total of 10 mL RNA was primed with UNI12 primer [10 mM; GCCGGAGCTCTGCAGATATCAGCGAAAGCAGG], 10 mM dNTP and incubated at 65°C for 5 mins and then quickly cooled on ice. A mixture of 10 x RT buffer, 25 mM MgCL₂, 0.1 M DTT, 1 ml of RNASE out (40 U/μL), 1 mL superscript III RT (200 U/μL) was added to each sample and incubated at 50°C for 50 mins following an incubation at 85°C for 5mins. 1 mL RNASE H was added and incubated for 20 mins before amplification of PCR.

cDNA amplified samples were then run using Phusion High fidelity DNA polymerase PCR kit and 10μM of HA_UniF (GGGGGAGCAAAAGCAGGGGA) and HA_UniR (CCGGGTATTAGTAGAAACAAGGGTG).

Thermocycling was performed in an Thermo PCR machine with the following cycling conditions: 98°C for 30 seconds once and then 25 cycles of 20 sec at 98°C, 30 sec at 59°C, 7 mins at 72°C. This was followed by a final 72°C for 7 mins. Each reaction was analysed by agarose gel electrophoresis before clean up using the QIAquick PCR Purification Kit (QIAGEN) before sequencing. Samples were sent to the Australian Genomics Research Facility (AGRF) located in Perth, WA, Australia for quality control, library preparation, sequencing and data acquisition. Amplicons were run on MiSeq 150bp PE sequencing, with a minimum of 10,000 reads per sample. Paired end FASTQ files were received and processed using fastp (Galaxy v. 0.24.0). Reads were mapped to H7N6 emu genome using Bowtie2 (Galaxy v. 2.5.3). Output bam and bai files were visualised manually using Integrative Genomics Viewer (IGV v2.6.2) for mutations. Total mapped reads were visualised using Samtools depth (Galaxy v 1.15.1) and plotted using Graphpad Prism 10.

2.2.5 Calculating fitness distributions of viral mutations from serial passaging

Fitness values were calculated as the normalised growth rate s_M such that e^{s_M} is the number of descendants of a single variant M at the end of the passage growth cycle. This rate is directly calculated as

$$s_M = \log \left(\frac{C_M(i)/C_{WT}(i)}{C_M(i-1)/C_{WT}(i-1)} \right)$$

where $C_M(i)$ represents the counts of the M th variant at passage i normalised to the wildtype fitness by counts $C_{WT}(i)$ to centralise the fitness distribution. Both the i and $(i-1)$ passage are used to estimate the rate of growth.

The Generalised Pareto Distribution (GPD) was calculated by

$$F(x|k, \sigma) = \begin{cases} 1 - \left(1 + \frac{kx}{\sigma}\right)^{-1/k} & x \geq 0, k > 0 \\ 1 - \left(1 + \frac{kx}{\sigma}\right)^{-1/k} & 0 \leq x < -\frac{\sigma}{k}, k < 0 \\ 1 - e^{-x/\sigma} & x \geq 0, k = 0 \end{cases}$$

with shape parameter k and scale parameter σ . All calculations and statistical fittings were performed in MATLAB 2023a.

2.3 Results

Avian influenza was passaged on chicken and duck endothelial cells and after 6 passages we investigated the number of mutations that arose in the influenza virus HA. After 6 passages an increased number of mutations were recorded in the influenza HA passaged in chicken cells (Figure 1).

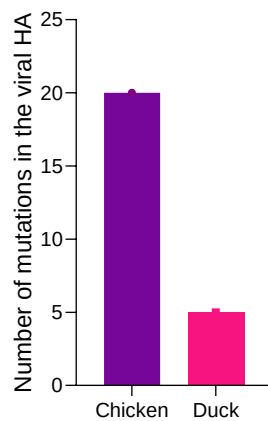


Figure 1 The number of mutations recorded in the influenza HA after 6 passages in chicken or duck endothelial cells

The number of mutations detected is often contingent upon the sequence coverage, i.e. improved coverage can increase the number of mutations. To confirm that the above results were not simply the result of differential sequential coverage we compared the sequence coverage after passaging on chicken and duck cells. There was no notable species-dependent difference in sequence coverage, suggesting that this did not confound the results (Figure 2).

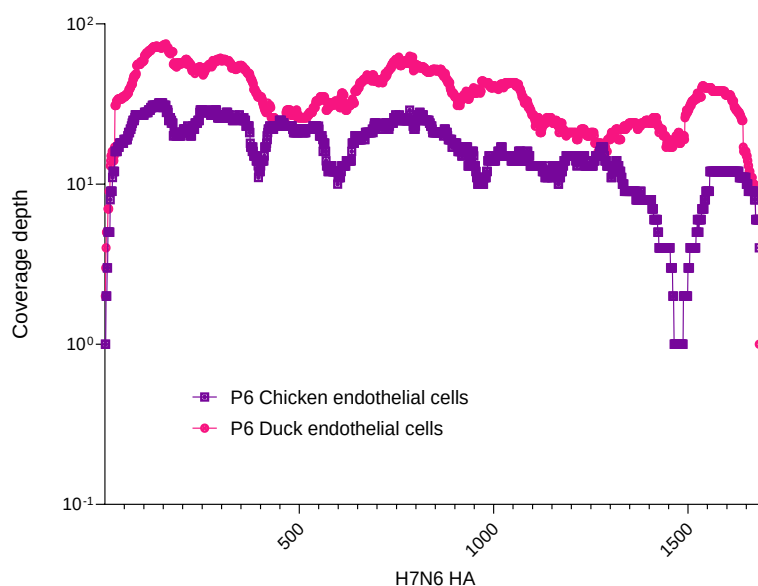


Figure 2 The sequence coverage of the influenza HA after six passages in chicken or duck endothelial cells

Together, these results suggest that influenza virus has a higher mutation rate in chicken cells compared to those of ducks. We next sought to use mathematical modelling to assess the fitness of the mutations that emerged in each species. In this *in vitro* context a high fitness mutation is defined as one associated with increased viral reads, i.e. increased viral replication. A generalised Pareto fit for chicken fitness distribution shows that it is much lower than duck fitness distribution (Figure 3). These results indicate that higher fitness mutations are more likely to occur in duck cells, even though chicken cells allow for the faster emergence of mutations. Indeed, when calculating the probabilities

and returns of mutations with higher than wild-type fitness estimated from the Pareto distributions, the return time (i.e. number of passages required) in chicken cells for a mutation to emerge with a 0.5 increased fitness is lower than that of duck cells (Table 1). However, this is not the same for mutations with a higher fitness value (i.e. 1.25) where the return time is 125 in chickens and only 6 in ducks (Table 1).

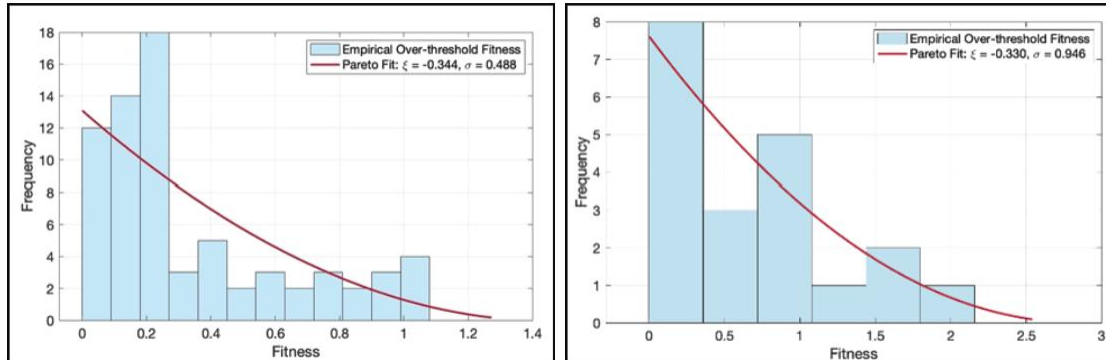


Figure 3 Fitted generalised pareto distributions for viral mutations with fitness above the wild-type in chicken (left) and duck (right) cells

Table 1 Probabilities and returns of mutations with higher than wild-type fitness estimated from the pareto distributions

Fitness value(s)	Probability chicken	Probability duck	Proportional difference	Return time chicken (number of passages)	Return time duck (number of passages)
0.5	0.28	0.56	1.98	1	2
0.8	0.09	0.37	4.14	3	3
1	0.03	0.27	9.47	8	4
1.25	0.002	0.18	86.12	125	6

2.4 Discussion

Our results indicate that higher fitness HA mutations are more likely to occur in duck cells, even though chicken cells allow for the faster emergence of mutations. We hypothesise that these data reflect the differential immune response of chicken and duck endothelial cells. Specifically, duck endothelial cells mount a strong anti-viral, interferon response to avian influenza⁵. Whilst this may mean the total number of mutations to emerge is less, it may mean that when a mutation emerges it is more likely to be of a higher fitness, as otherwise it would not survive in the viral population. These data have important implications for avian influenza surveillance. To date, surveillance for high fitness mutations has focussed on chickens due to the emergence of a MBCS in this species². However, our data suggest that wild-birds surveillance may be equally important to detect the emergence of high fitness mutations.

3 Chapter 2: The role of Sema3A in virus evolution

3.1 Introduction

There are multiple known differences in the immune response between chickens and ducks, which may account for the increased rate of viral mutations in chickens observed in Chapter 1. For example, chickens are known to lack a gene called RIG-I which is essential in the anti-viral immune response. It is hypothesised that this genetic difference contributes to the increased inflammatory response and severity of avian influenza in chickens compared to ducks⁵. Species-dependent differences in the expression of the gene Semaphorin 3A (Sem3A) are less well defined. Sem3A, originally identified as a neural guidance cue, also plays a significant role in immune modulation⁶. It is involved in the regulation of both innate and adaptive immune responses through its interaction with receptors such as neuropilin-1 (NRP1) and plexins, which are expressed on various immune cells including T cells, dendritic cells, endothelial cells and macrophages. Sem3A can exert immunosuppressive effects by inhibiting T cell activation and proliferation, modulating dendritic cell maturation, and promoting the development of regulatory T cells⁶. Additionally, it influences cytokine production and can limit excessive inflammatory responses, thereby maintaining immune homeostasis⁶. Through these mechanisms, Sem3A acts as a critical regulator in preventing immune overactivation and maintaining tolerance. Accordingly, differential expression of Sem3A may influence the avian immune response and thus affect the evolution of influenza virus.

As described above, Sema3A mediates its immune regulatory properties in part through binding to the cell surface receptor NRP1 (expressed on cells such as endothelial cells). Independent of any role in immune modulation, NRP1 may also play an important independent role in providing a selective advantage for nascent HPAIVs in chickens. In SARS-CoV-2 infection (another virus that contains a multi-basic cleavage site in the cell surface glycoprotein), cleavage of the multi-basic cleavage site by furin facilitates the binding of the SARS-CoV-2 spike protein binds to NRP1, amplifying infection⁷. Specifically, cleavage of the multi-basic site by furin exposes the C-end rule (CendR) motif identified as a ligand for NRP1⁷. Highly pathogenic avian influenza (HPAI) viruses, such as H5N1, can conform to the CendR when their hemagglutinin (HA) proteins acquire polybasic cleavage sites, which, upon proteolytic activation, expose C-terminal arginine or lysine motifs that interact with NRP(1). This interaction enhances viral entry and systemic spread by promoting endocytosis and vascular leakage, contributing to the virus's high pathogenicity⁸. We therefore propose that an ability to bind to NRP1 (and therefore facilitate endothelial cell infection and systemic spread) drives the evolution of avian influenza and specifically the selection of HPAIV (Figure 4). Specifically, we hypothesise that viral variants emerge by random mutations in domestic birds. From here, HPAIVs have a selective advantage due to the receptor-ligand interaction between the CendR motif and NRPs and the subsequent infection of endothelial cells (Figure 4).

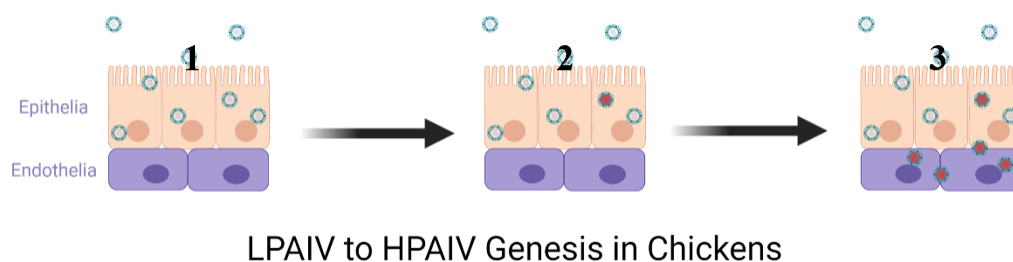


Figure 4 Proposed hypothesis for the role of NRP1 in the evolution of avian influenza virus

Blue virions represent LPAIV whilst red virions represent HPAIV.

Together, these data suggest that the role of Sem3A (and its ligand NRP1) in the evolution of avian influenza warrant further investigation.

3.2 Methods

3.2.1 H7N6 stock generation

LPAIV H7N6 was isolated from an infected emu in Victoria in 2020. Viral stocks were amplified in embryonated chicken eggs, approved by the University of Queensland Animal Ethics Committee (AE000089)

3.2.2 Cell culture

Chicken (ChAEC) and duck (DuAEC) aortic endothelial cells were previously isolated and characterised⁴. Briefly, 17-day-old embryonated chicken eggs (*Gallus gallus domesticus*) and 21-day-old Pekin duck embryonated eggs (*Anas platyrhynchos domesticus*) were purchased from Darling Downs Hatchery (Queensland, Australia). Primary aortic endothelial cells were cultured from the aortic arches of chicken and duck embryos as described previously using EGM-2MV medium (Lonza, Basel, Switzerland) with 10% FBS (Gibco, Waltham, MA, USA). Collection of avian cells was approved by the University of Queensland's Animal Research Ethics Committee (002/18). All avian cells were cultured at 40°C 5% CO₂.

3.2.3 Western blotting of aortic endothelial cells

ChAEC and DuAEC cells were seeded in a 24-well plate as described above. To harvest lysates, everything was done on ice. Firstly, cells were washed with cold PBS and 80 mL of RIPA buffer (50 mM Tris HCl, 150 mM NaCl, 1 mM EDTA, 1% Triton-X-100, 1% Sodium Deoxycholate, 0.1% SDS) with protease (Complete tablet; Roche) and phosphatase inhibitors (PhoSTOP; Roche). Samples were then stored at -80°C. Once thawed, samples were clarified at 14000 g for 5 mins to pellet cell debris and supernatant was subjected to BCA protein assay (BCA Protein Assay Kit; Pierce) to standardise protein levels. LDS sample buffer 4X (Novex) and reducing agent was added to 10 mg of sample before boiling at 100°C for 10 mins and samples were loaded on an SDS-PAGE gel and run at 120 V for 60 mins. Membranes were electroblotted using iBlot2 Dry Blotting System (Thermo Fisher Scientific) and blocked for 2 h at room temperature (rtp) using 5% milk in TBS-T (1% Tween-20). Membranes were incubated with either mouse α -tubulin (1:800; CST) or rabbit SEMA3A (1:800 Invitrogen) overnight at 4°C in 2.5% milk TBS-T. The next day, membranes were washed 3 x in TBS-T for 10 mins on a shaker and subjected to either goat-anti mouse or goat-anti rabbit HRP secondary antibody (1:1000, Invitrogen) at rtp for 1.5 h. After washing, membranes were subjected to 1 mL of Clarity ECL reagent (Bio-Rad) for 1 min before visualising activity on an Amersham Imager 600.

3.2.4 Infection of avian endothelial cells with LPAIV

All infectious work with LPAIV was conducted in a PC3 certified facility at the University of Queensland (UQ) and approved by the UQ Institutional Biosafety Committee (UQ IBC, approval IBC/525Bv2/SCMB/IMB/2022). Briefly, 24-well plates were coated with 0.2% gelatin in PBS (Sigma-Aldrich) for 5 mins at 4°C. Plates were washed and avian cells were seeded at a density of $\sim 1.25 \times 10^5$ cells/well and left to adhere overnight. Once 80% confluent, cells were taken into PC3, washed with PBS and 100 μ M Sema3a peptide (758; Genscript) or EGM (FCS-free) was added as a control for 30 mins at 40°C. Following that, 300 μ L of virus or EGM was added to triplicate wells and left to incubate for 2 days. This represented passage 1 (P1). Once CPE was observed (data not shown), inoculum was

pooled and clarified by centrifugation at 1,000 g for 5 mins at 4°C. 300 mL of supernatant was added to new plates constituting the next passage. After 48 hours of infection 100 mL of the cell culture supernatant was added to 300 mL of RLT Buffer and b-mercaptoethanol, and prepared for PCR.

3.2.5 PCR, sequencing and analysis

RNA was isolated with QIAamp viral RNA mini kit (QIAGEN GMBH) and incubated according to the instructions from the manufacturer, with minor modifications. A 100 ml sample of supernatant containing virus was lysed with 350 ml of lysis-binding buffer. After binding to the column and washing, the RNA was eluted in 20 ml of nuclease-free double-distilled water.

Complementary DNA (cDNA) was reverse transcribed using Invitrogen Superscript iii (Invitrogen) as per the manufacturer's protocol with minor modifications. A total of 10 ml RNA was primed with UNI12 primer [10 mM; GCCGGAGCTCTGCAGATATCAGCGAAAGCAGG], 10 mM dNTP and incubated at 65°C for 5 mins and then quickly cooled on ice. A mixture of 10 x RT buffer, 25 mM MgCL₂, 0.1 M DTT, 1ml of RNASE out (40 U/μL), 1 ml superscript III RT (200 U/μL) was added to each sample and incubated at 50°C for 50 mins following an incubation at 85°C for 5 mins. 1 ul RNASE H was added and incubated for 20 mins before amplification of PCR.

cDNA amplified samples were then run using Phusion High fidelity DNA polymerase PCR kit and 10 μM of HA_UniF (GGGGGGAGCAAAAGCAGGGGA) and HA_UniR (CCGGGTATTAGTAGAAACAAGGGTG).

Thermocycling was performed in an Thermo PCR machine with the following cycling conditions: 98°C for 30 seconds once and then 25 cycles of 20 seconds at 98°C, 30 seconds at 59°C, 7 mins at 72°C. This was followed by a final 72°C for 7 mins. Each reaction was analysed by agarose gel electrophoresis before clean up using the QIAquick PCR Purification Kit (QIAGEN) before sequencing. Samples were sent to the Australian Genomics Research Facility (AGRF) located in Perth, WA, Australia for quality control, library preparation, sequencing and data acquisition. Amplicons were run on MiSeq 150bp PE sequencing, with a minimum of 10,000 reads per sample. Paired end FASTQ files were received and processed using fastp (Galaxy v. 0.24.0). Reads were mapped to H7N6 emu genome using Bowtie2 (Galaxy v. 2.5.3). Output bam and bai files were visualised manually using Integrative Genomics Viewer (IGV v2.6.2) for mutations. Total mapped reads were visualised using Samtools depth (Galaxy v 1.15.1) and plotted using Graphpad Prism 10.

3.3 Results

To investigate whether differential Sem3A expression in chickens and ducks could account for species-dependent differences in influenza virus mutations, the protein levels of Sema3A in duck and chicken endothelial cells were compared. Figure 5 shows that duck cells express higher levels of Sem3A than chicken endothelial cells.

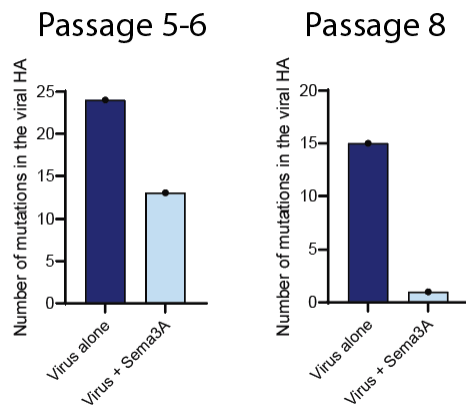


Figure 7 The number of mutations recorded in the influenza HA at different passages in the presence or absence of Sema3A

Figure 8 shows that the coverage for Virus + Sema3a was consistently higher than that for virus alone, indicating that the observed results were not due to poor coverage of the Sema3A samples.

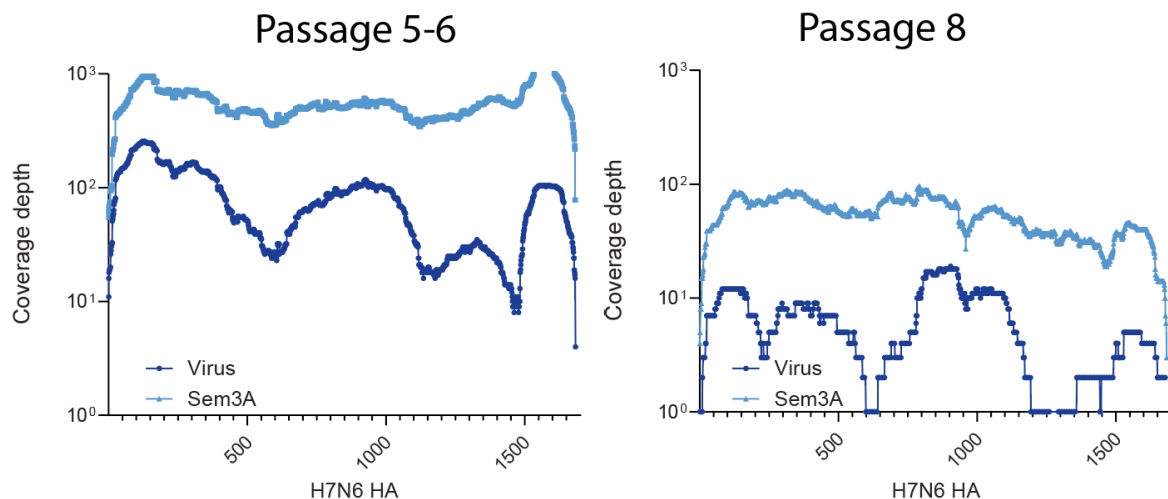


Figure 8 The sequence coverage of the influenza HA at different passages in the presence or absence of Sema3A

We next sought to identify if, as hypothesised, the reduced number of mutations in the Sema3A samples was due to the emergence of an MBCS in the virus alone samples. Surprisingly, no changes to the MBCS were observed in any of the sequenced samples (Figure 9).

```

Query  302  PFQININSRAVGKCPRYVKQKSLLLATGMKNVPEIPRKRGLFGAIAAGFIENGWEGLVDGWY  361
Sbjct  302  PFQININSRAVGKCPRYVKQKSLLLATGMKNVPEIPGKRGLFGAIAAGFIENGWEGLVDGWY  361

```

MBCS

Figure 9 No changes were observed in the multibasic cleavage site (MBCS) in the influenza virus HA in any of the sequenced samples

Figure shows amino acid alignment of input virus and representative sample sequence.

3.4 Discussion

Together, these data suggest a reduced number of influenza virus mutations in the presence of exogenous Sema3A. Contrary to our initial hypothesis, this was not associated with the emergence of mutations in the multibasic cleavage site and the selective pressure exerted by NRP1 on selecting viruses with a CendR motif. It is therefore interesting to speculate the alternate mechanisms by which Sema3A may influence the evolution of avian influenza virus. Sema3A typically functions through binding class A plexins and require neuropilins as obligate co-receptors for this interaction⁶. This interaction can trigger a variety of different physiological effects, one of which is immune modulation⁶. Specifically, Sema3A overexpression can reduce the levels of inflammatory cytokines and oxidative stress markers in endothelial cells in response to lipopolysaccharide stimulation⁹. It is therefore possible that this immunoregulatory property alters the selective pressure exerted on influenza virus and hence the number of mutations detected. Future studies are possible to investigate this hypothesis by examining the inflammatory profile of LPAIV infected cells exposed to exogenous Sema3A.

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5 Plain English Summary

Project Title:	Genetic restoration in poultry
Australian Eggs Limited Project No	INNO008
Researchers Involved	K.R. Short
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Objectives	This project was conducted to investigate methods to understand and ultimately reduce the mutations that arise in avian influenza in chickens.
Background	Bird flu is a serious threat to Australia's poultry industry, with recent outbreaks in Victoria, NSW, and the ACT leading to the culling of over 1.8 million birds. There is growing concern that the H5N1 strain, which has already spread worldwide, could reach Australia this spring. Bird flu viruses can change over time, sometimes becoming more deadly and spreading more easily to both poultry and humans.
Research	This project was designed to use chicken and duck primary cells to investigate the role of Sem3A in the evolution of bird flu.
Outcomes	These data present the first evidence that exogenous Sema3A reduces the number of mutations that arise <i>in vitro</i> in the influenza HA. This could include mutations associated with increased transmission and/or transmission from poultry to poultry workers. Our results indicate that higher fitness HA mutations are more likely to occur in duck cells, even though chicken cells allow for the faster emergence of mutations.
Implications	These data raise the intriguing possibility that the addition of exogenous Sema3A (e.g. in feed) may reduce the number of mutations that emerge in avian influenza viruses upon infection of poultry. Namely, these data further suggest that whilst increased mutations emerge in chickens, surveillance for mutations of increased fitness is best targeted at wild bird populations.
Key Words	Avian influenza, mutation
Publications	Pending