**Genome-wide Analysis Reveals Class and Gene Specific
Codon Usage Adaptation in Avian Paramyxoviruses 1**

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ABSTRACT

In order to characterize the evolutionary adaptations of avian paramyxovirus 1 (APMV-1) genomes, we have compared codon usage and codon adaptation indexes among groups of Newcastle disease viruses that differ in biological, ecological, and genetic characteristics. We have used available GenBank complete genome sequences, and compared codon usage of class I (CI-29 sequences containing 132,675 codons) and class II (CII-259 sequences containing 1,184,925 codons) APMV-1 genomes. We also compared available complete fusion protein gene sequences (CI-175 sequences containing 96,775 codons; CII-1166 sequences containing 644,798 codons). Adaptation to *Gallus gallus* was compared among the different classes of viruses, among different genomic regions based on transcriptional levels, or on the fusion gene. Interestingly, distinctive codon usage determined by differences in relative synonymous codon usage and by codon adaptation indexes was observed for the two APMV-1 classes and for different transcriptional regions within classes. Furthermore, differential use of the third codon position and preferential use of codon pairs were seen for the two different classes and for selected genotypes of class II despite the fact that there were no large differences in nucleotide composition. The data suggest that codon usage has changed significantly since the two APMV-1 classes diverged, however, these changes are not significantly pronounced among viruses of the same genotype, suggesting that codon adaptation in APMV-1 occurs through a slow evolutionary process.

Keywords: APMV-1 classes, RSCU, class II Genotypes, CAI, fusion gene

1. Introduction

Remarkably adaptable viruses, the avian paramyxoviruses of serotype 1 (APMV-1) are capable of infecting over 200 avian species and cause a diverse range of clinical outcomes in birds (Kaleta and Baldauf, 1988). APMV-1 is synonymous with Newcastle disease virus (NDV), with the virulent forms of the virus causing Newcastle disease (Afonso and Miller, 2013). They belong to the genus *Avulavirus* of the family *Paramyxoviridae* (Mayo, 2002), are endemic in specific geographic locations, and have also been identified in wild birds worldwide (Dimitrov et al., 2016). The genome of APMV-1 is a single-stranded, negative-sense, non-segmented RNA molecule with the following 6 genes arranged in this 3' to 5' order: the nucleocapsid (NC), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and RNA polymerase (L) (Alexander et al., 2012). Although these six transcriptional units all contain their own conserved 3' start and 5' stop sequence, the main viral transcription promoter is found in the 3' leader sequence (Gao et al., 2008). The result of this sole promoter is transcriptional polarity, where the genes at the 3' end of the genome are transcribed more efficiently than those at the 5' end (Collins et al., 1978; Wilde et al., 1986).

These six transcriptional units are then translated into essential proteins that allow NDV to infect and cause disease. The nucleoprotein is essential for viral replication as it binds NDV genomic RNA and makes up the core structure of the nucleocapsid (Buchholz et al., 1994; Collins et al., 1982). The phosphoprotein binds the ribonucleocapsid, where it positions and stabilizes the RNA polymerase (Buchholz et al., 1994; Hamaguchi et al., 1983). The matrix protein stabilizes the entire virus structure by linking the nucleocapsid to the two important, functional glycoproteins (HN and F) on the surface of the viral envelope (Coronel et al., 2001). The HN protein plays an important role in viral infection through recognizing and attaching to

host red blood cells, which allows the cleaved F protein, to fuse the virus to the host cell or host cell to host cell, where the viral transcription unit can enter (Huang et al., 2004). These two viral envelope projections have been shown necessary for infection, and are the neutralizing and protective antigens of NDV (Kim et al., 2011; Nagai et al., 1976). However, it is the amino acid sequence at the fusion protein cleavage site that is believed to be the major determinant of virulence (Glickman et al., 1988; Kim et al., 2011).

APMV-1s are divided into two separate classes: class I and class II. Class I is comprised of only one genotype (CI-G1); whereas, class II contains at least 18 different genotypes (CII-GI-XVIII) (Courtney et al., 2013; Diel et al., 2012b; Snoeck et al., 2013). While certain groups of viruses are preferentially maintained in specific host species, the majority of the virulent genotypes are capable of infecting in chickens (Dimitrov et al., 2016). Viruses of class I are predominantly reported in wild aquatic birds (Kim et al., 2007), and except genotypes I, II and X, the virulent viruses of all other class II genotypes are preferentially isolated from chickens. Class II genotypes VI and VII are responsible for the majority of the most recent outbreaks in poultry; viruses of class II genotype VI are preferentially isolated from *Columbiformes* (Dimitrov et al., 2016; Dortmans et al., 2010).

Patterns of codon usage normally vary among animal species, and because these species are hosts to viruses, it is expected that viruses would adapt to the translational codon usage of different host organisms by changing its codon usage to achieve maximum fitness (Bahir et al., 2009; Knight et al., 2001). However, other factors such as nucleotide compositional bias, transfer RNA (tRNA) abundance, RNA stability and expression levels have been found to be responsible for non-random codon usage (Knight et al., 2001). One form of non-random codon usage is the suboptimal use of codons to negatively regulate expression of genes for which

excessive expression decreases fitness (Coleman et al., 2008). Here, we have used the *Gallus gallus* codon dataset available in the Data Analysis in Molecular Biology and Evolution (DAMBE v5.5.2) software package to search for evidence of differential host adaptation and codon usage in APMV-1 (Xia, 2013). We have compared the coding sequences of the fusion gene (CI-175 sequences containing 96,775 codons; CII-1166 sequences containing 644,798 codons) and the coding sequences of complete genomes (CI-29 sequences containing 132,675 codons and CII-259 sequences containing 1,184,925 codons).

Previously, synonymous codon usage of the fusion protein gene (Kumar and Kumar, 2014) and the complete genome (Wang et al., 2011) in NDV was analyzed, and both studies found only random mutational pressure and a low amount of codon usage bias. In the light of those results, here, we have performed genome wide and gene specific comparisons of codon usage, focusing mainly on two distinct groups of viruses: viruses of low virulence of class I that are known to be maintained in waterfowl and viruses of class II, the majority of which are virulent. Among virulent viruses, we have compared viruses normally expected to be maintained in chickens (genotype VII) versus viruses that are normally maintained in pigeons (genotype VI). In addition, we have determined the relative synonymous codon usage (RSCU), the codon adaptation index (CAI) to *Gallus gallus*, the effective number of codons, the nucleotide composition and the di-codon frequency, and compared these analyses among class I and class II viruses. Our data demonstrates differences in codon usage within the fusion gene and in the full genome between class I and class II viruses, and the differences in codon bias in different genotypes of class II. This work also provides further investigation into the relationship between levels of APMV-1 gene expression and codon usage.

2. Materials and methods

2.1 Sequence data

Complete genome sequences of 330 APMV-1 isolates were downloaded from GenBank (<http://www.ncbi.nlm.nih.gov/nuccore>; as of June 20th 2016); in addition, 1,819 sequences of the complete coding region of the fusion protein gene were also available and downloaded. Duplicate and recombinant sequences, sequences of man-made clone viruses and sequences that were obtained from vaccine-like viruses were removed from the datasets in order to analyze only viruses that represent natural evolutionary and adaptation processes. Some additional sequences for which background data was missing were also removed. As a result, 288 complete genome sequences and 1,341 fusion gene sequences were used for subsequent analyses. The coding sequences for both the complete genome and the fusion gene were grouped into datasets according to class and genotype using the criteria described by Diel et al. (Diel et al., 2012b). The accession numbers, as well as location, year, and host of isolation are also provided in the Supplementary materials (Tables S1 and S2). To look at differentially expressed genomic regions, the APMV-1 genome was divided into 3 regions. The regions were conditionally named as follows: nucleocapsid-phosphoprotein-matrix (NC-PHO-M), fusion-haemagglutinin-neuraminidase (F-HN), and polymerase (POL). All coding sequences were aligned using the MAFFT v7.222 alignment tool as implemented in Geneious v9.1.5 (Kato et al., 2002).

2.2. Calculation of relative synonymous codon usage (RSCU) and codon adaptation index (CAI)

Relative synonymous codon usage was calculated for each sequence, as previously described (Sharp and Li, 1986). Equation 1 below was used as implemented in DAMBE v5.5.2 software package (Xia, 2013).

$$RSCU = \frac{x_{ij}}{\sum_j^n x_{ij}} n_i \quad (1)$$

RSCU is the value of the observed codon number (X) divided by the expected codon number (Σ); the expected codon number assumes an occurrence where the codons (i) for the same amino acid (j) were all used equally (n_i is the number of synonymous codons). RSCU values of greater than 1 have a positive codon usage bias and were considered abundantly used codons. Those that have RSCU values less than 1 have a negative codon usage bias and were used less. Codons with RSCU values of 1 lack any bias and were used equally within the genome or that particular genomic region.

In order to study the preference of codon usage in APMV-1, we used the codon adaptation index calculated using DAMBE (Sharp and Li, 1987; Xia, 2013). Based on the coding sequences, this method predicts expression levels, and compares the codon usage of the different APMV-1 isolates to a predetermined reference set, in this particular case, *Gallus gallus*. All 1,341 fusion gene sequences were used to analyze the adaptation of class I and class II viruses to *Gallus gallus*. In addition, the 288 complete genome sequences and each of the 3 designated genomic regions (NC-PHO-M, F-HN, and POL) were used to determine the CAI value to *Gallus gallus* and to compare between the two classes. Codons with RSCU values of 1 and stop codons were excluded from the analysis.

2.3. Correlation analysis

Pearson correlation analysis was carried out on the fusion gene, the three genomic regions (NC-PHO-M, F-HN, and POL), and the complete genome for both class I and class II viruses, using the GraphPad Prism 6 software, to determine the linear relationship among the samples (r). The r coefficient was used to determine the presence and magnitude of the

correlation, and the two-tailed P test ($P < 0.01$) was used to determine significance. Correlation plots with a Pearson r coefficient of 0.5 to 1 have a high correlation, 0.3 to 0.5 have a medium correlation, 0.1 to 0.3 have low correlation, and less than 0.1 have no correlation. In addition, linear regression analysis was also performed to support correlation among the different samples. Those samples with R^2 values close to 0 indicates no relationship between the two variables, and the closer the R^2 value gets to 1, the stronger the relationship between the two variables.

2.4. Nucleotide composition and di-codon frequency

Class I (n=175) and class II (n=1,166) fusion gene coding sequences and class I (n=29) and class II (n=259) complete genome coding sequences were submitted to DAMBE for nucleotide composition analysis. For both class I and class II fusion gene sequences, 1,659 nucleotides were analyzed with 553 nucleotides for each of the three positions, and for the complete genome of both classes, 13,725 nucleotides were analyzed with 4,575 nucleotides for each of the three positions. Values were given in percent with the total nucleotide count divided by the total count of each specific nucleotide at each of the three positions. In addition, the total GC content and GC content for the 3rd codon position (GC3) for each sequence were also determined by DAMBE.

Di-codon frequency was also determined using the DAMBE software employing all 1,341 fusion gene and 288 complete genome sequences. The observed count for each codon pair (4,096 pairs total) was divided by the expected counts to determine the di-codon frequency given in percentage. To obtain the expected counts, the total of codon pairs that encode the same set of amino acids was divided by the value given when the codon degeneracy of each of the amino

acids in the set was multiplied. This value assumes that each codon pair codes for the amino acid set equally.

2.5. Effective Number of Codons (ENC)

The effective number of codons for the fusion gene and complete genome of each sequence was calculated using Equation 2 as implemented in DAMBE.

$$ENC = 2 + s + \{29/[s^2 + (1 - s^2)]\} \quad (2)$$

ENC values range from 20 to 61, with 20 being exceptionally rare, indicating only one synonymous codon for each amino acid was used, where an ENC value of 61 corresponds to the use of all synonymous codons equally (Wright, 1990). The *s* in Equation 2 equals GC3. The average ENC and GC3 values of all sequences were calculated, and the average of each class and genotype were used to create ENC vs GC3 scatter plots. In addition, the average ENC and CAI value of all of the sequences were calculated, and the average of each class and genotype were used to create CAI vs ENC plots. The *r* coefficient was used to determine the presence and magnitude of the correlation, and the two-tailed *P* test (*P* < 0.01) was used to determine significance. Linear regression analysis was also performed to support correlation among the different samples, with a 95% confidence level, and the line was constrained to go through the origin.

2.6 Statistical Analysis

The 2-way analysis of variance (ANOVA) method, with Dunn-Sidak's multiple test correction was used to perform statistical analysis on the different experimental results through the GaphPad Prism 6 software with significance defined as *P* < 0.01.

2.7 Time to Most Recent Common Ancestor (MRCA)

Bayesian time scaled analyses were conducted by the Bayesian Markov Chain Monte Carlo (BMCMC) method implemented in BEAST v1.8.2 (Drummond et al., 2012) program utilizing a polymerase gene sequence dataset. The polymerase gene was selected for molecular clock estimations because, as an internal gene, it is expected to be less influenced by selective pressure and is known to have a high level of genetic conservation (Kim et al., 2008; Fuller et al., 2010). The L-gene also occupies almost half of the APMV-1 genome. The used dataset was parsed from the available complete genome sequences (Supplemental Table S2). Sequences from the same year and genotype were removed, resulting in a final dataset of 155 full L-gene sequences isolated between 1933 and 2014. General time reversible and Hasegawa, Kishino, and Yano with gamma distribution nucleotide substitution models were applied separately for comparison (GTR+ Γ_4 and HKY+ Γ_4 , respectively). Relaxed clock model (uncorrelated lognormal distribution) was tested with different demographic models (nonparametric Bayesian skyline plot, parametric exponential growth and constant coalescent size), and the best model was selected based on marginal likelihood estimations using path sampling/stepping-stone sampling (Baele et al., 2012). An input file for BEAST analysis was prepared using Bayesian evolutionary analysis utility software v.1.8.2, in which sequences were annotated with year of collection. Three independent chains were run to get output of 10,000 trees. Convergence was assessed in Tracer v1.6.0 program (<http://beast.bio.ed.ac.uk/Tracer>). Uncertainties in BMCMC estimates were reflected by the ranges of the 95% highest posterior density (95% HPD). Maximum clade credibility trees were generated using the Tree Annotator program from the BEAST package, and the FigTree program v1.4.2 (<http://tree.bio.ed.ac.uk/software/figtree>) was used for the visualization of the annotated trees.

3. Results

3.1. Differences in the full fusion coding sequence RSCU between class I and class II viruses

After we removed recombinant viruses and viruses that were apparent vaccines and vaccine escapes, 1,341 available sequences were used to extend our study across class I (175) and class II (1,166) viruses (Supplemental Table S1) to study the fusion gene RSCU. RSCU is defined as the number of times a codon appears divided by the number of expected occurrences when all codons are equally used. RSCU values were determined for each sequence from both APMV-1 classes, and the averages for each class are reported in Supplemental Table S3. Values of RSCU greater than 1 indicate more frequent use of that codon than expected; whereas, values of RSCU less than 1 indicate use of that codon less than expected. Figure 1A shows only the average RSCU values of the fusion gene for the codon usage that was found to be statistically different, using 2-way ANOVA, between the class I and class II viruses (bold font in Table S3), and clear differences in average RSCU values were seen in multiple codons. In addition, our analysis led us to question whether there were any codons whose usage was statistically different between class I and class II viruses in the fusion gene that were not found in the complete genome, and fifteen codons were discovered to be differentially used between class I and class II viruses only in the fusion gene (boxed values in Table S3).

To study whether the fusion gene provided a good representation of the differences of codon usage between class I and class II viruses, a Pearson Correlation plot was made to visualize the relationship between class I and class II RSCU (Fig. 1B). Correlation plots with a Pearson r coefficient of 0.5 to 1 have a high correlation, 0.3 to 0.5 have a medium correlation, 0.1 to 0.3 have low correlation, and less than 0.1 have no correlation. A Pearson r coefficient of 0.35 was observed when analyzing the RSCU values of class II versus class I sequences,

indicating a weak relationship among codon usage between the two classes of viruses. This weak relationship was due to the statistically significant differences in codon usage between class I and class II viruses (Table S3). In contrast, when class II sequences were compared against other class II sequences, a high, positive correlation is displayed with r coefficients of 0.8 (data not shown).

We wanted to determine if adaptive pressures have affected the differences in codon usage of the fusion gene between the two classes of viruses and among the genotypes of class II. ENC measures codon usage bias, but this measure does not distinguish codon usage bias that was due to A/T bias or GC3 content (Vicario et al., 2007). The relationship between ENC and GC3 can be used to decipher between codon usage bias that was due to A/T bias or preferred GC content at the wobble position. For that purpose, the ENC and GC3 values for the fusion gene of all sequences were also calculated and used to create a scatter plot for each class and genotype (Fig 1C). The average ENC value was 56 for both classes. Average GC3 values ranged from 43.3 to 50.1, with class I having the highest GC3 value, indicating an evolutionary decrease in the use of GC at position 3 in class II sequences.

To understand whether gene expression was correlated with codon usage, CAI vs ENC scatter plots were created (Figs. 1D and 1E). Whereas ENC is a non-directional measure that observes the unequal use of codons across the coding regions, CAI is a directional measure of codon usage, and will explain deviation from the expected codon usage in comparison to the codon usage of a reference genome (Vicario et al., 2007). The ENC and CAI values for the fusion gene of all sequences were calculated and plotted. Class I has a negative correlation with an r value of -0.8983 (Fig. 1D). Class II (Fig. 1E) has a loss of correlation between CAI and ENC with an r value very close to zero (-0.09976).

To further compare codon usage in the fusion gene between class I and the class II viruses, we analyzed di-codon frequency, and discovered major differences between class I and class II sequences, as well as differences between class II genotypes VI and VII (Table S4). A di-codon is six consecutive nucleotides in a row, and there are 4,096 possible di-codon pairs. Di-codon frequency, the di-codon pairs that were observed versus the number that should be expected, can be used to indicate further codon usage bias. The 35 di-codon pairs with the highest frequency within the fusion gene across class I and class II sequences are represented in Supplemental Table S4.

Additionally, we studied the codon usage patterns of the fusion gene in class II sequences based on predicted virulence (deduced amino acid motif of the cleavage site)(n=1,341, number of viruses isolated from 1933 to 2014), location (n=59, number of countries), and geography. However, we did not find any evidence of adaptation within classes or significant differences due to virulence and/or geographic location. No significant difference in codon usage was found between virulent and non-virulent viruses of the same genotype or viruses isolated in different continents (data not shown).

3.2. Variation in codon adaptation is observed among the three genomic regions of class I and class II

We divided the genome into three regions (NC-PHO-M, F-HN, and POL) based on transcriptional polarity and studied the codon usage per genomic region in 288 complete genomes. We began by comparing the RSCU within the different genomic regions, and in addition to the variation found within the fusion gene, the existence of additional variation within each region between class I and class II sequences was determined. In all 3 regions, codons that

282 encode for amino acids G, I, L, P, Q, R, S, T, and V have statistically different RSCU values
283 between class I and class II sequences (Fig S1). Fig S1 represents the statistically significant
284 differences on distribution and bias on the RSCU data from Table 1, which shows that despite all
285 regions utilizing GCA (A), UUC (F), and AAG (K) similarly, the codon usage bias among
286 different regions is evident. In addition, no association between the number of synonymous
287 codons per amino acid and chemical composition was observed (data not shown).

288 To understand the relationship between the different transcriptional regions and codon
289 usage, we looked at those codons that were statistically different between class I and class II in
290 the three differentially expressed genomic regions (Table 1), since there were minor (not
291 statistically significant) differences between the RSCU values among the regions specifically. In
292 the NC-PHO-M region, there were 38 codons used significantly different between class I and
293 class II (bold in Table 1); the codon usage of five of those codons (UGU, UGC, AUA, CUC, and
294 ACA) was specifically only found to be significant in the NC-PHO-M region (blue in Table 1).
295 In the F-HN region, there were 35 codons used significantly different between class I and class II
296 (bold in Table 1); the codon usage of five of those codons (GAG, GAA, GGC, UCG, and GUA)
297 was specifically only found to be significant in the F-HN region (red in Table 1). In the POL
298 region, there were 40 codons, used significantly different between class I and class II (bold in
299 Table 1); the codon usage of seven of those codons (GCU, GCG, GCC, GAU, GAC, AGC, and
300 AGU) was specifically only found to be significant in the POL region (green in Table 1). It is
301 interesting to note, that AUC (I), CUA (L), AAC (N), AAU (N), CGC (R), UCU (S), ACC (T),
302 UAC (Y) and UAU (Y) were found to be statistically different between class I and class II in
303 both the NC-PHO-M and POL regions but not within the F-HN region.

To expand our analysis on codon usage bias, the CAI values determined for the class I and class II sequences were also used to visualize codon adaptation to *Gallus gallus* in Fig. 2A. Those sequences with higher CAI values are considered to be more adapted towards the host. CAI values of all three genomic regions were statistically different ($P < 0.01$) when comparing adaptation between class I and class II viruses to *Gallus gallus* (corresponding letters in Fig. 2A). In addition, all regions were statistically different within each class; however, the class II F-HN genomic region has a lower CAI value compared to the class II complete genome but the same trend was not observed in class I. Due to this result and to further understand more about how gene expression is correlated with codon usage bias in the F-HN genomic region, we once again determined the CAI (to *Gallus gallus*) and ENC values for each class I (n=29) and class II (n=259) sequence, and plotted the values in CAI vs ENC scatter plots per class (Figs. 2B-C). The class I F-HN region (Fig. 2B) had a negative r value of -0.9033, with average ENC values around 56.6 and average CAI values around 0.743. The class II F-HN region (Fig. 2C) had a positive r value of 0.1828, with average ENC values around 56.6, and average CAI values around 0.730.

The ENC and GC3 value for all sequences in each genomic region and for each class was calculated, and the average for each region and class is plotted in Figure 2D (NC-PHO-M = blue; F-HN = red; POL = green). In agreement with the other panels in Figure 2, Figure 2D reiterates that overall class II codon usage is consistently different for class I and II in all regions. The GC3 content of the regions in class II were numerically lower than the class I regions, with the class II F-HN region having the lowest GC3 content among all regions in both classes. The ENC values were very similar (between 56-57) for all regions in both classes, with only a slight difference in the NC-PHO-M regions. Interestingly, the ENC values of the F-HN region of Class I and II, were nearly identical.

3.3. Codon usage of the complete genome does not match the codon usage of the fusion gene

We have compared the relative synonymous codon usage patterns of the complete genome between class I and class II sequences. We have analyzed the number and frequencies of codon usage in 288 complete genome datasets (Table S2). Figure 3A displays the average RSCU value of all complete genome sequences of that class that is statistically different between class I and class II viruses, which includes codons that encode amino acids A, F, G, L, N, P, Q, R, S, T and V. Complete genome RSCU values for both class I and class II are found in supplementary Table 3, and those codons in bold indicate the codon usage that is statistically different between class I and class II. Values of RSCU greater than 1 indicate more frequent use of that codon than expected and was found for codons CUA (L), UUG (L), AGG (R) CGC (R), and UAU (Y) in class I only, and UUA (L), CCU (P), AGA (R), CGG (R), ACU (T), and GUC (V) in class II only (Table S3). Significance of differences in codon usage was determined by comparing RSCU values among the numbers of class I and class II sequences using 2-way ANOVA, with Dunn-Sidak's multiple test correction, with significance defined as $P < 0.01$.

To study whether the complete genome provided a more representative pattern of codon usage between class I and class II viruses, a Pearson Correlation plot was made to visualize the relationship between class I and class II RSCU (Fig. 3B). The average RSCU of all sequences for each class and genotype is represented on the graph. The complete genome of class I and class II viruses show a high, positive correlation with a Pearson r coefficient of 0.84 for each comparison ($P < 0.0001$).

We wanted to determine if the differences seen for the codon usage in the fusion gene of the class II genotypes also existed in the complete genome. Therefore, the ENC and GC3 values

for the complete genome of all sequences were calculated and used to create a scatter plot for each class and genotype in Fig. 3C. The average ENC value was 57-58 for both classes, closer to the maximum 61, the latter indicating use of most synonymous codons. Average GC3 values ranged from 46.6 to 53.7, with class I having the highest GC3 value, indicating an evolutionary decrease in the use of GC at position 3 in class II sequences, in agreement with the fusion gene. The regression line provides a way to associate the effect ENC and GC3 have on each of the groups (Gerdol et al., 2015; Wright, 1990).

We analyzed di-codon frequency to further compare codon usage in the complete genome between class I and class II viruses, including class II genotypes VI and VII, and discovered differences in the di-codons used most often between class I and class II viruses and among class II genotypes (Table S5). The 35 di-codon pairs with the highest frequency within the complete genome across class I and class II sequences are represented in Supplemental Table S5, which are different from the highest frequency di-codon pairs seen in Supplemental Table S4 for the fusion gene.

3.4 Codon usage adaptation in APMV-1 is a slow process.

Because of the small differences in codon usage among different genotypes, we speculated that codon usage adaptation is a slow process that may require long periods of evolution. In order to estimate the time to MRCA of class I and class II viruses and between best represented genotypes within class II, we used a BMCMC approach as implemented in the BEAST package (Drummond et al., 2012). The exponential growth model was the best fit to the data (Griffiths and Tavaré, 1994). Similar values were estimated using both GTR+ Γ_4 and HKY+

372 Γ_4 models (Table 2). Maximum clade credibility trees revealed that class I and class II APMV-1
373 have evolved from ancestors that existed between 1772 and 1782 (Table 2). Indeed, the long
374 period to the separation of class I and class II (approximately 234-244 years before the most
375 recent isolates included in this study) and the small differences in CAI of these two classes
376 provides evidence to suggest that the codon adaptation of APMV-1 is a slow process. As
377 expected, class II genotype VI emerged from common ancestors with genotype VII (and
378 genotypes XIII, XIV, XVII and XVIII) sometime between 1947 and 1951 (Table 2), that is in
379 agreement with previously reported estimates (Chong et al., 2013; Sonora et al., 2015). Despite
380 the differences in host of isolation for viruses of genotypes VI and VII, there are no large
381 differences in codon usage or host adaptation, as it would be expected from a slow process of
382 adaptation. Genotypes VII and VI separation is estimated to have occurred approximately 69-73
383 years ago. While genotype VI viruses are the cause of the third panzootic and maintained in
384 pigeons and doves, viruses of genotype VII belong to the fourth and fifth panzootics and have
385 been mainly isolated in chickens with occasional spillovers in other domestic and wild species
386 (Dimitrov et al., 2016).

4. Discussion

While codon usage bias has been studied in other viruses of the *Paramyxoviridae* family (Belalov and Lukashev, 2013), and in AMPV-1 (Kumar and Kumar, 2014; Wang et al., 2011), this is the first study that shows that there are preferential codon usage within the two different APMV-1 classes and that there are different codon usages among different genomic regions. Preferential codon usage occurs across all domains of life, including viruses, in which this codon usage preference enables viruses to increase or decrease replication rates (Behura and Severson, 2012; Moura et al., 2007). Previous studies have concentrated on codon usage bias in viral families such as *Orthomyxoviridae* (Wong et al., 2010), *Flaviviridae* (Moratorio et al., 2013), and *Picornaviridae* (Aragones et al., 2010) to show that viruses differ in their specificity of codon usage depending on their host organism. Previously, codon usage bias was studied for 201 fusion genes (Kumar and Kumar, 2014) and 31 complete genomes (Wang et al., 2011) of APMV-1 to determine if there were any host specific changes in codon usage; however, the authors did not find any host adaptation in APMV-1. Here, using 1,341 fusion and 288 complete genome sequences, we have extended those studies, and found differences between the two AMPV-1 classes and among the different genomic regions of class I and class II.

The detection of codon usage adaptations in this manuscript may have been possible due to the separation of sequences into recognized classes (41 to 46% nucleotide distance between class II genotypes and class I), the higher number of codons utilized here, and the recognition that the fusion gene may not best represent the mechanisms of codon usage adaptation in APMV-1. The data indicates that codon usage bias for the fusion gene is distinct from other genes and there is not a good correlation between class I and II fusion gene codon usage for this gene. The observed low correlation when comparing the fusion gene RSCU between class I and

class II sequences (Fig. 1B) indicates gene specific differences in codon usage bias, suggesting the existence of different yet unknown types of evolutionary pressures affecting that bias in this gene. The ENC vs CAI and GC3 data provides information that suggests selection plays a predominant role in codon usage variability in class I fusion gene, whereas the codon usage relations for class II fusion are more consistent with random mutations or with unknown pressures. The fusion protein is a surface protein that has been shown to be affected by positive selection and antibody selective pressures (Choi et al., 2010; Miller et al., 2009; Yusoff et al., 1989); therefore, it is possible that fusion-specific selection may affect codon usage of this gene.

This study indicates that there are specific codon usage adaptations to yet unknown pressures in the fusion and HN coding regions. Codon usage differences in three different APMV-1 genomic regions appear to be related to expected transcriptional levels. In both classes, the CAI of the highest expressed genes to *Gallus gallus* is higher than the CAI of the lowest expressed genes (except for F and HN), suggesting a possible role of codon usage on the control of APMV-1 gene expression (Quax et al., 2015). The increased adaptation of the NC-PHO-M to *Gallus gallus* is consistent with a higher use of host tRNAs, as this region would be translated faster and more efficiently. Although different codons are preferentially used across the entire genome and the positive correlation seen for the codon usage among the three genomic regions indicate that class I and class II are varying together in the same magnitude and direction. Most interesting are the deviations observed for the F and HN genes of class II viruses. A CAI gradient from highly transcribed regions to low transcribed regions is clearly observed in viruses that are mostly isolated from wild birds (class I) however the gradient in the CAI is interrupted in viruses generally associated with chickens (class II) at the F and HN genes. This observation is relevant because the evolution of F and HN genes of class II viruses is likely to be influenced by

intensive vaccination in poultry. As the F and HN genes encode for surface proteins that contains the sites of neutralization, it is possible that those two genes may evolve under different type of pressures than the rest of the genome. It is possible that the discrepancies seen in codon usage between the fusion protein and other genomic regions may be partially attributed to positive selection caused by immune selective pressures on this gene because of its function as a surface viral protein (Miller et al., 2009).

We infer that the fusion gene of class II viruses does not follow the same evolutionary trend as the complete genome, which leads us to propose that any future analysis dealing with codon usage bias should be done on both complete genomes and individual, essential genes. Although all APMV-1s would have the same ancestral host, our data suggest that these two classes of viruses have separated over two hundred years ago, thus it is likely that the different classes may have adapted to replicate in different host species. As APMV-1s are reliant on the avian host's translational machinery to synthesize the necessary proteins they need to replicate (Walsh and Mohr, 2011), it would make sense that these viruses would evolve to use the host's tRNA pool more efficiently (Pavon-Eternod et al., 2013). The differences in codon usage and the differences in di-codon frequencies support this result. It is widely believed that virulent Class II viruses (most of available sequences in GenBank) are maintained in vaccinated chicken, and therefore, it would be expected that recent viruses would have significantly changed to adapt to the host translational machinery. Surprisingly our codon adaption index data did not reveal any significant increased adaptation on class II codon usage to chickens except for a minor increase on the polymerase gene. Our results suggest that codon usage adaptation to chicken was not a significant force in the rapid NDV evolution that has occurred in the last 40 years since

vaccination started. It remains to be determined if other types of adaptations, such as adaptations to receptors or adaptation to the host translational machinery operate at the protein level.

To characterize the codon usage bias in the genotypes of class II viruses, we have separated the genotypes that are genetically related but clearly defined by the occurrence of significant genetic changes (above 10% nucleotide differences between different genotypes) (Diel et al., 2012a; Dimitrov et al., 2016). Most viruses from class I are normally isolated from waterfowl, while viruses from class II have been isolated from a wide range of species. Some of those differences may be due to the existence of a maintenance cycle in other possible host organisms; with these hosts being different for the two classes and possibly different for the genotypes. However, a definitive identification of additional hosts is still lacking for some genotypes (e.g. genotype VII), and further investigation into the adaptation mechanisms of these viruses to their avian hosts need to be done.

Due to the high mobility of the viruses, the broad host ranges, and the slow rate of change, codon usage geographic or temporal changes are not likely to be easily detected. First, apart from a few exceptions in APMV-1, it is difficult to ascribe viruses to hosts. Spillovers between chickens and wild birds or among different wild bird species have been reported (Ayala et al., 2016; Cardenas Garcia et al., 2013; Dimitrov et al., 2016; Kim et al., 2007) and are likely to occur due to the wide host ranges of APMV-1s (Kaleta and Baldauf, 1988). Thus, it is possible to isolate identical or highly similar viruses from different host species, masking results on the adaptation to a host. A similar situation is likely when studying codon usage association with geographic or temporal factors. Our data and previous work suggest that efforts should be made to identify and exclude sequences from spillovers from any evolutionary studies (Ayala et al., 2016; Dimitrov et al., 2016), which may account for the failure of detection of patterns by others.

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Figure Legends:

Fig. 1. Class I and class II avian paramyxoviruses 1 display differences in codon usage

within the fusion gene. A) The average relative synonymous codon usage (RSCU) value for each codon in the fusion gene (X axis) was calculated using all available sequences (175 for CI and 1166 for CII), and only the codon usage (Y axis) that was statistically different ($P < 0.01$) between class I (blue) and class II (yellow) viruses is shown with standard error bars. Values can be found in Table S3; **B)** The average RSCU value for each codon in the fusion coding region of avian paramyxoviruses 1 (APMV-1) was used to assess the correlation of codon usage among class I (X axis) and class II viruses (Y axis). **C)** The ENC value (X axis) and GC3 value (Y axis) were calculated for the fusion gene of each APMV-1 sequence, and average values for all class I sequences ($n=175$) and the more prominent class II genotypes (104-GI, 18-GII, 87-GV, 261-GVI, 474-GVII, 11-GX, 41-GXIII) are plotted on the graph. The 95% confidence levels are indicated by the dotted lines; linear regression analysis provides a slope of 0.8086 ± 0.01507 with a significant P value < 0.0001 . **D)** The fusion gene CAI (X axis) and ENC (Y axis) values were calculated and plotted for all class I sequences; **D)** The scatter plot of CAI vs ENC for class II sequences was created similarly to the plot in D.

Fig. 2. Avian paramyxoviruses 1 display differences in codon usage between the three

transcriptional genomic regions. A) The average CAI value, for the complete genome and each genomic region (NC-PHO-M, F-HN, and POL) for class I ($n=29$) and class II ($n=259$) viruses, was determined using *Gallus gallus* as a reference genome. The complete genome result is in black, NC-PHO-M is in blue, F-HN is in red, and POL is in green color. Standard error bars are shown and statistically significant differences between the same regions between classes are

shown ($P < 0.01$) with corresponding letters; **B**) The F-HN region CAI (X axis) and ENC (Y axis) values were calculated and plotted for all class I sequences; **C**) The F-HN region CAI (X axis) and ENC (Y axis) values were calculated and plotted for all class II sequences; **D**) For each region, the ENC (X axis) and GC3 (Y axis) values were determined for each class I and II sequence, and average values for each region from each class are plotted on the graph. NC-PHO-M sequences are in blue, F-HN sequences are in red, and POL sequences are in green color.

Fig. 3. Differences and correlations in relative synonymous codon usage (RSCU) in the complete genome between class I and class II avian paramyxoviruses 1. **A**) The average RSCU value for each codon across the complete avian paramyxoviruses 1 (APMV-1) genome (X axis) was calculated for all available sequences (29 for class I and 259 for class II), and only the codon usage (Y axis) that is statistically different ($P < 0.01$) between class I (blue) and class II (yellow) viruses are shown with standard error bars. Values can be found in Table S6; **B**) The average RSCU value for each codon in the complete genome of APMV-1 was used to assess the correlation of codon usage among class I (X axis) and class II viruses (Y axis); **C**) The ENC (X axis) and GC3 (Y axis) values were calculated for the complete genome of each APMV-1 sequence, and average values for all class I sequences ($n=29$) and the more prominent class II genotypes (28-GI, 5-GII, 17-GV, 73-GVI, 87-GVII, 8-GX, 14-GXIII) are plotted on the graph. Linear regression analysis provides a slope of 0.8348 ± 0.01521 with a significant P value < 0.0001 .

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Table 1. Relative synonymous codon usage values of the three genomic regions in class I and class II avian paramyxoviruses 1.

Codon	AA	NC-PHO-M		F-HN		POL	
		Class I	Class II	Class I	Class II	Class I	Class II
GCU	A	0.87	0.89	0.84	0.87	0.83	0.98
GCG	A	0.45	0.54	0.42	0.48	0.37	0.49
GCC	A	1.09	1	0.99	0.91	0.94	0.7
GCA	A	1.6	1.57	1.75	1.74	1.85	1.83
UGU	C	0.82	0.63	1.09	1.08	1.08	1.06
UGC	C	1.18	1.37	0.91	0.92	0.92	0.94
GAU	D	0.89	0.91	1.15	1.08	1.07	1.16
GAC	D	1.11	1.09	0.85	0.92	0.93	0.84
GAG	E	1.16	1.25	1.2	0.79	1.1	1.15
GAA	E	0.84	0.75	0.8	1.21	0.9	0.85
UUU	F	0.72	0.96	0.82	0.96	0.83	0.87
UUC	F	1.28	1.04	1.18	1.04	1.17	1.13
GGU	G	0.76	0.69	0.76	0.95	0.75	0.9
GGG	G	1.31	1.3	1.53	1.42	1.53	1.2
GGC	G	0.78	0.75	0.6	0.8	0.79	0.72
GGA	G	1.14	1.26	1.11	0.83	0.94	1.18
CAC	H	1.11	0.78	0.68	0.87	0.83	0.83
CAU	H	0.89	1.22	1.32	1.13	1.17	1.17
AUU	I	1.02	0.81	0.78	0.68	0.99	1.1
AUA	I	0.78	0.66	1.16	1.26	0.94	0.92
AUC	I	1.2	1.53	1.05	1.07	1.07	0.98
AAA	K	0.85	0.8	0.86	0.86	0.88	0.94
AAG	K	1.15	1.2	1.14	1.14	1.12	1.06
CUA	L	0.83	0.71	1.01	0.93	1.32	1.08
CUC	L	1.22	1.38	0.97	1	1.01	0.99
CUG	L	0.92	0.9	1.18	1	0.91	1.04
CUU	L	1.03	1.02	0.84	1.07	0.76	0.88
UUA	L	0.78	1.17	0.9	1.24	0.87	1.1
UUG	L	1.22	0.83	1.1	0.76	1.13	0.9
AAC	N	1.22	0.89	0.78	0.74	0.95	0.82
AAU	N	0.78	1.11	1.22	1.26	1.05	1.18
CCA	P	1.42	1.15	1.35	0.89	1.44	1.53
CCC	P	0.95	0.74	0.86	1.09	0.46	0.66
CCU	P	0.88	1.24	1.22	1.36	0.98	1.23
CCG	P	0.76	0.87	0.56	0.66	1.12	0.58
CAA	Q	0.79	0.93	0.84	1.23	0.99	0.92
CAG	Q	1.21	1.07	1.16	0.77	1.01	1.08
AGA	R	0.92	1.01	0.79	1.03	0.85	1.01
AGG	R	1.08	0.99	1.21	0.97	1.15	0.99
CGA	R	0.33	0.96	1.72	0.8	1.49	1.23
CGC	R	1.14	0.9	1.12	1.11	1.02	0.5
CGG	R	1.54	1.34	0.65	1.32	0.85	1.18

CGU	R	0.99	0.81	0.5	0.77	0.64	1.08
AGC	S	1.25	1.23	0.96	1.06	1.25	1.03
AGU	S	0.75	0.77	1.04	0.94	0.75	0.97
UCA	S	1.67	1.07	1.11	1.45	1.56	1.45
UCC	S	0.79	1.02	0.97	0.76	0.64	0.91
UCG	S	0.43	0.47	0.52	0.36	0.42	0.36
UCU	S	1.11	1.43	1.41	1.44	1.39	1.28
ACC	T	1.11	1.25	0.97	1	0.94	0.79
ACA	T	1.45	1.2	1.55	1.5	1.55	1.61
ACG	T	0.36	0.24	0.42	0.32	0.63	0.39
ACU	T	1.08	1.3	1.07	1.18	0.88	1.21
GUU	V	0.42	0.75	0.51	0.84	0.56	0.63
GUG	V	1.87	1.22	1.3	0.97	1.14	1.11
GUC	V	0.96	1.19	0.76	1.11	1.18	1.1
GUA	V	0.75	0.84	1.43	1.09	1.12	1.16
UAC	Y	1.29	1.09	0.96	0.91	0.9	1.02
UAU	Y	0.71	0.91	1.04	1.09	1.1	0.98

* Codons in **bold font** indicate the codon usage that is statistically different between class I and class II

*Values in color indicate the codon usage that is statistically different between class I and class II only in that specific region (NC-PHO-M = blue; F-HN = Red; POL = Green)

*NC-PHO-M: Class I = 36,163 codons; Class II = 322,973

*F-HN: Class I = 32,596; Class II = 291,116

*POL: Class I = 63,916; Class II = 570,836

Table 2. Bayesian estimates of demographic parameters as inferred from Bayesian Markov Chain Monte Carlo analyses of 155 APMV-1 polymerase gene sequences using relaxed lognormal molecular clock.

Group	Model	Demographic model	Date (years)	Time to MRCA ^a In years	Time to MRCA In years (95% HPD ^b)
Class I and class II	GTR+ Γ_4	Exponential	1782	234	191/259
	HKY+ Γ_4	Exponential	1772	244	176/336
Genotype VI and Genotype VII	GTR+ Γ_4	Exponential	1945	71	63/73
	HKY+ Γ_4	Exponential	1949	67	58/70

^a MRCA = most recent common ancestor

^b HPD = highest posterior density

Figure(s) 1

Figure(s) 2

Figure(s) 3