Reassortant influenza A viruses in wild duck populations: effects on viral shedding and persistence in water

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Wild ducks of the genus *Anas* represent the natural hosts for a large genetic diversity of influenza A viruses. In these hosts, co-infections with different virus genotypes are frequent and result in high rates of genetic reassortment. Recent genomic data have provided information regarding the pattern and frequency of these reassortant viruses in duck populations; however, potential consequences on viral shedding and maintenance in the environment have not been investigated. On the basis of full-genome sequencing, we identified five virus genotypes, in a wild duck population in northwestern Minnesota (USA), that naturally arose from genetic reassortments. We investigated the effects of influenza A virus genotype on the viral shedding pattern in Mallards (*Anas platyrhynchos*) and the duration of infectivity in water, under different temperature regimens. Overall, we found that variation in the viral genome composition of these isolates had limited effects on duration, extent and pattern of viral shedding, as well as on the reduction of infectivity in water over time. These results support that, in wild ducks, functionally equivalent gene segments could be maintained in virus populations with no fitness costs when genetic reassortments occur.

**Keywords:** avian influenza virus; genetic reassortments; wild birds; mallards; water-borne transmission; experimental infections

1. INTRODUCTION

Influenza A viruses (IAV) represent a major threat to human and veterinary health. The emergence of the highly pathogenic (HP) H5N1 virus in domestic birds in southeastern Asia [1] and the introduction of the swine-origin H1N1 virus in human populations [2] have shown IAV ability to spread beyond species barriers and adapt rapidly to new hosts and environmental conditions. Genetic reassortment between viruses co-infecting the same host is a key process for the emergence of IAV in humans [3]. For instance, the genomic characterization of the swine-origin H1N1 virus has revealed that successive reassortment between virus genes circulating in swine, human and wild birds, occurred several years before its detection in 2009 [4]. Although it is recognized that genetic reassortment is of primary importance for host shifts and adaptation to humans, swines and domestic birds, its significance in wild birds remains unclear.

Wild ducks of the genus *Anas* represent the natural hosts for a large genetic diversity of low pathogenic (LP) IAV, with no evidence for species-specific associations [5,6]. In wild duck populations, host co-infections with different virus genotypes are frequent [7] and result in high rates of genetic reassortment, with no clear pattern of gene segment association [8,9]. It has been suggested that a large pool of functionally equivalent gene segments could co-circulate in duck populations, without strong selective pressure to be maintained as linked genomes [9]. The increasing genomic data provide novel information regarding the pattern and frequency of genome reassortment in wild ducks, however, its consequences on viral shedding and maintenance in the environment have not been investigated.

Shedding pattern and persistence in water represent two key components of LP IAV transmission dynamics and dispersal in wild duck populations [10–15]. Viral replication mainly occurs in the epithelial cells of the intestinal tract, resulting in high virus concentration in faeces [16,17]. Infected birds contaminate aquatic environments in which IAV could persist for extended periods of time, depending on water temperature and physico-chemical characteristics [18–20]. In addition to LP IAV, it has been suggested that patterns of viral excretion and environmental persistence could also represent important factors for the spread of domestic bird-adapted HP viruses by migratory waterfowl [14,15,21–24].

In this study, we investigated the effects of LP IAV genome reassortment on the viral shedding pattern in ducks and...
persistance in water. We hypothesized that reassortment could generate genotype-related differences, favouring the selection for particular viruses, potentially leading to a temporal and spatial heterogeneity in the prevalence and diversity of virus genotypes, as commonly reported in wild duck populations [25–27]. Alternatively, non-significant differences among virus genotypes would support the maintenance of functionally equivalent gene segments, with no fitness costs associated with reassortments, as suggested by recent genomic studies [9,28].

To test these hypotheses, we focused on wild bird-origin viruses co-circulating in a duck population in northwestern Minnesota, USA. First, we performed the full-genome sequencing of five viruses identified as potential reassortants (based on the virus subtype; i.e. haemagglutinin and neuraminidase combination). For each virus genotype, we then experimentally characterized the viral shedding pattern (duration, viral load and excretion route) based on infections of mallards (Anas platyrhynchos). Finally, the reduction of infectivity in water over time was assessed under different temperature regimens to identify genotype-related differences in the persistence in aquatic habitats.

2. MATERIAL AND METHODS

(a) Virus selection

Isolates were obtained from an ongoing long-term surveillance of IAV circulation in wild birds in Minnesota, USA [27]. In this study, five viruses isolated from dabbling duck (adults and juveniles) during the summer of 2007 (between 14 and 16 September), at Roseau River Wildlife Management Area (48°58′39.77″ N, 96°00′32.08″ W) were selected: A/Mallard/MN/Sg-00169/2007 (H3N8); A/Mallard/MN/Sg-00107/2007 (H6N2); A/Mallard/MN/Sg-00219/2007 (H4N8); A/Mallard/MN/Sg-00170/2007 (H6N1); and A/Green-winged teal/MN/Sg-00197/2007 (H6N8) (referred to hereafter as their respective subtype). Relative abundance of the five virus subtypes (i.e. prevalence ± 95% confidence intervals) in the waterfowl population at the sampling site was 22.5 ± 9.7% (H3N8), 9.9 ± 6.9% (H6N1), 5.6 ± 5.4% (H6N2), 2.8 ± 3.9% (H6N8) and 1.4 ± 2.7% (H4N8) (cf. [27] for details).

Second passages of stock viruses were propagated in 9- to 11-day-old specific-pathogen-free (SPF) embryonating chicken eggs [29]. Endpoint dilutions were performed in SPF embryonating chicken eggs and Madin–Darby canine kidney (MDCK) cell line to determine the median embryo infectious dose (EID50) and the median tissue culture infectious dose (TCID50) [29,30].

(b) Full-genome sequencing

Sample preparation, RNA extraction and amplification of all gene segments were performed using a single-step multiplex PCR [31]. cDNA libraries were prepared from PCR-purified multiplex amplicons with the QIAquick PCR Purification kit (Qiagen, Valencia, CA, USA) and subjected to adaptor lig- ation and whole genome sequencing using 454 FLX technology [32]. Sequencing reads were aligned with IAV genomes using BlastN algorithm of NCBI BLAST v. 2.2.16, assembled in GS De Nova Assembler v. 2.0.00.20 (454 Life Sciences, Branford, CT, USA) and mapped in GS Reference Mapper v. 2.0.00.20 (454 Life Sciences), as described previously [32]. Contigs were reassembled in SEQUENCER v. 4.9 (Gene Codes, Ann Arbor, MI, USA) and annotated based on BLAST analyses. Nucleotide sequences generated in this study have been deposited in GenBank under the accession numbers listed in the electronic supplementary material, table S1.

Nucleotide and amino acid sequences of each gene segment were aligned with CLC sequence viewer 6.4 (CLC bio, Aarhus, Denmark), and similarity matrix was calculated using the program PHYLIP v. 3.68 [33]. Concatenated sequences, including the eight gene segments were also generated to estimate the overall level of similarity between virus genotypes.

(c) Experimental infections

All work was conducted at an enhanced Biosafety Level 2 facility at the Poultry Diagnostic Research Center, Athens, GA, USA.

One-day-old mallards were purchased from a commercial source (Murray McMurray Hatchery, Webster City, IA, USA) and raised for one month under indoor confinement until the beginning of the experiments. Three days before inoculation, 30 ducks were randomly assigned to one of six infection groups (H3N8, H4N8, H6N1, H6N2, H6N8 and a positive control: A/Mallard/MN/199106/1999 H3N8 (second passage of stock virus), referred to as H3N8-C hereafter) and transferred to isolation units ventilated under negative pressure with high-efficiency particulate air filters.

Ducks were inoculated in the choanal cleft and the trachea with a volume of 0.2 ml (split evenly between the two routes) containing an infectious titre of approximately 105.6 EID50 ml−1: (based on back titres: 105.5 EID50 ml−1 for H6N1 and H6N2, 106.3 EID50 ml−1 for H4N8 and H6N8, 105.78 EID50 ml−1 for H3N8, and 105.53 EID50 ml−1 for H3N8-C). Oropharyngeal (OP) and cloacal (CL) swabs were collected from all ducks before inoculation, and at 1, 2, 3, 4, 5, 7, 9, 11 and 14 days post-inoculation. Swab samples were stored at −80°C. Virus isolations were performed on CL and OP swab samples using 9- to 11-day-old SPF embryonating chicken eggs [29]. For each sample, RNA was extracted the same day as the samples were inoculated in eggs, with the MagMAX-96 ALND Viral RNA Isolation kit (Ambion, Austin, TX, USA), using the Thermo Electron KingFisher magnetic particle processor (Thermo Electron Corporation, Waltham, MA, USA) [34]. Real-time PCR (RT-PCR) targeting the Matrix gene was conducted with the Qiagen OneStep RT-PCR kit (Qiagen, Valencia, CA, USA) and the Cepheid SmartCycler System (Cepheid, Sunnyvale, CA, USA) [35].

Blood samples were collected from the right jugular vein, before inoculation and the last day of the experiment (14 days post-inoculation), to ensure that ducks successfully became infected and produced IA-specific antibodies. Samples were centrifuged for 30 min at 1500 r.p.m and sera were stored at −20°C until tested with a commercial blocking ELISA kit (IDEXX FlockCheck IA Multi-Screen Antibody Test; IDEXX Laboratories, Westbrook, ME, USA).

(d) Water-persistence trials

Distilled water buffered with 10 mM HEPES was adjusted with 1 N solutions of NaOH or HCl to provide a pH of 7.2. For each virus (H6N1, H6N2, H6N8, H3N8 and H4N8), we tested the effect of five constant (4°C, 10°C, 17°C, 23°C, 28°C) and two variable (−20°C/4°C and 17°C/23°C) temperature regimens, on infectivity over time.
For each virus, infectious amnio–allantoic fluids were diluted 1 : 100 in the distilled water. Inoculated water samples were divided into 2 ml aliquots in 5 ml polystyrene round-bottom tubes and placed in incubators set to the appropriate temperature. For variable temperature regimens, samples were transferred daily into incubators set to different fixed temperatures (i.e. 14 h at low temperature: −20 °C or 17 °C; 10 h at high temperature: 4 °C or 23 °C).

For each temperature regimen, the virus concentration was measured at time of inoculation (day 0) and nine to 23 times post-inoculation. Duplicate 0.5 ml inoculated water samples were diluted 1 : 1 by addition of 0.5 ml of 2× minimal essential medium (MEM). Serial 10-fold dilutions (from 10−1 to 10−6) of the samples were made in 1× MEM supplemented with antibiotics (10 000 U ml−1 penicillin G, 10 mg ml−1 streptomycin and 25 μg ml−1 amphotericin) and titrated on MDCK cells [20,30].

(e) Statistical analyses
All analyses were conducted in r v. 2.12.1 [36]. Percentage agreement and Cohen’s kappa coefficient (κ) were calculated to estimate agreement between virus isolation and RT-PCR for virus detection in CL and OP swab samples. Kappa coefficients were interpreted as followed: κ ≥ 0.8 indicated a substantial agreement and Cohen’s kappa coefficient (k), κ ≥ 0.6 indicated a moderate agreement, κ ≥ 0.4 indicated a fair agreement, κ ≥ 0.2 indicated a slight agreement, 0.2 < κ < 0.4 indicated a small agreement, 0.4 < κ < 0.6 indicated a moderate agreement, 0.6 < κ < 0.8 indicated a substantial agreement and κ > 0.8 indicated a perfect agreement [37].

One-way analysis of variance (ANOVA) was used to test the effect of virus genotype on: (i) the total shedding duration (number of days from the first to the last day of successful virus isolation) and (ii) the restricted shedding duration (number of consecutive days for which viruses were isolated). The total shedding duration accounts for the intermittent viral shedding sometimes reported in Mallard [38], whereas the restricted shedding duration considers only the continuous excretion. Separate ANOVAs were performed for the CL and OP shedding durations. Fligner–Killeen tests were used prior to the ANOVA to check for homogeneity of variance [39].

A standard curve was generated to estimate the viral shedding load based on the Matrix gene copy number (hereafter M-gene copy) in samples tested by RT-PCR (available upon request). Successive dilutions (from 10−1 to 10−5) were performed on a Matrix gene transcript containing 9.1 × 1011 gene copies per microlitre (National Veterinary Services Laboratory, Ames, IA, USA). As the cycle threshold (Ct) value and the log10(M-gene copy) were significantly correlated (Pearson’s correlation coefficient r = −0.98, d.f. = 70, p < 0.001), a simple regression was performed (n = 72; adjusted r² = 0.96, p < 0.001) and Ct values were transformed in log10(M-gene copy) for the tested samples. Linear mixed models (LMMs) were used to investigate the effect of virus genotype and time on the CL and OP shedding load. Generalized linear mixed models (GLMMs) were used with a binomial error structure and logit link function were used to examine the effect of virus genotype and time on the probability of successfully isolating viruses from CL and OP swab samples. Full models included time and virus genotype as explanatory variables, and their interaction. Time and virus genotype were specified as fixed factors. We included duck identity as a random factor to account for the repeated viral measurements performed on individual ducks. Terms significance were estimated using models comparison based upon the likelihood ratio test (LRT) statistics.

An analysis of covariance was used to test the effects of time, virus genotype and temperature regimen on virus infectivity in water. Results from duplicate titrations were averaged and log10 transformed prior to analyses. Linear regressions were also used to calculate the time required for a 90 per cent reduction of infectivity titre in water (i.e. time required for a decrease of the viral titre by 1 log10 TCID50 ml−1; [18,20]).

3. RESULTS
(a) Genomic characterization
Nucleotide sequences comparison between viruses showed a level of similarity between gene segments ranging from 70.7 per cent to 100 per cent for the internal gene segments (PB2, PB1, PA, NP, MP and NS), and from 51.5 per cent to 99.9 per cent for the HA and NA genes (table 1). The same pattern was observed when considering amino acid sequences: PB2, PB1, PA, NP

Table 1. Genome constellation of the five influenza A viruses. Different coloured squares represent gene segments with at least 95% nucleotide sequence similarity between virus genotypes. (For example, for the NP: the nucleotide sequence similarity between H3N8 and N6N2, and between H4N8 and H6N1, is higher than 95%).
and MP exhibited a high level of similarity (97–100%) when compared with NS (67–100%), HA (43–99%) and NA (40–100%). When analysing concatenated nucleotide sequences, the highest genome similarity was found between H6N2 and H6N8 (91.8%); the lowest one was observed between H4N8 and H6N1 (82.7%). Overall, these results suggest that these five viruses arose from a limited gene pool, likely resulting from genetic reassortment between circulating viruses in the studied duck population.

(b) Viral shedding in mallard
No ducks in any of the groups were shedding virus at the time of inoculation and all tested negative for IAV antibodies. All ducks seroconverted by the end of the experiment (14 days post-inoculation), confirming that all were successfully infected. For one of the five ducks inoculated with H6N2, virus was not isolated before day 9 post-inoculation in CL swabs. This late excretion pattern contrasted with the rapid shedding observed in all other ducks. Because this individual may have been infected from contact transmission within the isolation unit rather than from the primary inoculation, this duck was excluded from the statistical analyses.

Viruses were successfully isolated from both CL and OP swabs collected from all ducks inoculated with H3N8-C (control group) during the first 5 days post-infection, consistent with a previous study performed in one-month-old mallards with the same virus strain [38]. Overall, a substantial agreement was found between viruses isolated in embryonating chicken eggs and detected by RT-PCR (85.8% agreement, $\kappa = 0.70$), indicating that when a virus was detected by PCR it was also likely successfully isolated.

Total and restricted CL shedding durations were $8.8 \pm 0.4$ and $8.1 \pm 0.5$ days (mean ± s.e.; figure 1a,b), respectively. There was no effect of virus genotype on shedding durations (total: $F_{5,23} = 1.7, p = 0.18$; restricted: $F_{5,23} = 0.31, p = 0.9$). Total and restricted OP shedding duration were $6.1 \pm 0.4$ and $4.7 \pm 0.3$ days (figure 1c,d), respectively. A marginally non-significant effect of virus genotype was found on the total shedding duration ($F_{5,23} = 2.6, p = 0.056$) and a significant effect was found for the restricted shedding duration ($F_{5,23} = 17.5, p < 0.001$). However, this effect was driven by a single virus genotype: when ducks inoculated with H6N2 were excluded from the analysis, no significant differences were observed between virus genotypes ($F_{4,20} = 2.2, p = 0.11$).

Finally, although there was no significant relationship between the total and the restricted OP shedding duration ($F_{1,27} = 2.9, p = 0.1$), a positive association was found between the total and the restricted CL shedding duration ($F_{1,27} = 29.5, p < 0.001$).

Overall, the viral shedding load was higher in the cloaca than in the oropharynx of infected ducks (paired Student’s t-test, $t = 7.4$, d.f. = 260, $p < 0.001$; figure 2a,b), and the CL and OP shedding were positively correlated (LMM, LRT $\chi^2 = 53, p < 0.001$). There was a strong effect of time on the viral shedding in the cloaca (LRT $\chi^2 = 81.2, p < 0.001$; figure 2a) with a sharp increase from day 1 to day 3 post-inoculation followed by a slow and continuous
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Figure 2. (a) Cloacal and (b) oropharyngeal viral shedding load over time. Each colour line represents a LOESS curve (with s.e. of the mean) fitted to the values of a virus genotype (green, H3N8; red, H4N8; light blue, H6N1; purple, H6N2; dark blue, H6N8; black, H3N8-C).

The probability of isolating a virus in the oropharynx over time was significantly different between virus genotypes (virus × time interaction: LRT $\chi^2 = 5.9$, $p = 0.31$). However, only ducks infected with H6N2 displayed a stronger effect of shedding pattern (i.e. all virus genotypes displayed a similar temporal shedding dynamics).

(c) Infectivity in distilled water

Overall, viral infectivity in distilled water decreased over time ($F_{1,556} = 218$, $p < 0.001$; figure 3). This decrease strongly varied with water temperature; the warmer the water, the faster virus infectivity decreased (temperature regimen × time interaction, $F_{6,556} = 200$, $p < 0.001$; figure 3 and table 2). Variable temperature regimens also affected virus infectivity in water ($F_{4,556} = 5.4$, $p < 0.05$) with viral titres quickly reduced when exposed to repeated freeze–thaw cycles ($-20\, ^\circ\text{C}$/4°C) but not when exposed to a milder temperature variation (17/23°C). The main effect of temperature regimen was also significant ($F_{6,556} = 193$, $p < 0.001$), with reduced persistence time observed at high temperatures or for the freeze–thaw variations. We found a significant main effect of virus genotype on viral persistence in water ($F_{4,556} = 711$, $p < 0.001$); however, a note of caution is warranted, because slightly different initial doses of virus were used for each virus genotype. There was no virus by temperature regimen interaction ($F_{24,552} = 1.5$, $p = 0.08$) but a significant time by virus interaction ($F_{4,556} = 3.5$, $p < 0.01$) was found, indicating that the decrease in infectivity over time varied among virus genotype. This interaction was, however, no longer significant when the 10°C temperature regimen was excluded from the analysis.
analysis ($F_{4,443} = 1.5, p = 0.19$). At this temperature, the H6N2 virus exhibited a significantly lower persistence than the H4N8, H6N1 and H6N8 virus, but not H3N8. No differences were found between the other four virus genotypes. Finally, there was no significant three-way interaction between time, virus and temperature regimen ($F_{24,508} = 1.4, p = 0.11$) indicating that the effect of temperature regimen on virus infectivity over time was similar among the different virus genotypes.

### 4. DISCUSSION

On the basis of full-genome sequencing, we identified five IAV that naturally arose from genetic reassortments in a wild duck population in northwestern Minnesota, USA. The genomic characterization revealed variable level of nucleotide and amino acid sequence similarities, for the HA and NA genes but also for internal gene segments (e.g. NS). In this study, we considered that these viruses differed not only by the subtype (i.e. genetic differences in the HA and NA proteins) but that they overall corresponded to different genotypes, although genotypic variations were largely driven by HA- and NA-related genetic differences. We further investigated the effects of the genotype on the viral shedding pattern in mallards and the infectivity in water over time under different temperature regimes. We hypothesize that genetic reassortments could generate genotype-related differences in these two components of IAV fitness, favouring the selection for particular viruses.

CL shedding pattern did not significantly differ between the five viruses studied, suggesting that naturally occurring genetic reassortments may not affect viral shedding by this route. As our analysis was based on a limited number of tested viruses, such a finding should be reinforced with additional studies focusing on other virus genotypes, at other locations and in different avian reservoir hosts (e.g. gulls, waders). This result nevertheless suggests that CL shedding may not differ among LP IAV co-circulating in a single duck population.
The absence of variation between virus genotypes contrasts with host-related effects that can significantly affect the outcome of virus shedding. Recent studies have for instance highlighted the effect of bird host species [40], age [38,41], body condition [42] and pre-exposure to other IAV [43,44], in the CL shedding duration and viral load. Such host-related variations may have direct implications in the transmission dynamics of IAV in wild duck populations. In addition, it may also affect long-distance virus dispersal during duck migrations [14,42,45] and be critical for the spread of HP viruses [21,22,24,46].

Compared with CL shedding, genotype-related differences may exist for OP shedding, as one of the five virus genotype we studied exhibited a significantly different pattern. Although this result suggests that genetic re-assortment potentially had a significant effect on the dominant route of viral shedding in the duck host, we cannot exclude the possibility of mutations in the H6N2 virus responsible for this reduced OP shedding. In dabbling duck species, viral load shed in the oropharynx is usually lower than in the cloaca [40]. In other bird species such as geese and gulls, the opposite pattern has been described [40,47]. The route of viral shedding is likely to be a determinant for virus transmission in wild bird populations. For instance, faeces represent an important source of contamination of aquatic habitats and subsequent infection of dabbling ducks. In gulls, however, respiratory transmission may represent a more adapted route, particularly during the breeding seasons when there are very high bird densities on colonies and contact between adult and juvenile birds. In gallinaceous poultry, respiratory transmission also is the dominant route of IAV transmission, particularly under intensive farming conditions [48,49]. Respiratory transmission is unlikely to be selected for in wild dabbling ducks; however, when IAV are introduced to other wild and domestic avian populations, respiratory transmission can represent a better-adapted route than faecal–oral transmission and favour the selection of virus genotypes inducing shedding from the respiratory tract.

Our viral shedding results are consistent with previous experimental trials (cf. [15] for synthesis) with the highest probability of virus isolation success in the cloaca during the first 8 days of infection. The potential effect of sampling time on the probability to detect viruses has been previously discussed along with aspects related to sampling technique, sample conservation and detection method, which all influence IAV detection [50–52]. Our study provided no evidence for differences in IAV detection among co-circulating viral genotypes, based on CL swab sampling. This result suggests that subtype-related differences in the probability of virus detection in the field are likely to be driven by sampling biases [53] and differences in laboratory techniques rather than genotype-related differences in the CL shedding. When considering OP shedding however, virus detection in duck populations may be biased by genotype-related differences, suggesting that only OP swab sampling may not represent an appropriate technique to estimate subtype diversity in virus populations.

LP IAV have been shown to persist for extended periods of time in distilled water, depending on temperature and physico-chemical characteristics [18–20]. Similar patterns have been reported for viral infectivity in field water samples [16,18,54] and viruses isolated from surface lake waters [31]. The five virus genotypes we studied exhibited a similar response to different temperature regimens. Temperature had a strong effect on the viral infectivity in water over time: at cold temperatures (4°C, 10°C) infectivity was maintained for several months, whereas under high temperature regimens (23°C, 28°C) it remained only for a few weeks. These results suggest that genetic reassortment may not induce important variations in persistence in aquatic habitats. Previous studies have proposed that subtype-related variations may exist, especially at low temperatures [19,20]; these studies however mainly tested viruses isolated in different host species, locations and time. Differences in the persistence of IAV in water may be limited when considering co-circulating viruses in a single duck population; however, differences observed between sampled locations may reflect adaptive responses to local water characteristics and the ability for IAV to rapidly evolve towards an optimal level of persistence in changing environments.

Persistence at cold temperature and in frozen lakes has been invoked as a possible mechanism of overwinter persistence of IAV in the environment and as a source of contamination for migratory ducks in breeding grounds [55,56]. Although viral RNA has been found in different compartments of aquatic habitats, only a limited number of viruses have been isolated from the environment [57] and the understanding of the mechanisms involved in the long-time environmental persistence of IAV in high-latitude habitats or its significance remains limited. Along with a previous study [31], we suggest that freeze–thaw cycles could be a limiting factor for the persistence of IAV in shallow waters that undergo important temperature changes before and after ice formation in lakes.

In this study, we highlighted that the virus genome composition in naturally circulating reassortants has limited effects on the viral shedding in mallards, as well as on the persistence in water under different temperature regimens. These findings support that functionally equivalent gene segments could be maintained in IAV populations, with limited fitness costs when reassortment occurs [9]. Indeed, our results suggest that genetic reassortment may not affect viral shedding pattern and persistence in aquatic habitats, although a larger set of host- and environment-related factors, as well as virus genotypes, would need to be tested to confirm these findings.

General care was provided in accordance with an animal use protocol (AUP no. A2010-6-101) approved by the Institutional Animal Care and Use Committee at the University of Georgia, Athens, GA, USA.

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