INTRODUCTION

Low-pathogenicity avian influenza viruses (LPAIVs) have been identified in many bird species, but primarily from the orders Anseriformes (ducks, geese, and swans) and Charadriiformes (gulls, terns, and shorebirds). Mallards (Anas platyrhynchos) and other dabbling duck species are important LPAIV hosts, and transmission between ducks occurs through the fecal-oral route involving contaminated water. However, gulls also are susceptible and can contribute to geographic spread, reassortment, and the evolution of AIVs. Surveillance data indicate that the prevalence of AIV and subtype diversity vary significantly between different genera and species. All HA subtypes have but detected in ducks and gulls, but the H3 and H4 subtypes predominate in ducks, and H13 and H16 in gulls. In this study, our goals were to understand AIV infectivity and pathogenesis in gulls, through clinical assessment, viral shedding patterns, and seroconversion, to related findings to potential mechanisms of transmission and ecological maintenance. Additional infectivity and pathogenesis studies are needed to confirm this replication difference for LPAI viruses in gulls.

KEYWORDS
avian influenza, laughing gulls, low-pathogenicity avian influenza, mallard, pathogenesis, pathogenicity, wild birds
2 | METHODS

North American LPAIVs used in experiments are listed in Table 1; they were propagated in specific pathogen-free (SPF) 9- to 11-day-old embryonating chicken eggs (ECE) following standard procedures. Low-passage virus stocks were used for challenge.

Laughing gulls (7-10-days-of-age) were obtained under federal permit and reared for 12 weeks in captivity until challenged. Ten- to 16-week-old mallards were purchased from a commercial hatchery (Chenoa Waterfowl). For challenge (Table 1), birds were grouped and housed in negative pressure high-efficiency particle air (HEPA) ventilated cabinets with ad libitum access to feed and water.

Laughing gulls and mallards were divided in groups and inoculated with respective gull- and mallard-origin LPAIV (Tables 1 and 2) via the choanal cleft, which provides exposure to upper respiratory tract and drainage into oral cavity for swallowing and exposure to gastrointestinal tract, with approximately 10^6 mean embryo infectious doses (EID_{50}) in 0.1 ml per bird. Back titers were reported in Table 1. Oropharyngeal (OP) and cloacal (CL) swabs were collected on 1, 2, 3, 4, 7, and 10 days of post-inoculation (dpi) and placed in Becton-Dickinson BBL brain heart infusion (BHI) medium with 2× concentration of antibiotics (10,000 U/ml Penicillin G, 10,000 μg/ml Streptomycin, 25 μg/ml Amphoterin B) (HyCone Laboratories, Inc). Samples were stored at −8°C until tested. Blood was collected pre- (0-day) and post- inoculation (10 days) to assess serum antibody responses. Birds were observed daily for clinical signs and euthanized at 10 dpi following approved protocols. These studies were reviewed and approved by the USNPRC Institutional Animal Care and Use Committee (IACUC) and conducted with appropriate biocontainment and biosafety measures.

OP and CL swabs were processed to determine viral shedding titers by quantitative real-time PCR (RRT-PCR). Briefly, the RNA was extracted using MagMAXTM-96 Al/ND Viral RNA Isolation Kit® (ThermoFisher Scientific) following the manufacturer’s instruction. Further, RRT-PCR that targets the matrix gene of avian influenza was performed with the AgPath-ID OneStep RT-PCR kit (ThermoFisher Scientific) using 7500 FAST Real-time PCR System (Applied Biosystems), as previously described. Virus quantity was established with a standard curve from RNA extracted from 10-fold dilutions of the challenge virus in duplicate.

Serum was tested for anti-AIV antibodies using hemagglutination inhibition (HI) assay and blocking enzyme-linked immunosorbent assay (bELISA). The homologous antigens were prepared as previously described and the HI assay performed following standard procedures. Titors were calculated as the reciprocal of the last HI positive serum dilution and were converted to log2. Titors were expressed as geometric mean titers (GMT-log₂). Samples were considered positive for the presence of AI antibodies with titers ≥3 log₂ GMT. The blocking- ELISA, the Avian Influenza Virus Antibody Test Kit, MultiS-Screen Ab (IDEXX, Westbrook, Maine) was used in duplicate following the manufacturer’s instruction.

Statistical analyses were performed using Prism 8 (GraphPad Software).

3 | RESULTS

The sham controls were not infected based on lack of pre- and post-challenge AIV antibodies and negative virus detected in OP and CL swabs after challenge. Clinical signs or mortality were not observed in any inoculated laughing gulls and mallards.

### TABLE 1 Avian influenza viruses and back titers used in the experimental challenge of laughing gulls and mallards

| Avian species | Number of birds | Experimental Group Abbreviation | Inoculated LPAI virus strain | GenBank accession numbers (Hemagglutinin gene) | Back Titers of inoculum (EID_{50}/0.1ml) |
|---------------|----------------|--------------------------------|----------------------------|---------------------------------------------|----------------------------------------|
| Laughing gulls | 3              | LG/H7N3                        | A/laughing gull/New York/AI00-2455/2000 -H7N3* | CY144292.1 | 10^6.6 |
|               | 3              | LG/H6N4                        | A/laughing gull/New York/AI00-470/2000 -H6N4* | CY144162.1 | 10^5.5 |
|               | 3              | LG/H3N8                        | A/laughing gull/New Jersey/768/2005 H3N8* | GU186466.1 | 10^5.5 |
|               | 3              | HG/H2N3                        | A/herring gull/New York/AI00-532/2000 -H2N3* | CY144178.1 | 10^5.6 |
|               | 2              | SHAM                           | NA                         | NA                                          | NA                                     |
| Mallards      | 3              | M/H5N6                         | A/mallard/Wisconsin/34/1975 -H5N6* | U79451.1 | 10^5.3 |
|               | 3              | M/H4N8                         | A/mallard/Ohio/338/1986 -H4N8* | DQ021863.1 | 10^5.7 |
|               | 2              | SHAM                           | NA                         | NA                                          | NA                                     |

Note: LPAI virus isolates were provided by Southeast Cooperative Wildlife Disease Study, Athens, GA (*), and Department of Veterinary Preventive Medicine, Columbus, OH (#).

Abbreviation: NA, not applicable.
Viral shedding patterns, including respiratory versus gastrointestinal tracts and duration, varied between the LPAIVs and individual birds (Figure 1A-F). Gulls inoculated with LG/H7N3, and LG/H6N4 had the highest shedding titers, with mean OP titers reaching peaks of 4.5 and 6.3 log₁₀ EID₅₀/ml, respectively (Figure 1A-B). Only LG/H7N3- inoculated gulls shed to 10 dpi while LG/H6N4- inoculated gulls shed to 3 dpi. In contrast, laughing gulls infected with LG/H3N8 and HG/H2N3 (Figure 1C-D) had low OP virus titers (highest mean virus titer detection of 1.5 and 1.7 log₁₀ EID₅₀/ml, respectively), and virus was not detected in CL swabs.

Overall, in laughing gulls viral shedding was predominantly associated with OP swabs, with highest titers observed in the first two days of post-inoculation. Virus was detected less frequently in CL swabs and titers were low (Figure 1). Mallards inoculated with M/H5N6 (Figure 1E), and M/H4N8 (Figure 1F) had viral shedding detected over the 10-day study. In the first three dpi, the mean virus shedding was higher in the OP than in the CL samples for M/H5N6- inoculated mallards (Figure 1E). This shedding pattern changed after 4 dpi with M/H5N6 virus excretion in the CL reaching peaks as 4.7 log₁₀ EID₅₀/ml (Figure 1E). Mallards inoculated with M/H4N8 had a constant mean shedding titer in the OP samples during the 10 dpi, and CL shedding oscillated between days and birds with titers similar between OP and CL samples or CL slightly lower than OP swabs. None of the LPAIV-infected gulls or mallards in our experiments experienced morbidity or mortality (Figure 1 and Table 2) which was expected with LPAIV in these host species. 1,8 Gulls inoculated with LG/H7N3, LG/H6N4 and LG/H3N8, all become infected based on detection of their respective LPAIV in one or more OP or CL swabs, but only one laughing gull became infected with LG/H3N8.

### Table 2

| Avian Species | Experimental Group Abbreviation | Virus Detection/ Total of birds | OP Swab | CL Swab | AIV antibody+ /Total |
|---------------|---------------------------------|--------------------------------|---------|---------|---------------------|
| Laughing Gulls | LG/H7N3                          | 3/3                            | 10^4.5 (1 dpi) | 1-10    | 10^1.6 (4 dpi) | 2-4 |
|                | LG/H6N4                          | 3/3                            | 10^6.3 (1 dpi) | 1-4     | 10^2.9 (2 dpi) | 2-3 |
|                | LG/H3N8                          | 3/3                            | 10^1.5 (1 dpi) | 1-2     | -       | -   |
|                | HG/H2N3                          | 1/3                            | 10^1 (1 dpi) | 1-2     | -       | -   |
|                | SHAM                             | 0/2                            | -       | -       | -       | -   |
| Mallards       | M/H5N6                           | 3/3                            | 10^6.1 (4 dpi) | 1-10    | 10^7 (4 dpi) | 2-10|
|                | M/H4N8                           | 3/3                            | 10^2.4 (2 dpi) | 1-10    | 10^2.4 (2 dpi) | 1-10|
|                | SHAM                             | 0/2                            | -       | -       | -       | -   |

- Mean Peak Titer are report as EID₅₀/ml.
- HI titers expressed as geometric mean titers (GMT-log2). The HI results were determined using challenge virus as antigen. Samples with titers below 3 log₂ GMT were considered negative, and then assigned as 2 log₂ GMT for statistical purpose.

**DISCUSSION**

Studies of LPAIV are crucial to provide an understanding of the active association between LPAIVs, avian host, and the environment. This knowledge is needed to identify mechanisms related to LPAIV maintenance, subtype diversity, and evaluate the risk factors that contribute to LPAIV spread to new geographic regions. The results from our study, which includes other wildlife species, poultry, domestic animals, and humans, provide valuable insights into the role of LPAIVs in the transmission of the virus to new hosts. The data collected in this study will inform future research efforts aimed at understanding the spread and evolution of LPAIVs.

Overall, the shedding patterns observed in this study highlight the importance of continued monitoring of avian influenza viruses to better understand their ecological and epidemiological impacts.

**TABLE 2** Summary of OP and CL virus shedding and anti-influenza antibodies post-LPAIV virus inoculation in laughing gulls and mallards.
when inoculated with HG/H2N3 obtained from a herring gull (Larus argentatus), a related gull species. The HI or bELISA antibody tests confirmed such infections in most inoculated gulls except for two gulls (Table 2), which despite having low-virus replication and shedding titers, no anti-AIV antibodies were detected by either method.

The most interesting outcome was subtle differences in virus shedding patterns and their implications on virus transmission and maintenance in gulls compared to mallards. In this study, and others, involving laughing, silver, and ringed-billed gulls, predominant shedding of LPAIV from the oropharynx has been observed. With black-headed gulls, peak prevalence of H13 and

**FIGURE 1** Evaluation of virus shedding in the oropharyngeal (OP) and cloacal (CL) swabs after different days post-inoculation (dpi) of LPAIV in laughing gulls and mallards. Variation in viral shedding patterns observed in laughing gulls (A to D) and mallards (E and F) experimentally infected with different LPAI virus strains at dose $10^{6.0}\,\text{EID}_{50}/0.1\,\text{ml}$. Virus shedding titers, represented as $\log_{10}\,\text{EID}_{50}/\text{ml}$, were evaluated by RRT-PCR on 1, 2, 3, 4, 7, and 10 dpi. Black (OP) and dashed gray (CL) lines indicated means per sampling day. Squares (OP) and dots (CL) indicate values for individual birds (n=3 birds per day). For each experiment, birds 1, 2, and 3 are shown in magenta, blue, and green color, respectively. Plotted data from each bird had a nudge of 0.05 in the Y direction for dataset visualization.
H16 viruses is associated with fledged birds during the breeding season and predominant OP shedding may represent an adaptation for efficient transmission during this period. Previous studies demonstrated LPAIVs infect and replicate in both respiratory and intestinal epithelial cells of mallards and domestic ducks (Anas platyrhynchos domesticus). In ducks, the high volume of feces containing high titers of LPAIV and the long duration of shedding would both contribute to contaminate aquatic habitats and facilitate fecal/oral transmission. This predominant shedding pattern was reproduced in mallards in this study where ducks were experimental infected by intrachoanal inoculation, which simulates exposure during natural feeding behavior; both intrachoanal and direct gastrointestinal exposure have resulted in productive LPAIV infection in mallards. The observed differences in shedding by mallards and gulls inoculated by the same route, suggesting different mechanisms for transmission and maintenance of LPAIV in gulls and dabbling ducks.

It is possible that differences in shedding patterns between ducks and gulls may be related to the expression of α2,3-linked sialic acid (SA) receptors in tissues. Ducks show similar expression of SA receptors in the respiratory and intestinal tract, which may explain the equal respiratory and fecal shedding pattern of our study. In vitro studies demonstrated that SA receptors’ stronger expression in respiratory tract of ring-billed gulls and laughing gulls which was consistent with predominant respiratory shedding in our study. However, other factors present in the host and the virus strain may be also involved in the differences in AIV prevalence, viral shedding, and the disease’s outcome, which are beyond this study’s scope.

The comparative study of different taxa of migratory aquatic birds, especially Chadriiformes and Anseriformes, offers a unique opportunity to understand how different LPAIV subtypes evolved and are maintained in diverse avian ecosystems. Our data demonstrated different patterns of viral shedding associated with relevant LPAIV subtypes in laughing gulls as compared to current understanding in mallards. Further studies are needed to understand the pathophysiological and ecological mechanisms of AIVs transmission cycles in gulls to better understand cross-species transmission and environmental maintenance.

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CONFLICT OF INTEREST
Authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS
Miria F. Criado: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Validation (equal); Visualization (equal); Writing-original draft (lead); Writing-review & editing (supporting). Kira A. Moresco: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Investigation (equal); Methodology (equal); Validation (equal); Visualization (equal); Writing-original draft (supporting); Writing-review & editing (supporting). David E. Stallknecht: Conceptualization (supporting); Data curation (supporting); Formal analysis (supporting); Investigation (equal); Methodology (equal); Validation (equal); Visualization (equal); Writing-original draft (supporting); Writing-review & editing (supporting). David E. Swayne: Conceptualization (equal); Data curation (equal); Formal analysis (equal); Funding acquisition (lead); Investigation (equal); Methodology (equal); Project administration (lead); Resources (lead); Supervision (lead); Writing-review & editing (supporting).

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are provided in the figures and tables of the article. Additional information is available from the corresponding author upon reasonable request.

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