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Research paper

Ambient ammonia does not appear to inhibit the immune response to infectious bronchitis virus vaccination and protection from homologous challenge in broiler chickens

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ABSTRACT

Commercial broilers are commonly exposed to gaseous ammonia (NH_3) originating from degradation of nitrogen-containing excreta in the litter during the grow-out period. Ammonia concentrations in the air are higher in poorly ventilated houses and appear to coincide with the elevated incidence of respiratory disease occurring during the winter months. This study examined the effect of NH_3 on the immune response to infectious bronchitis virus (IBV) vaccination and protection against homologous serotype challenge in commercial broiler chickens. One-day-old chicks were administered IBV vaccine and exposed to 30–60 ppm of NH_3. At 28 DOA, birds were challenged oculonasally with a pathogenic homologous IBV, and protection was measured by viral detection, clinical signs, ciliostasis, and presence of airsacculitis. IBV-specific serum IgG and lacrimal fluid IgA titers, as well as Harderian gland (HG) immune cell phenotypes, were evaluated. Ammonia exposure was associated with an increased incidence of airsacculitis among non-vaccinated, challenged birds. Vaccinated, NH_3-exposed birds were completely protected from IBV challenge. Ammonia had subtle effects on cilia morphology and function but did not affect vaccine or challenge virus replication and clearance, clinical signs, ciliostasis, tracheal histopathology scores, or immune responses. In the HG of vaccinated birds, the percent of leukocytes, MHC I^+/MHC II^{hi} expression, IgM^+ expression, and $CD8^+$ expression was increased, while mucosal IgA and serum IgG titers were nominal. Non-vaccinated, IBV-challenged birds exhibited an increased percent of leukocytes, MHC I^+/MHC II^{hi} expression, and IgM^+ expression in the HG at 5 dpc, followed by increased mucosal IgA and serum IgG titers and $CD8^+$ expression at 10–14 dpc. In contrast, vaccinated, IBV-challenged birds had a minimal increase in MHC I^+/MHC II^{hi} expression, and serum IgG antibody titers in vaccinated birds increased rapidly. The results indicate that commercial broilers exposed to moderate levels of ambient NH_3 are equally protected against IBV challenge if appropriately vaccinated, and the absence of robust immune activation in vaccinated, challenged birds suggests that the challenge virus was efficiently neutralized before establishing infection. In contrast, ambient NH_3 exposure was associated with a higher incidence of airsacculitis in non-vaccinated, challenged birds, despite the apparent lack of differences in the immune response between birds in the NH_3-exposed and NH_3 control groups.

Abbreviations: NH_3, ammonia; dpv, days post-vaccination; dpc, days post-challenge; HG, Harderian gland; hpc, hours post-challenge; IBV, infectious bronchitis virus; –/NAm/+, non-vaccinated, no ammonia, challenged group; –/Am/+, non-vaccinated, ammonia-exposed, challenged group; –/NAm/–, non-vaccinated, no ammonia, nonchallenged group; –/Am/–, non-vaccinated, ammonia-exposed, nonchallenged group; ppm, parts per million; Rh, relative humidity; +/NAm/+, vaccinated, no ammonia, challenged group; +/Am/+, vaccinated, ammonia-exposed, challenged group; +/NAm/–, vaccinated, no ammonia, nonchallenged group; +/Am/–, vaccinated, ammonia-exposed, nonchallenged group

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1. Introduction

Commercial broiler chickens are commonly exposed to ambient ammonia (NH_3), an irritant gas released from poultry manure, during the grow-out period. The mechanisms of NH_3 emissions from manure have been summarized in Ni (1999), in which uric acid in feces is converted to gaseous NH_3 by bacterial activity within poultry litter. Ammonia concentrations in a poultry house are positively correlated with moisture content, temperature and pH, and NH_3 concentrations increase with reduced ventilation (Carr et al., 1990; Knizatova et al., 2010; Liu et al., 2007). Elevated NH_3 levels tend to occur during cold weather, but NH_3 in a broiler house may present a year-round challenge to bird health and production (Miles et al., 2014).

Surprisingly few reports have investigated the effect of NH_3 on respiratory tissues. In general, those reports have described deciliation, goblet cell hypertrophy, epithelial hyperplasia, and inflammatory cell infiltrates in the upper-respiratory tract associated with NH_3 exposure (Al-Mashhadani and Beck, 1985; Anderson et al., 1966; Xiong et al., 2016). However, it is uncertain whether NH_3 exposure alone increases inflammation in these tissues. Miles et al. (2006) reported no increase in lymphocytic or heterophilic infiltrates in tissue from the trachea, lungs, and air sacs following a 28-day exposure period with up to 75 ppm of NH_3, and the authors speculated that the harmful effects of NH_3 may not always be apparent until the bird experiences additional environmental or microbial challenges. For example, some studies have demonstrated that respiratory tract lesions (i.e. deciliation, increase in mucus-secreting goblet cells, inflammatory cell infiltration, air-sacculitis) are exacerbated when NH_3 is combined with environmental stressors, such as dust and carbon dioxide (Anderson et al., 1966), or microbial challenges, such as *Escherichia coli* (Oyetunde et al., 1978). Acute exposure (72 h) to 20 ppm of NH_3 has also been linked to increased susceptibility to Newcastle disease (Anderson et al., 1964). In addition, some studies documented the occurrence of airsacculitis in NH_3-stressed birds vaccinated with live attenuated infectious bronchitis virus (IBV) (Kling and Quarles, 1974; Quarles and Caveny, 1979).

At least one study has addressed the mechanisms by which NH_3 influences immunity. Xiong et al. (2016) showed that broilers exposed to 75 pm of NH_3 for 20 days experienced an increase in the production of reactive oxygen species (ROS), downregulation in the immune response proteins responsible for antigen recognition and presentation, and upregulation in the proteins involved in muscle contraction and mucin production. However, it remains unknown whether NH_3 impacts immunity in the context of vaccination.

IBV is a common upper respiratory tract pathogen of economic significance in the commercial poultry industry and may lead to reduced weight gain and feed efficiency, drops in egg production, stunted growth, and secondary bacterial infection resulting in airsacculitis (Cavanagh and Gelb, 2008). IBV initially infects the upper respiratory tract, including the Harderian gland (HG), nasal turbinates, and the trachea, followed by systemic replication in the reproductive tract and some strains can cause lesions in kidney (Cavanagh and Gelb, 2008). IBV-infected birds may exhibit nasal discharge, coughing, sneezing, and tracheal rales (Cavanagh and Gelb, 2008).

Mucosal respiratory responses are important in protection against IBV, and the HG is a major paraocular gland that contains IgA-, IgG-, and IgM-expressing plasma cells (Baba et al., 1990; Ohshima and Hiramatsu, 2002). Previous studies have reported that IgA is locally synthesized in the HG, and IgG is both synthesized and transduced to the respiratory tract from systemic circulation (Baba et al., 1990; Davelaar et al., 1982; Toro et al., 1993). IgA is involved with protection of mucosal surfaces, and local IBV-specific IgA responses, in contrast to systemic IBV-specific IgG responses, have been associated with protection from IBV infection (Toro and Fernandez, 1994). In addition to immunoglobulin-secreting plasma cells, the HG also contains macrophages, lymphocytes, dendritic cells, and heterophils (Bejdić et al., 2014; Oláh et al., 1996).

Upon primary exposure to either an IBV vaccine or virulent strain, a cell-mediated immune response also develops. The primary cell type responsible for early viral clearance is the $CD8^+$ αβ T lymphocyte (Seo et al., 2000). The humoral response appears to be important in clinical disease resolution and viral clearance (Cook et al., 1991). Furthermore, neutralizing antibodies are important in preventing IBV re-infection (Guo et al., 2008; Holmes, 1973). Recent advances in the parallel assessment of lymphocyte and monocyte/macrophage phenotype from mucosal (conjunctiva-associated lymphoid tissue, Harderian gland) and systemic immune tissues (spleen) serially collected from birds offer new insight into the dynamics of mucosal and systemic immune responses to respiratory viral disease (Krunkosky et al., 2018). These advances also provided additional tools in understanding the dynamics of immunity relative to infection in this study.

The objective of this study was to analyze the effect of moderate levels (30–60 ppm) of ambient NH_3 on local and systemic immune response to IBV vaccination and protection against homologous challenge in commercial broilers.

2. Materials and methods

2.1. Viruses

A commercial Massachusetts (Mass) type vaccine (strain MILDVAC-Ma5™, Merck Animal Health, Summit, NJ) was used in this study. The vaccine was diluted in phosphate-buffered saline (PBS) according to the manufacturer's recommendations. The challenge virus used was Massachusetts Mass/Mass41/41 (Mass41). Virus titers were calculated by Reed and Muench (Villegas, 2004).

2.2. Chickens

Maternal antibody-positive broiler eggs from a commercial source were obtained at 18 days of incubation and hatched at the Poultry Diagnostic & Research Center, Athens, GA. Methods used in this study were approved by the Institutional Animal Care and Use Committee at the University of Georgia, Athens, GA (AUP #: A2015 05-001-R2).

2.3. Experimental design

This study consisted of three *in vivo* experiments to investigate the effect of ambient NH_3 on the immune response to IBV vaccination and protection against homologous challenge in commercial broilers. Experiment 1 was a survey to gather preliminary data for the immune response post-vaccination (10–14 dpv), pre-challenge (24–28 dpv), and post-challenge (10–14 dpc). Experiment 2 included a negative control group post-challenge, as well as a –/Am/+ group to mimic incomplete or partial vaccination, a common result of mass vaccination in which some birds received a partial vaccine dose or no vaccine at all. Experiment 3 was designed similar to Experiment 2 but incorporated a larger sample size and additional collection times post-challenge.

2.3.1. Experiment 1

One day-of-age commercial broilers (n = 339) were arbitrarily divided into 3 groups: negative control/no NH_3 (–/NAm, n = 113), vaccinated-only (+/NAm, n = 113), and vaccinated, NH_3-exposed (+/Am, n = 113) groups, and were placed in three climate-controlled rooms. Birds in groups receiving no NH_3 were placed on fresh shavings, while +/Am birds were placed on used litter, which served as a source of NH_3. To mimic field conditions, ventilation was controlled by three-speed 20-inch box fans, the stocking density was 0.6 ft^2/bird, and standard heat sources and feed were used. Within each room, all birds received the same treatments, but sample collections involved only a subset of these birds. More birds were required to maintain a stocking density appropriate for mimicking field conditions. At one-day of age birds were vaccinated with one dose each of IBV-Mass vaccine via

oculonasal route (left eye/left nares), in a total volume of 50 µL. Temperature and relative humidity (Rh) were monitored in each room, and NH_3 levels were recorded in the +/Am room. Measuring NH_3 levels in all rooms was cost-prohibitive, so olfaction, with a mean odor detection threshold of 2.6 ppm (Smeets et al., 2007), was used to confirm the absence of detectable levels of NH_3 in control rooms. Birds were observed daily for clinical signs. The immune response to vaccination was measured at 7, 10, 12, 14, 24, 26, and 28 days of age using two birds from each group per collection day. The choanal cleft was swabbed for vaccine virus detection, and swabs were stored in PBS at −80 °C. Granulated NaCl was added to the left eye to collect 50 µL of tears, and samples were stored at −20 °C. Blood was collected by cardiac puncture in a heparinized (1000 U/mL) syringe and added to a microcentrifuge tube to collect plasma for antibody detection. Birds were humanely euthanized, and the HG was extracted and stored in PBS at room temperature for flow cytometry. The trachea was removed and placed in 10% neutral buffered formalin. At 28 DOA, birds were transferred to Horsfall Bauer isolation units under positive pressure and provided with feed and water *ad libitum*. Birds were sub-divided into nonchallenge groups and challenge groups, and challenges were administered with pathogenic Mass41 virus at a dose of 10^4 EID_{50}/bird in a volume of 100 µL, which was applied oculonasally (left eye/left nares). Five days post-challenge (dpc), necropsies were performed on the following groups: –/NAm/– (n = 5), –/NAm/+ (n = 10), +/NAm/– (n = 5), +/NAm/+ (n = 10), +/Am/– (n = 5), +/Am/+ (n = 10). Birds were observed for respiratory rales, as previously described (Toro et al., 2012): 0 = absent; 1 = mild; 2 = moderate; 3 = severe. Abdominal air sacs were assessed for airsacculitis, which was defined by the presence of opacity or foamy material or both in the air sacs at necropsy, as previously described (da Silva et al., 2017). Tracheas were collected in formalin for histopathology and cell culture media (Dulbecco's Modified Eagle's Medium, DMEM) for the ciliostasis test described below. Post-challenge immune responses were measured at 10, 12, and 14 dpc using the same methods described above.

2.3.2. Experiment 2

One day-of-age commercial broilers (n = 452) were arbitrarily divided into negative control (–/NAm, n = 113), NH_3-only (–/Am, n = 113), vaccinated-only (+/NAm, n = 113), and vaccinated, NH_3-treated (+/Am, n = 113) groups, and were placed in four climate-controlled rooms. All environmental conditions and vaccine and challenge viruses and doses remained the same as in Experiment 1. Birds were monitored daily for clinical signs. At 7 dpv, 5 birds/group were swabbed via the choanal cleft, euthanized, and HG, blood, and trachea were collected as described above. Tears were collected from 5 separate birds/group. The immune response to vaccination was measured at 10, 12, 14, 24, 26, and 28 DOA using two birds from each group per collection day by applying the same methods described in Experiment 1. At 28 DOA, birds were transferred to isolation units and challenged with Mass41 as described above. Necropsies were performed at 5 dpc on the following groups: –/NAm/– (n = 5), –/NAm/+ (n = 10), –/Am/– (n = 5), –/Am/+ (n = 5), +/NAm/– (n = 5), +/NAm/+ (n = 10), +/Am/– (n = 5), +/Am/+ (n = 10). Birds were observed for respiratory rales and air sacs were assessed for airsacculitis. Tracheas were collected in formalin and DMEM. Post-challenge immune responses were measured at 4, 10, 12, and 14 dpc using the same methods described above.

2.3.3. Experiment 3

One day-of-age commercial broilers (n = 452) were divided into negative control (–/NAm, n = 113), NH_3-only (–/Am, n = 88), vaccinated-only (+/NAm, n = 113), and vaccinated, NH_3-treated (+/Am, n = 138) groups, and were placed in four climate-controlled rooms. Due to a number tracking error at placement, only 88 birds were placed in the –/Am room, and 138 birds were placed in the +/Am room. The population was later adjusted in the +/Am room to approximate 113, to maintain the same stocking density used in each experiment. All environmental conditions and vaccine and challenge viruses and doses were the same as in Experiment 1. Birds were monitored daily for clinical signs. In addition to swabbing birds via the choanal cleft at 5 dpv, the immune response to vaccination was measured at 7, 10, 12, and 14 dpv using five birds from each group per collection day, according to the methods outlined in Experiment 1. At 28 DOA, birds were transferred to isolation units and challenged with pathogenic Mass41 virus as described in Experiment 1. Necropsies were performed at 5 dpc on the following groups: –/NAm/– (n = 5), –/NAm/+ (n = 10), –/Am/– (n = 5), –/Am/+ (n = 10), +/NAm/– (n = 5), +/NAm/+ (n = 9), +/Am/– (n = 5), +/Am/+ (n = 10). Birds were observed for respiratory rales and air sacs were assessed for airsacculitis. Tracheas were collected in formalin and DMEM. Post-challenge immune responses were measured at 18 and 48 h post-challenge (hpc), and 3, 5, 10, 12, and 14 dpc using the same methods described in Experiment 1. At each time point, four birds per group were collected until 5 dpc, after which two to three birds per group were obtained.

2.4. Air quality measurements

The air quality parameters measured during this study included temperature, Rh, and NH_3. Temperature and Rh inside each room were measured and were recorded at 15-minute intervals using a Temperature/Relative Humidity Smart Sensor (Onset, Bourne, MA, S-THB-M002) connected to a HOBO® RX3000 Monitoring Station (Onset, Bourne, MA). NH_3 levels, measured in parts per million (ppm), were recorded at 15-minute intervals at the level of the drinkers in NH_3-treated rooms only by a Chillgard® RT Refrigerant Monitor (MSA, Cranberry Township, PA), which was connected to the same HOBO® monitoring station. Air quality parameters were measured from hatch until challenge at 28 DOA. A target range (30–60 ppm) for NH_3 was achieved by either adding water to the litter until it was damp and then turning the litter or adjusting the ventilation rate. Temperature was adjusted according to the temperature requirements of the birds.

2.5. Ciliostasis test

The ciliostasis test was performed on harvested tracheas collected in cell culture media (Dulbecco's Modified Eagle's Medium) at 37 °C. For each trachea, five tracheal rings measuring approximately 1 mm thick were cut and represented the proximal, middle, and distal portions of the trachea (41, 54). Cilia activity was observed using an inverted microscope (Olympus, Center Valley, PA). The scoring system was as follows: 0 = all cilia beating; 1 = 75% of cilia beating; 2 = 50% of cilia beating; 4 = no cilia beating. Each ring was scored by three individuals, and the average total score for each trachea was calculated. The ciliostasis protection score for each group was determined by the following formula: 100 − [(total of the individual scores for the group)/(the number of individuals in the group × 20) × 100], as previously described (Cook et al., 1999), and a score ≥50 was considered protected. The binomial protection score was calculated to assess the proportion of chickens that were protected in each group, as outlined in the European Pharmacopoeia (European Pharmacopoiea 8.0). Briefly, a tracheal ring with ≥50% of the cilia showing vigorous activity was considered protected, and a chicken was considered protected when 90% of the rings were showing ≥50% of cilia beating.

2.6. Tracheal histopathology

A section of each trachea was fixed in 10% neutral buffered formalin, processed into paraffin, and 5-µm sections were cut for hematoxylin and eosin staining. Epithelial hyperplasia, lymphocyte infiltration, and epithelial deciliation were scored for each trachea on the basis of severity and distribution, severity and distribution scores for each parameter were averaged, and the three averaged scores were added to

determine a total tracheal histopathology score for each trachea. Scores were determined as follows: 1 = normal, 2 = mild/focal, 3 = moderate/multifocal, and 4 = severe/diffuse, as described previously (Jackwood et al., 2015).

2.7. RNA extraction and qRT-PCR

Viral RNA extraction from 50 µl of the PBS from each swab was conducted using a 5X MagMAX-96 Viral Isolation Kit (Thermo Fisher, Waltham, MA) on a MagMAX™ Express-96 Deep Well Magnetic Particle Processor (Thermo Scientific, Waltham, MA), according to the manufacturer's instructions. The qRT-PCR was performed with the AgPath-ID™ One-Step RT-PCR kit (Thermo Fisher, Waltham, MA), following the manufacturer's protocol. Each 25-µL reaction mixture contained 12.5 µL of 2× RT-PCR buffer, 10 µM of each primer, 4 µM of probe, 1 µl of 25× RT-PCR enzyme mix, and 5 µL of viral RNA. The qRT-PCR reactions were run on the Applied Biosystems® 7500 Fast Realtime PCR system (Life Technologies Ltd., Carlsbad, CA) under the following conditions: one cycle of 50 °C for 30 min and 95 °C for 15 min, followed by 40 cycles of 94 °C for 1 s and 60 °C for 60 s. The primers and probe for the qRT-PCR were previously published (Callison et al., 2006), and are comprised of a forward primer IBV5′GU391 (5′-GCT TTT GAG CCT AGC GTT-3′), a reverse primer IBV5′GL533 (5′-GCC ATG TTG TCA CTG TCT ATT G-3′), and a Taqman® dual-labeled probe IBV5′G probe (5′-FAM-CAC CAC CAG AAC CTG TCA CCT C-BHQ1-3′). The primers were obtained from Integrated DNA Technologies (Coralville, IA), and Taqman probe was synthesized by BioSearch Technologies (Novato, CA). Real-time RT-PCR components and thermocycler parameters were previously described (Callison et al., 2006). The data were expressed as the average cycle threshold (CT) value for all samples in each group, with positive CT values based on the limit of detection for this test associated with virus detection in eggs (Roh et al., 2014).

Each qRT-PCR reaction plate included a standard curve as an RNA extraction control and as a positive control. Mass41-type IBV isolated from allantoic fluid was used as the template for the standard curve. Negative controls were also included in each plate and consisted of PCR reagents with no RNA.

2.8. Serum and plasma IBV-specific IgG antibody titers

IBV-specific IgG titers were detected using an indirect commercial IgG ELISA IBV antibody test kit (IDEXX, Westbrook, ME). Briefly, serum or plasma samples (stored at −20 °C) were diluted 1:500, and the procedure was performed according to the manufacturer's protocol. Results were reported as $\log_2$ of the titer.

2.9. Tear secreted IBV-specific IgA antibody titers

Tear IBV-specific IgA was detected using a commercial IgG ELISA IBV antibody test kit (IDEXX, Westbrook, ME) as the source of the capture antigen. Briefly, tears were serially diluted two-fold in PBS and 100 µL was added in duplicate to wells and incubated overnight at 4 °C. All manual wash steps were performed using PBS-Tween 20 (0.05% Tween 20). Plates were washed, incubated at 23 °C for 2 h with 100 µL of monoclonal mouse anti-chicken IgA-BIOT (1:1000, clone A-1, Southern Biotech, Birmingham, AL) per well, washed a second time, incubated at 23 °C for 1 h with 100 µL of Streptavidin-HRP (1:4000, Southern Biotech, Birmingham, AL) per well, and washed a final time. Final antibody detection steps were completed using the substrate in the kit, according to the manufacturer's instructions. Endpoint titers were determined by reporting the lowest dilution at which the optical density (OD), recorded at 650 nm wavelength, was at least three standard deviations above the mean of 12 control wells incubated with no tear samples. Data from wells with a pinpoint color change due to residual substrate or air bubbles were excluded from analysis, and results were reported as $\log_2$ of the endpoint titer.

2.10. Leukocyte enrichment and enumeration

Single-cell suspensions were obtained from the HG by mechanical disruption using 60 µm mesh screens (Sigma-Aldrich, St. Louis, MO). Cell suspensions were centrifuged at 23 °C for 7 min at 300 x g. Single cell suspensions of leukocytes from 10, 12, and 14 dpv were re-suspended in PBS containing 1% BSA. HG in young birds (10–14 dpv) were not yet well vascularized and yielded too few cells to justify separation by density gradient centrifugation. However, HG in older birds (24–28 dpv and 10–14 dpc) were highly vascularized and yielded greater cell counts, so leukocytes were enriched by Histopaque density gradient (1.077 g/mL) (Sigma-Aldrich, St. Louis, MO) at 23 °C for 30 min at 450 × *g*. Cells were collected from the interface, washed as described above, and suspended in PBS containing 1% BSA. Cells were enumerated and viability was determined using trypan blue exclusion on a Cellometer Mini (Nexcelcom Bioscience, Lawrence, MA) with final cell dilutions set at 4×10^6cells/mL. To standardize the identification of the percent of leukocytes in the HG, the data for leukocytes isolated over a Histopaque gradient were normalized to the data for cells collected by resuspension without Histopaque enrichment by determining the difference between the mean percent of leukocytes of negative controls from Histopaque-enriched HG at each collection point and HG not enriched by Histopaque. The difference was then subtracted from the mean percent of Histopaque-enriched HG leukocytes in all groups at each respective collection.

2.11. Flow cytometry

The following antibodies (at the previously determined minimum saturating condition) were used in pairs to stain the enriched cells: mouse anti-chicken MHC Class I-FITC, mouse anti-chicken MHC Class II-PE, mouse anti-chicken CD4-PE, mouse anti-chicken CD8α-FITC, and mouse anti-chicken IgM-PE (Southern Biotechnology, Birmingham, AL) diluted in PBS, 1% bovine serum albumin, and 0.1% sodium azide at 4 °C. Stained cells were incubated on an orbital shaker for 30 min at 4 °C, and 100 µL PBS, 1% bovine serum albumin, and 0.1% sodium azide was added to each well. The plate was then centrifuged at 7 °C for 7 min at 400 x g, and fixed in 100 µL PBS, 1% bovine serum albumin, and 0.1% sodium azide, and 100 µL IC fixation buffer (Thermo Fisher, Waltham, MA) overnight at 4 °C. Flow cytometric analysis was performed using a BD Accuri™ C6 (BD Biosciences, San Jose, CA) for Experiments 1 and 2, and a BD Accuri™ C6 Plus (BD Biosciences, San Jose, CA) for Experiment 3. Data were analyzed with BD Accuri C6 (or C6 Plus) software. Leukocytes were identified by gating on singlets using forward angle area vs. height and then discriminated by forward and side scatter gating. Avian lymphocytes were further discriminated by gating on a moderate forward and low side scatter distribution. Identification of leukocytes based on forward and side scatter was confirmed in a follow-up experiment by CD45 staining (not included in this study). All groups were represented in three experiments, except –/Am (Experiments 2 and 3). Each time point represented the average of three experiments, except 18 hpc, 48 hpc, and 5 dpc (Experiment 3 only).

2.12. Statistical analysis

Data were analyzed using Prism v.6.0 software (GraphPad Software, Inc., La Jolla, CA; www.graphpad.com). For data in which $n \geq 5$, a Kruskal-Wallis test with Dunn's multiple comparisons posttest was used to compare treatment groups. For airsacculitis data, a Fisher's exact test, followed by a post-hoc test to yield probability values for each combination of independent category levels with a Bonferroni correction for type I error inflation, was performed using SPSS Statistics v.17.0 (SPSS, Inc., Chicago, IL). Significant differences were determined at $p < 0.05$.

3. Results

3.1. Air quality measurements

Continuous measurements of NH_3, Rh, and temperature for each group were recorded until challenge at 28 days. As expected, NH_3 was not detected in rooms housing the +/NAm and –/NAm groups at any point during the experiment. During the first 14 days, NH_3 concentrations for both +/Am and –/Am groups were generally within the target concentration of 30–60 ppm (Fig. 1). From 14 days until challenge, target NH_3 levels were maintained in Experiment 1 but declined to the low end of the range or below the target range in Experiments 2 and 3. No large differences between +/Am and –/Am groups were apparent, though NH_3 levels in the –/Am group tended to be lower than in the +/Am group in the final six to ten days.

Rh trends in the NH_3-treated rooms in Experiment 3 were initially higher compared to Rh in the no-NH_3 control rooms, and differences were negligible after 12 days (Fig. 2). Rh trends in Experiments 1 and 2 and temperature in all three experiments, however, were comparable between NH_3-treated rooms.

3.2. Vaccine virus RNA loads post-vaccination

Intrachoanal swabs were collected from birds on various days following vaccination. Vaccine virus was first detected at 18 hpv in vaccinated birds (data not shown). Vaccine virus RNA load was highest at 5 and 7 dpv and declined by 10–14 dpv (Fig. 3). By 24–28 dpv, vaccine virus load was weakly positive (experiments 1 and 2) or below the limit of detection (experiment 3), determined at CT value 36.59 (Roh et al., 2014). No significant differences were observed between NH_3 treatment groups among both non-vaccinated and vaccinated birds. Non-vaccinated birds were negative for vaccine virus.

3.3. Post-challenge virus RNA loads

Intrachoanal swabs were collected from birds on various days following challenge. Post-challenge peak viral load in non-vaccinated, challenged birds occurred at 5 dpc (Fig. 4). By 10 dpc, virus loads had declined below the limit of detection in all challenge groups except –/Am/+ birds in Experiment 2, though the difference between virus loads in the –/Am/+ group and the rest of the challenge groups was not significant. All vaccinated birds were protected from IBV replication, and viral loads were below the limit of detection at all time points. Overall, the trends in viral replication and clearance did not appear to be influenced by NH_3.

3.4. Clinical signs, airsacculitis, tracheal histopathology, and ciliostasis

Typical signs of IBV infection, which include watery eyes and tracheal rales, and gross lesions, such as mucus in the nares and trachea, were observed in the non-vaccinated, challenged birds at 5 dpc (Table 1). Tracheal rales were assessed, and mean scores in non-vaccinated, challenged birds were significantly higher than scores in vaccinated, challenged birds. NH_3 did not influence clinical sign scores. No clinical signs were observed in the negative controls. Trends in clinical sign scores were comparable among the three experiments.

Airsacculitis, denoted by the presence of opacity or foamy material or both in the air sacs at necropsy, was observed in several non-vaccinated, challenged birds. Airsacculitis was associated with –/NAm/+ birds in Experiment 1 only and with –/Am/+ birds in both Experiments 2 and 3 (Table 1). Airsacculitis was absent in all vaccinated birds, regardless of NH_3 exposure, as well as in the negative controls.

Post-challenge tracheal histopathology scores, scored on the basis of inflammation, epithelial hyperplasia, and deciliation, were higher in non-vaccinated birds compared to vaccinated birds, and NH_3 had no impact on these scores (Table 1). Compared to scores from vaccinated, challenged birds and negative control birds, scores from –/Am/+ birds

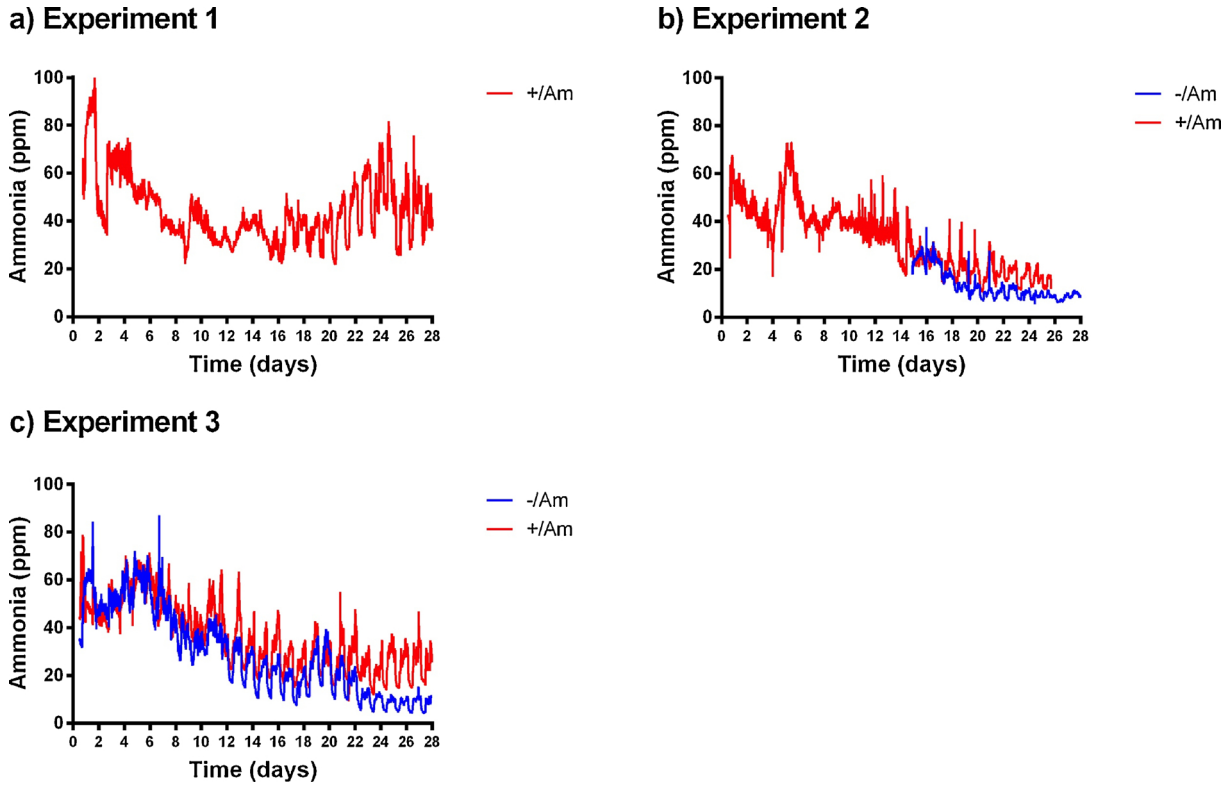

Fig. 1. NH_3 concentrations in NH_3-treated rooms. a) Experiment 1, b) Experiment 2, c) Experiment 3. Due to technical difficulties, experiment 2 NH_3 recordings in the –/Am room were not started until 14 dpv. –/Am = non-vaccinated, NH_3-exposed; +/Am = vaccinated, NH_3-exposed.

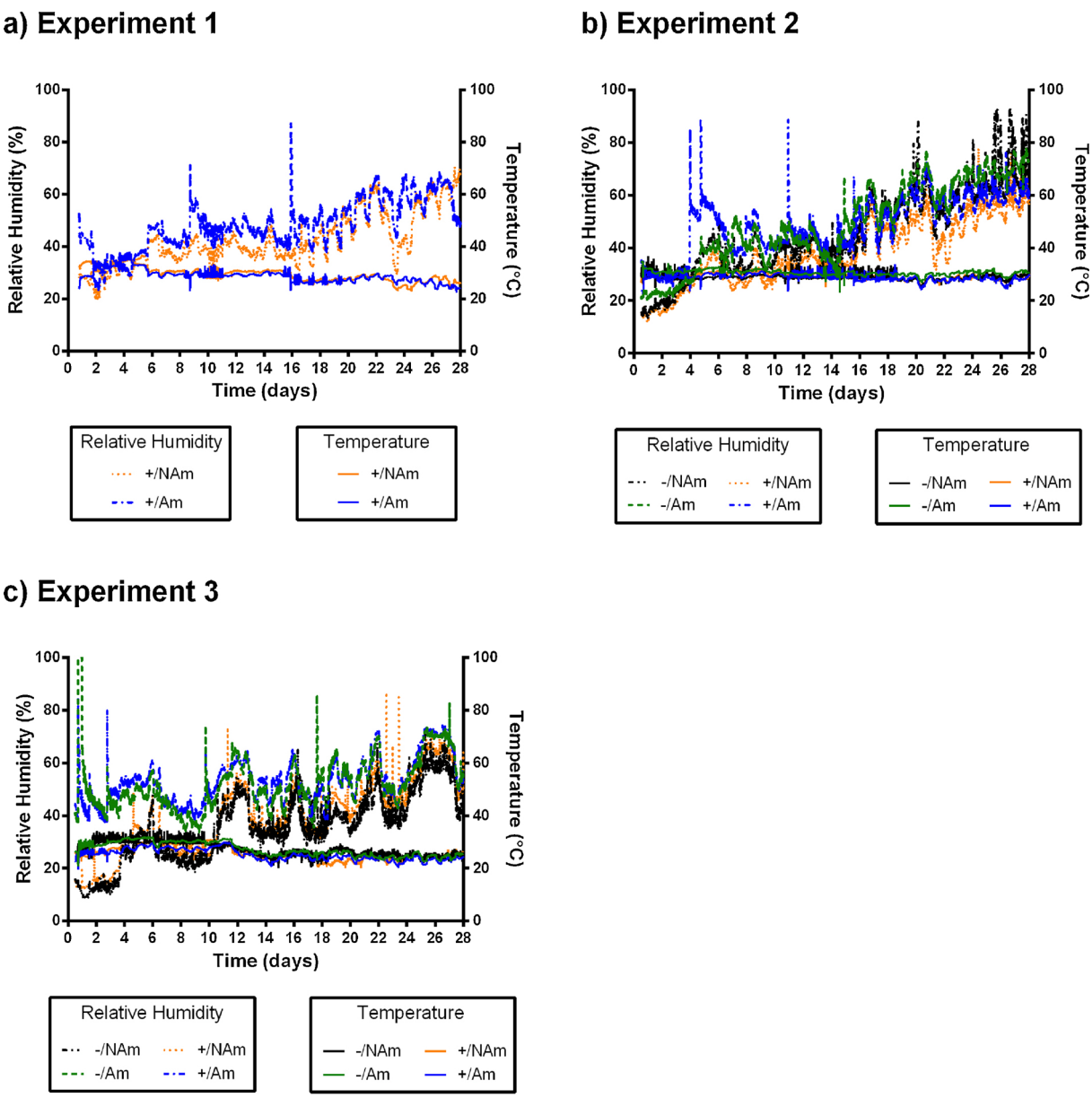

Fig. 2. Relative humidity (Rh, %) and temperature (°C). a) Experiment 1, b) Experiment 2, c) Experiment 3. –/NAm = non-vaccinated, no-NH_3; –/Am = non-vaccinated, NH_3-exposed; +/NAm = vaccinated, no-NH_3; +/Am = vaccinated, NH_3-exposed.

were significantly higher in Experiments 2 and 3, and scores from –/NAm/+ birds were significantly higher in Experiments 1 and 3. Tracheas were also scored post-vaccination (7, 10–14, and 24–28 dpv) and at other times post-challenge (18 and 48 hpc, 10–14 dpc), and no NH_3-related differences were observed (data not shown).

At 5 dpc, trachea cilia activity was measured under a microscope. All non-vaccinated, challenged birds exhibited complete ciliostasis, and no effect on ciliostasis from NH_3 exposure could be observed (Table 1). However, cilia from the –/Am/+ birds appeared blunted and to have slower ciliary beating compared to the cilia from –/NAm/+ birds. All negative controls and all birds from vaccinated, challenged groups, independent of NH_3 exposure, passed the ciliostasis test. These observations were similar for all three trials.

3.5. IBV-specific IgG serum titers

Serum IBV-specific IgG maternal antibodies were present until at least 10–14 dpv in all experiments, and neither vaccine nor NH_3 status had an effect on titers (Table 2). Prior to challenge at 24–28 dpv, IgG titers in vaccinated birds trended above baseline but were not significant, and titers were not influenced by NH_3. By 18 hpc, titers had increased dramatically among vaccinated, challenged birds and remained high for the duration of the study (data not shown for 18 hpc, n = 4, and 48 hpc, n = 4). Compared to negative control titers, significantly higher titers were observed in –/NAm/+ birds (Experiment 1) and –/Am/+ birds (Experiments 1 and 3). In contrast, titers in non-vaccinated, challenged birds did not increase until 10–14 dpc, when they were significantly higher than negative control titers. NH_3 had no effect on post-challenge IgG titers at any collection time.

3.6. IBV-specific IgA tear titers in tears

At all times post-vaccination, IBV-specific IgA titers in tears among vaccinated birds were not significantly different from background titers, nor were they influenced by NH_3 (Table 3).

In the first week post-challenge, no significant differences in titers among bird groups were observed, regardless of vaccination or NH_3 status (data not shown for 18 hpc, 48 hpc, 3 dpc, and 7 dpc). By 10–14

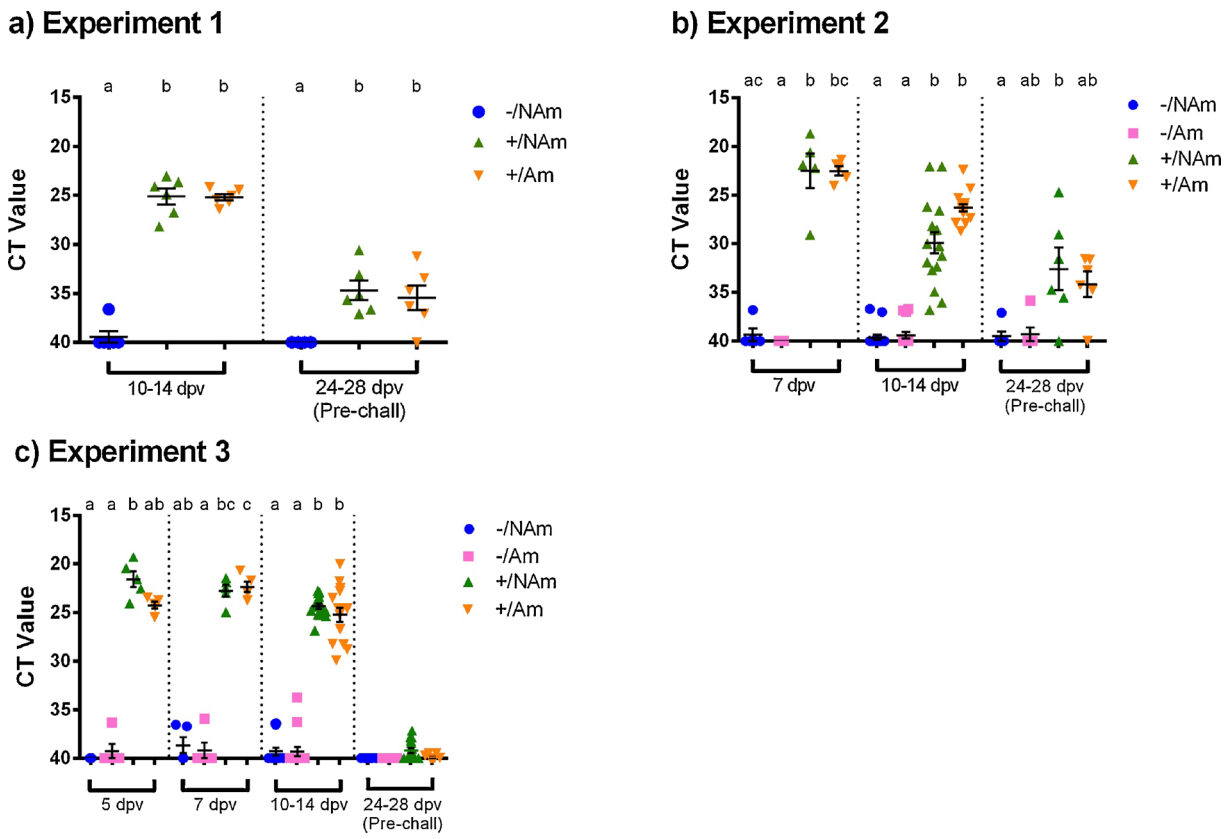

Fig. 3. qRT-PCR average CT values from choanal swabs collected post-vaccination. a) Experiment 1, b) Experiment 2, c) Experiment 3. Error bars indicate standard error. Letters (a–c) indicate significant differences at $p < 0.05$. –/NAm = non-vaccinated, no-NH_3; –/Am = non-vaccinated, NH_3-exposed; +/NAm = vaccinated, no-NH_3; +/Am = vaccinated, NH_3-exposed.

dpc, titers in –/NAm/+ birds were significantly higher than titers in +/NAm/+ birds (Experiments 1 and 2). No other significant differences were found, though non-vaccinated, challenged birds in all experiments had upward trends in titers compared to vaccinated and negative control birds.

3.7. Percent of leukocytes and MHC I^+/II^{lo} and MHC I^+/II^{hi} cells

Ambient NH_3 exposure had no impact on leukocyte percentages and leukocyte phenotypes in the HG, but vaccination- and challenge-related differences among treatment groups were apparent (Table 4). At 10–14 dpv, the percent of leukocytes was significantly higher in +/NAm birds (Experiments 2 and 3) and +/Am birds (Experiments 1, 2 and 3). The percent of MHC I^+/II^{hi} cells was significantly higher in +/NAm birds (Experiments 1 and 3) and +/Am birds (Experiments 1, 2 and 3), while the percent of MHC I^+/II^{lo} cells was significantly lower in +/NAm birds (Experiment 3) and +/Am birds (Experiment 1 and 3). By 24–28 dpv, fewer significant differences were detected, though the overall trends still persisted, which consisted of an elevated percent of leukocytes and MHC I^+/II^{hi} cells accompanied by a lower percent of MHC I^+/II^{lo} cells.

At 18 and 48 hpc, vaccinated birds tended to have a higher percentage of leukocytes and MHC I^+/II^{hi} cells, and a lower percentage of MHC I^+/II^{lo} cells, compared to non-vaccinated birds (data not shown). In non-vaccinated birds, an upward trend in the percent of MHC I^+/II^{lo} cells was observed relative to the pre-challenge levels. By comparison, there was no significant change in the percentage of MHC I^+/II^{lo} and MHC I^+/II^{hi} cells relative to the pre-challenge levels in vaccinated birds.

By 5 dpc, non-vaccinated birds experienced a strong peak in the percent of leukocytes and MHC I^+/II^{hi} cells, while the percent of MHC I^+/II^{lo} cells declined below the percent of MHC I^+/II^{lo} cells in negative controls. Vaccinated birds did not experience significant changes in the percentage of leukocytes or MHC I^+/II^{lo} cells, though a slight increase in the percent of MHC I^+/II^{hi} cells occurred. No statistical analysis could be performed due the low sample size (n = 4) at this time.

At 10–14 dpc, the percent of leukocytes in non-vaccinated, challenged birds had declined, and though the data were not significant, the birds maintained higher trends in the percent of leukocytes compared to vaccinated, challenged birds and negative controls. Similarly, vaccinated birds displayed trends of a higher percent of leukocytes compared to negative controls. There were no significant differences among challenge and negative control groups in the percent of MHC I^+/II^{lo} cells. With respect to MHC I^+/II^{hi} cells, no significant differences were detected among challenge groups. Compared to negative controls, the only group to have a significantly higher percent of MHC I^+/II^{hi} cells was the –/Am/+ group in Experiment 3, but all challenge groups in Experiments 2 and 3 showed trends of a higher percent of MHC I^+/II^{hi} cells compared to negative controls.

3.8. Percent of IgM^+ cells

Compared to the respective no-NH_3 groups, ambient NH_3 exposure in ammonia-treated groups did not significantly affect the percent of IgM^+ cells across treatment groups and over time (Table 4). Compared to the respective non-vaccinated controls at 10–14 dpv, only the +/Am birds had a significantly elevated percent of IgM^+ cells (Experiments 2 and 3), but +/NAm birds also displayed higher trends in the percent of IgM^+ cells (Experiments 2 and 3). By 24–28 dpv, the percent of IgM^+ cells was comparable across all treatment groups. At 18 hpc, the percent of IgM^+ cells remained comparable across all treatment groups, and at 48 hpc the percent of IgM^+ cells began to trend upward in non-

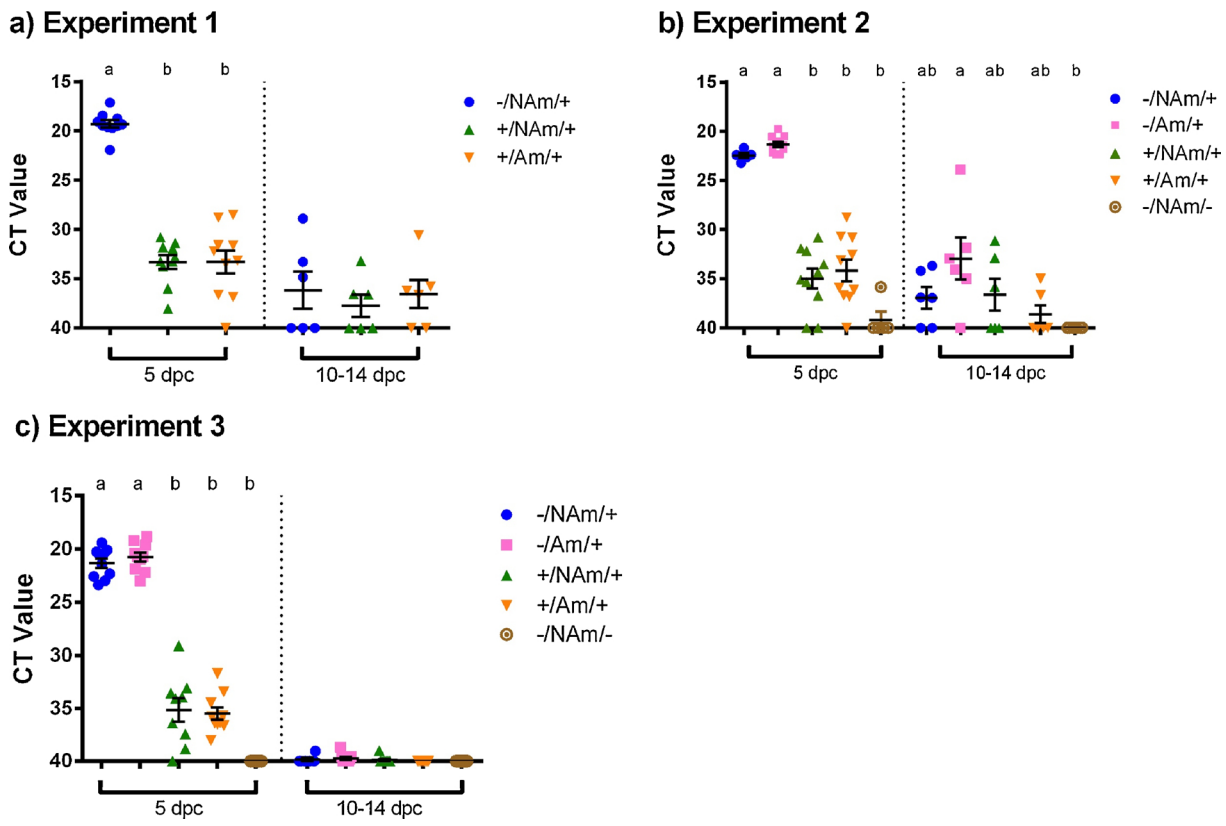

Fig. 4. qRT-PCR average CT values from choanal swabs collected post-challenge. a) Experiment 1, b) Experiment 2, c) Experiment 3. Error bars indicate standard error. Letters (a, b) indicate significant differences at $p < 0.05$. –/NAm = non-vaccinated, no-NH_3; –/Am = non-vaccinated, NH_3-exposed; +/NAm = vaccinated, no-NH_3; +/Am = vaccinated, NH_3-exposed.

vaccinated birds, but no differences were observed in the vaccinated birds (data not shown). By 5 dpc, non-vaccinated birds experienced a strong peak in the percent of IgM^+ cells relative to vaccinated birds and negative controls. In contrast, no clear changes were observed among vaccinated birds, though a higher trend was noted in +/Am/+ birds compared to +/NAm/+ birds and the controls. At 10–14 dpc, the percent of IgM^+ cells among all challenged birds, irrespective of vaccination or NH_3 treatment, were comparable to each other (all experiments) and negative controls (Experiments 2 and 3).

3.9. Percent of $CD4^+$ and $CD8^+$ cells

The HG was collected from 10 to 28 dpv and 18 hpc to 14 dpc and analyzed for the percent of T cell phenotypes via flow cytometry (Table 5). At 10–14 dpv, overall significant differences were not observed among treatment groups with respect to the percent of $CD4^+$ cells, though vaccinated birds showed upward trends in the percent of $CD4^+$ cells. The percent of $CD8^+$ cells was significantly increased ($p < 0.05$) in –/NAm birds (Experiment 3) and –/Am birds (Experiments 1, 2 and 3). Though no significant differences were detected in the CD4:CD8 ratio, vaccinated birds showed trends of a decreased CD4:CD8 ratio. Ambient NH_3 exposure did not affect any of these parameters. Prior to challenge at 24–28 dpv, the percent of $CD4^+$ cells in vaccinated birds was significantly lower (Experiment 1), and no differences in the percent of $CD8^+$ cells were observed. Overall, CD4:CD8 ratio was not affected with the exception of the +/NAm birds in which the ratio was significantly lower ($p < 0.05$, Experiment 1) compared to –/NAm birds. Although there were vaccine treatment-related trends in lower CD4:CD8 ratios and shifts in the percent of CD4 and CD8 cells at 18 hpc, 48 hpc and 5 dpc, there were no significant differences in the T cell phenotypes across treatments (data not shown for 18 hpc and 48 hpc). By 10–14 dpc, there were no significant differences in the percent of $CD4^+$ cells across groups. Although significant differences were not detected in the percent of $CD8^+$ cells among challenge groups, non-vaccinated, challenged birds trended toward a higher percent of $CD8^+$ cells compared to vaccinated, challenged birds. This pattern was also observed in all challenge groups compared to negative controls, though only –/Am/+ birds had a significantly elevated percent of $CD8^+$ cells (Experiment 3). Still, no significant differences in CD4:CD8 ratio were observed.

4. Discussion

In this study, we conducted three *in vivo* experiments to analyze the effect of litter-sourced ambient NH_3 on broiler immune response to IBV vaccination and protection against homologous IBV challenge. One challenge with NH_3 studies is maintaining exposure levels that are representative of NH_3 concentrations in commercial poultry operations. NH_3 levels vary considerably and may depend on season, time of day, geographic location, and individual poultry houses, and concentrations tend to be higher during cold weather as ventilation rates are reduced to conserve heat and reduce energy costs (Carr et al., 1990; Knizatova et al., 2010; Miles et al., 2014). NH_3 concentrations commonly average 39.9 ppm in the winter months, compared to 6.3 ppm in the summer months (Miles et al., 2014), and broiler houses are prone to NH_3 spikes leading to high levels of short duration (Czarick et al., 2014, 2015). Because of the wide variation in NH_3 levels in the field and the difficulties with maintaining constant NH_3 concentrations using litter-sourced NH_3, we chose a target range (30–60 ppm) that encompassed average wintertime concentrations. While NH_3 concentrations in Experiment 1 were within the target range throughout the entire study, NH_3 levels in Experiments 2 and 3 stayed within the target range for only the two first weeks, after which they declined to the low end of or below the target range. In spite of this observation, it is unlikely that

Table 1
Clinical signs, airsacculitis, histopathology scores, and ciliostasis five days post-challenge.

a) Experiment 1

Treatment			Clinical Signs (Mean ± SEM*)	Airsacculitis	Histopathology Scores (Mean ± SEM)	Ciliostasis	
Vaccine	Ammonia	Challenge				Ciliostasis Protection Score†	Binomial Protection Score‡
–	–	+	2.1 ± 0.4[a]	5/10	9.8 ± 0.1[a]	0.0	10/10[a]
+	–	+	0.1 ± 0.1[b]	0/10	6.4 ± 0.4[b]	86.2	1/10[b]
+	+	+	0.3 ± 0.2[b]	0/10	5.1 ± 0.5[b]	87.3	0/10[b]
–	–	–	0.0 ± 0.0[b]	0/5	6.0 ± 0.4[b]	97.7	0/5[ab]
+	–	–	0.0 ± 0.0[b]	0/5	5.9 ± 0.4[b]	82.0	0/5[ab]
+	+	–	0.0 ± 0.0[b]	0/5	5.2 ± 0.4[b]	68.7	1/5[ab]

b) Experiment 2

Treatment			Clinical Signs (Mean ± SEM)	Airsacculitis	Histopathology Scores (Mean ± SEM)	Ciliostasis	
Vaccine	Ammonia	Challenge				Ciliostasis Protection Score	Binomial Protection Score
–	–	+	3.0 ± 0.0[a]	2/5	9.5 ± 0.0[ab]	0	5/5[a]
–	+	+	3.0 ± 0.0[a]	9/10	9.6 ± 0.1[a]	0	10/10[a]
+	–	+	0.0 ± 0.0[b]	0/10	6.1 ± 0.1[bc]	90.7	0/10[b]
+	+	+	0.0 ± 0.0[b]	0/10	6.2 ± 0.3[abc]	88.3	0/10[b]
–	–	–	0.0 ± 0.0[b]	0/5	3.9 ± 0.4[c]	96.0	0/5[b]
–	+	–	0.0 ± 0.0[b]	0/5	5.1 ± 0.6[bc]	91.0	0/5[b]
+	–	–	0.0 ± 0.0[b]	0/5	4.8 ± 0.6[c]	92.0	0/5[b]
+	+	–	0.0 ± 0.0[b]	0/5	5.5 ± 0.3[bc]	87.0	0/5[b]

c) Experiment 3

Treatment			Clinical Signs (Mean ± SEM)	Airsacculitis	Histopathology Scores (Mean ± SEM)	Ciliostasis	
Vaccine	Ammonia	Challenge				Ciliostasis Protection Score	Binomial Protection Score
–	–	+	2.3 ± 0.3[a]	3/10	9.6 ± 0.2[a]	0	10/10[a]
–	+	+	2.7 ± 0.2[a]	7/10	9.6 ± 0.1[a]	0	10/10[a]
+	–	+	0.2 ± 0.1[b]	0/9	6.6 ± 0.2[b]	78.3	1/9[b]
+	+	+	0.0 ± 0.0[b]	0/10	6.5 ± 0.6[b]	82.2	1/10[b]
–	–	–	0.0 ± 0.0[b]	0/5	4.7 ± 0.5[b]	71.3	0/5[b]
–	+	–	0.4 ± 0.2[ab]	0/5	5.6 ± 0.4[b]	90.0	0/5[b]
+	–	–	0.4 ± 0.2[ab]	0/5	4.1 ± 1.1[b]	83.3	0/5[b]
+	+	–	0.4 ± 0.2[ab]	0/5	6.0 ± 0.0[ab]	88.3	0/5[b]

[a,b,c]Different letters indicate significant differences at $p < 0.05$.

* SEM = standard error of the mean.

† The protection score for each group was calculated using the following formula: 100 − [(total of the individual scores for the group)/(the number of individuals in the group × 20) × 100], groups with scores of > 50 are considered protected.

‡ The number of birds with ciliostasis per total in the group. Each ring was scored by three individuals. A tracheal ring with ≥50% of the cilia showing vigorous activity was considered protected, and a chicken was considered protected when 90% of the rings were showing ≥50% of cilia beating.

maintaining target concentrations for the duration of the experiment would have led to different outcomes, as few clear differences were detected among the three experiments with regards to the parameters measured.

A second challenge of NH_3 studies involving used litter as a source of NH_3 is the potential for greater microbial and dust exposure present in used litter. Since NH_3 production requires the presence of microbes and moisture in the litter (Ni, 1999; Carr et al., 1990; Knizatova et al., 2010; Liu et al., 2007), it is not possible to separate NH_3 from these factors without losing relevance to industry practices. However, fresh litter is quickly contaminated with fecal material, dander, and feathers, and birds placed on fresh litter are also exposed to microbes and dust. Finally, commercial broilers are raised on litter year-round, but the higher incidence of respiratory disease in wintertime associated with decreased ventilation and the accumulation of NH_3 suggests that the difference in air sacculitis, but not immune response or development of immunity as measured by protection from challenge, observed in this study are likely attributed to ambient NH_3.

Based on the immune parameters evaluated in this study, ambient NH_3 exposure from used litter had no discernible impact on the immune response to IBV vaccination. Furthermore, +/Am/+ birds were protected from challenge virus replication and demonstrated an immune response comparable to the response in +/NAm/+ birds. Despite being protected from challenge, birds in the +/Am/+ group showed subtle effects of NH_3 on cilia morphology and function, characterized by blunted cilia and slower ciliary beating, although these observations were not able to be measured. In the event of higher NH_3 levels, we speculate that the morphological and functional effects of NH_3 might become more prominent, perhaps predisposing birds to respiratory disease. Nevertheless, in the present study, airsacculitis was especially associated with –/Am/+ birds, which also showed trends toward increased clinical sign scores in Experiment 3. Airsacculitis resulting from NH_3 exposure combined with live attenuated IBV vaccination has been reported previously (Kling and Quarles, 1974; Quarles and Caveny, 1979). In the present study, vaccinated birds, irrespective of NH_3 exposure, were completely protected from challenge, and airsacculitis was not detected in either the vaccinated, challenged birds or the vaccinated, non-challenged birds. Our data therefore suggest that the negative effects of ambient NH_3 only become apparent when non-vaccinated birds are exposed to a virulent IBV challenge virus, resulting in airsacculitis.

With regard to ciliostasis and tracheal histopathology scores, which were determined by evidence of inflammation, epithelial hyperplasia, and deciliation, exposure to ambient NH_3 was not a factor as no NH_3-

related histological lesions were observed at any time post-vaccination or post-challenge. In this study, all of the non-vaccinated, challenged birds exhibited ciliostasis at 5 dpc, but it is possible that an NH_3 effect on the incidence of airsacculitis would be detectable should the birds be exposed to a less virulent IBV or smaller dose. Nevertheless, ciliostasis was not observed in the +/Am/+ birds, and scores were comparable to those for the +/NAm/+ birds. These results are in contrast with a report by Anderson et al. (1966), which described some deciliation in the upper trachea and nasal turbinates in chickens, however those birds were housed in an environment consisting of poultry house dust, carbon dioxide and NH_3 in which NH_3 concentrations were not recorded.

Except for some minor differences, ambient NH_3 had no significant effect on vaccine and challenge virus replication and clearance by the host, nor did it seem to impact IBV-specific local and systemic

Table 2
Serum IBV-specific IgG titer post-vaccination and post-challenge in maternal antibody-positive broilers.

a) Experiment 1

Day	Treatment			IgG Titer (Log_2, Mean ± SEM*)
	Vaccine	Ammonia	Challenge	
7 dpv†	–	–	–	9.3 ± 0.1
	+	–	–	10.7 ± 0.3
	+	+	–	11.5 ± 0.5
10-14 dpv	–	–	–	3.2 ± 0.8
	+	–	–	5.2 ± 1.2
	+	+	–	6.5 ± 0.9
24-28 dpv	–	–	–	0.4 ± 0.4^{a}
(Pre-Challenge)	+	–	–	6.0 ± 1.4^{ab}
	+	+	–	7.9 ± 0.8^{b}
5 dpc‡	–	–	+	1.5 ± 0.6^{ac}
	+	–	+	6.8 ± 0.6^{b}
	+	+	+	8.0 ± 0.4^{b}
	–	–	–	0.0 ± 0.0^{c}
10-14 dpc	–	–	+	10.6 ± 0.6
	+	–	+	7.4 ± 1.2
	+	+	+	8.2 ± 0.5

b) Experiment 2

Day	Treatment			IgG Titer (Log_2, Mean ± SEM)
	Vaccine	Ammonia	Challenge	
7 dpv	–	–	–	10.9 ± 0.5
	–	+	–	9.9 ± 0.5
	+	–	–	10.7 ± 0.2
	+	+	–	10.8 ± 0.5
10-14 dpv	–	–	–	7.8 ± 0.7
	–	+	–	6.5 ± 1.4
	+	–	–	5.1 ± 1.8
	+	+	–	6.3 ± 1.0
24-28 dpv	–	–	–	1.0 ± 1.0
(Pre-Challenge)	–	+	–	1.2 ± 1.2
	+	–	–	4.9 ± 0.9
	+	+	–	4.1 ± 1.4
5 dpc	–	–	+	4.1 ± 0.8^{ab}
	–	+	+	3.6 ± 0.6^{b}
	+	–	+	8.1 ± 0.6^{ac}
	+	+	+	8.5 ± 0.4^{c}
	–	–	–	5.1 ± 0.8^{abc}
10-14 dpc	–	–	+	9.6 ± 0.4^{a}
	–	+	+	9.0 ± 0.7^{a}
	+	–	+	4.3 ± 1.7^{ab}
	+	+	+	7.4 ± 1.3^{ab}
	–	–	–	1.2 ± 0.8^{b}

c) Experiment 3

Day	Treatment			IgG Titer (Log_2, Mean ± SEM)
	Vaccine	Ammonia	Challenge	
7 dpv	–	–	–	12.4 ± 0.3
	–	+	–	12.3 ± 0.2
	+	–	–	11.5 ± 0.4
	+	+	–	12.2 ± 0.1
10-14 dpv	–	–	–	11.5 ± 0.3
	–	+	–	11.1 ± 0.4
	+	–	–	10.7 ± 0.5
	+	+	–	10.8 ± 0.2

(*continued on next page*)

Table 2 (*continued*)

c) Experiment 3

Day	Treatment			IgG Titer (Log_2, Mean ± SEM)
	Vaccine	Ammonia	Challenge	
5 dpc	–	–	+	7.0 ± 0.4[a]
	–	+	+	7.1 ± 0.6[a]
	+	–	+	9.6 ± 0.3[ab]
	+	+	+	10.6 ± 0.3[b]
	–	–	–	4.1 ± 1.1[a]
10-14 dpc	–	–	+	13.1 ± 0.5[ab]
	–	+	+	13.5 ± 0.2[a]
	+	–	+	10.3 ± 0.4[bc]
	+	+	+	11.8 ± 0.5[ab]
	–	–	–	6.6 ± 0.4[c]

[a,b,c]All time points were analyzed and different letters indicate significant differences at $p < 0.05$. No letters are indicative of no statistical difference.

* SEM = standard error of the mean.

† dpv = days post-vaccination.

‡ dpc = days post-challenge.

serological responses as measured in the tears (IgA) and serum (IgG). Cell phenotypes of the local immune response measured in the HG, which included the percent of leukocytes, MHC I^+/II^{lo} and MHC I^+/II^{hi} cells, IgM^+ cells, and $CD4^+$ and $CD8^+$ T cells, also showed no NH_3-related differences. Although ambient NH_3 exposure had no apparent effect on the immune response to IBV vaccination and challenge, vaccination status alone had a measurable impact.

Commercial broiler chickens were selected for this study to maintain relevance to the poultry industry because the experimental conditions were tailored to a typical broiler grow-out period. Vaccination of maternal antibody-positive broiler chicks at hatch induced a weak-to-nonexistent serum IgG response and by 24–28 DOA; titers were only slightly higher than background titers in non-vaccinated controls. In contrast to IgG responses, post-vaccination IBV-specific IgA responses in tears were not detected. Non-vaccinated birds challenged at 28 DOA developed strong serum IgG responses by 10–14 dpc. As seen with serum IgG responses, non-vaccinated, challenged birds developed robust IgA titers in tears at 10–14 dpc. Orr-Burks et al. (2014) have reported similar timing in the development of plasma IgG and tear IgA titers in chickens following ocular IBV vaccination at 28 DOA. Though we did not evaluate the effect of maternal antibodies on active antibody production in this study, the absence of robust titers in vaccinated birds in this study could be explained by maternal antibody interference which diminished the young birds' epitope-specific antibody response following IBV vaccination. Maternal antibody modulation of IBV epitope-specific antibody production in young chicks has been well documented and yet protection against homologous challenge does not appear to be compromised suggesting that other arms of the immune system, not evaluated in this study, may be activated by the vaccine (Raggi and Lee, 1965; Toro et al., 1994).

Among the vaccinated birds in this study challenged with IBV, serum IgG titers increased rapidly, reaching high titers as early as 18 hpc. Titers were sustained for the duration of the study. In spite of these systemic IgG responses, it is worth noting that circulating antibody titers do not typically correlate with protection against IBV (Raggi and Lee, 1965). By comparison, average post-challenge IgA titers in vaccinated birds did not differ from negative control titers at any time post-challenge. The lack of a significant increase in the IgA titers among vaccinated birds after challenge has also been described (Guo et al., 2008; Joiner et al., 2007). On the other hand, other IBV challenge studies have suggested that local IgA responses may be a more reliable indicator of protection from active infection (Toro and Fernandez, 1994) and that mucosal IgA levels are greater than mucosal IgG levels (Baba et al., 1990; Toro et al., 1994). Additionally, Orr-Burks et al. (2014) demonstrated both IBV-specific IgG and IgA titers in tears following IBV vaccination. Because we did not examine IgG titers in tears, we could not make direct comparisons between IBV-specific IgG and IgA levels in tears. Nevertheless, our data provide novel insight into the role of tear IgA in IBV immunity.

The percent of leukocytes in the HG was higher in post-vaccinated birds and sustained until challenge, which implies that the viral vaccine stimulated colonization and clonal expansion of leukocytes in the HG. It is believed that B cell colonization of the HG occurs prior to two weeks-of-age (Gallego and Glick, 1988) and that the HG microenvironment orchestrates terminal B cell differentiation and immunoglobulin class switch (Mansikka et al., 1989). In the present study, MHC I^+/II^{lo} cells were the predominant phenotype in non-vaccinated birds whereas MHC I^+/II^{hi} cells were over-represented in vaccinated birds. This would infer that IBV vaccination induces an upregulation in MHC II expression as cells colonizing the HG become activated.

During the first 48 h post-IBV challenge, there was no evidence of leukocyte migration or activation in the HG. By 5 dpc, non-vaccinated, IBV-challenged birds had a significant increase in the percent of leukocytes, whereas no change occurred in vaccinated birds. This observation coincided with a dramatic shift in MHC I^+/II^{lo} to MHC I^+/II^{hi} cells among non-vaccinated, challenged birds, and a slight shift from MHC I^+/II^{lo} to MHC I^+/II^{hi} cells among vaccinated, challenged birds. These observations are not surprising given that at 5 dpc virus replication peaked in non-vaccinated birds but remained below the level of detection in vaccinated birds. By 10–14 dpc, the relative lack of differences in the percent of leukocytes and MHC I^+/II^{lo} and MHC I^+/II^{hi} cells between non-vaccinated and vaccinated birds would suggest the resolution of the infection.

Cells that express MHC II were reviewed in Glimcher and Kara (1992) and include B lymphocytes, macrophages, dendritic cells, thymic epithelium, and activated T cells. The level of MHC II expression is influenced by a large number of different stimuli. In addition, multiple cell types, including epithelial cells, may be induced to express MHC II by IFN-γ (Glimcher and Kara, 1992), a cytokine that is upregulated during IBV infection (Okino et al., 2017; Vervelde et al., 2013). In a follow-up experiment using CD45 and CD115 markers that identify leukocytes and cells of the monocyte-macrophage lineage, respectively, we confirmed that the MHC I^+/II^{lo} population consisted mostly of macrophages and propose that macrophages in the HG in their resting state are MHC I^+/II^{lo}. We theorize that the macrophages become activated and upregulate MHC II expression post-infection, although further research is needed. Mature B lymphocytes are the only major population of cells that constitutively express large amounts of MHC II (Glimcher and Kara, 1992), and MHC II expression is largely lost during terminal differentiation into plasma cells (Halper et al., 1978). Literature citing constitutive expression of MHC II in high quantities on B cells, combined with our observations of IgM^+ B lymphocyte dynamics following IBV infection, suggest that a large

Table 3
Tear IBV-specific IgA titer post-vaccination and post-challenge.

a) Experiment 1

Day	Treatment			IgA Titer (Log_2, Mean ± SEM*)
	Vaccine	Ammonia	Challenge	
10-14 dpv†	–	–	–	7.3 ± 0.3
	+	–	–	8.3 ± 0.8
	+	+	–	8.0 ± 0.4
24-28 dpv	–	–	–	8.5 ± 0.7
(Pre-Challenge)	+	–	–	8.3 ± 0.8
	+	+	–	10.5 ± 0.8
10-14 dpc‡	–	–	+	13.3 ± 0.5[a]
	+	–	+	10.8 ± 0.4[b]
	+	+	+	10.5 ± 0.8[b]

b) Experiment 2

Day	Treatment			IgA Titer (Log_2, Mean ± SEM)
	Vaccine	Ammonia	Challenge	
7 dpv	–	–	–	5.8 ± 0.5
	–	+	–	4.8 ± 0.2
	+	–	–	6.6 ± 0.4
	+	+	–	5.0 ± 0.4
10-14 dpv	–	–	–	6.5 ± 0.5
	–	+	–	7.3 ± 0.3
	+	–	–	9.3 ± 1.1
	+	+	–	8.0 ± 1.1
24-28 dpv	–	–	–	8.8 ± 0.7
(Pre-Challenge)	–	+	–	9.1 ± 0.3
	+	–	–	10.1 ± 0.4
	+	+	–	11.3 ± 0.7
5 dpc	–	–	+	9.1 ± 0.5
	–	+	+	10.6 ± 1.1
	+	–	+	8.9 ± 1.1
	+	+	+	11.1 ± 0.3
	–	–	–	11.4 ± 0.5
10-14 dpc	–	–	+	12.6 ± 0.4[a]
	–	+	+	12.8 ± 0.6[a]
	+	–	+	9.0 ± 0.7[b]
	+	+	+	10.1 ± 0.8[ab]
	–	–	–	10.1 ± 0.4[ab]

c) Experiment 3

Day	Treatment			IgA Titer (Log_2, Mean ± SEM)
	Vaccine	Ammonia	Challenge	
7 dpv	–	–	–	9.4 ± 0.6
	–	+	–	10.2 ± 0.2
	+	–	–	11.0 ± 0.4
	+	+	–	9.8 ± 0.6
10-14 dpv	–	–	–	10.4 ± 0.2
	–	+	–	10.1 ± 0.2
	+	–	–	10.7 ± 0.3
	+	+	–	10.8 ± 0.3
24-28 dpv	–	–	–	9.6 ± 0.3
(Pre-Challenge)	–	+	–	10.4 ± 0.3
	+	–	–	10.2 ± 0.4
	+	+	–	9.8 ± 0.5
5 dpc	–	–	+	8.6 ± 0.6
	–	+	+	9.2 ± 0.8
	+	–	+	9.8 ± 0.6
	+	+	+	9.8 ± 0.7
	–	–	–	9.2 ± 0.7
10-14 dpc	–	–	+	11.5 ± 0.5[ab]
	–	+	+	12.2 ± 0.6[a]
	+	–	+	9.8 ± 0.4[b]
	+	+	+	10.1 ± 0.6[ab]
	–	–	–	10.2 ± 0.4[ab]

[a,b]Different letters indicate significant differences at $p < 0.05$.
* SEM = standard error of the mean.
† dpv = days post-vaccination.
‡ dpc = days post-challenge.

Table 4
Percent leukocytes, MHC I/II^{lo} and MHC I/II^{hi} cells, and IgM^{+} lymphocytes in Harderian gland post-vaccination and post-challenge.

a) Experiment 1

Day	Treatment			% Leukocytes (Mean ± SEM*)	% MHC I/II^{lo} (of leukocytes)	% MHC I/II^{hi} (of leukocytes)	% IgM Expression (Mean ± SEM*)
	Vaccine	Ammonia	Challenge				
10-14 dpv†	–	–	–	1.3 ± 0.2^{a}	26.2 ± 3.9^{a}	10.8 ± 1.9^{a}	22.6 ± 2.6
	+	–	–	5.9 ± 1.8^{ab}	19.3 ± 2.4^{ab}	29.9 ± 2.1^{b}	20.7 ± 5.0
	+	+	–	6.2 ± 1.2^{b}	16.0 ± 2.9^{b}	36.4 ± 3.6^{b}	23.2 ± 4.5
24-28 dpv (Pre-Challenge)	–	–	–	1.6 ± 0.3^{a}	18.7 ± 2.7	18.4 ± 2.3	19.6 ± 1.7
	+	–	–	4.7 ± 1.2^{ab}	13.3 ± 3.0	30.0 ± 8.1	20.1 ± 6.0
	+	+	–	5.3 ± 1.1^{b}	11.2 ± 2.0	38.1 ± 6.8	32.2 ± 4.5
10-14 dpc‡	–	–	+	10.2 ± 1.0	10.6 ± 1.4	26.1 ± 2.8	31.6 ± 4.4
	+	–	+	7.4 ± 0.9	14.5 ± 3.4	28.0 ± 4.9	21.8 ± 1.4
	+	+	+	8.2 ± 1.3	14.4 ± 3.2	33.9 ± 4.5	29.3 ± 2.9

b) Experiment 2

Day	Treatment			% Leukocytes (Mean ± SEM)	% MHC I/II^{lo} (of leukocytes)	% MHC I/II^{hi} (of leukocytes)	% IgM Expression (Mean ± SEM)
	Vaccine	Ammonia	Challenge				
10-14 dpv	–	–	–	1.3 ± 0.1^{a}	14.1 ± 1.6	11.5 ± 1.5^{ab}	10.9 ± 0.8^{a}
	–	+	–	1.4 ± 0.2^{ab}	12.1 ± 1.7	10.3 ± 2.1^{a}	8.7 ± 2.3^{a}
	+	–	–	7.6 ± 2.0^{bc}	12.0 ± 1.8	28.0 ± 4.8^{b}	25.2 ± 6.6^{ab}
	+	+	–	11.6 ± 3.5^{c}	9.2 ± 1.5	28.8 ± 3.4^{b}	27.3 ± 1.7^{b}
24-28 dpv (Pre-Challenge)	–	–	–	3.2 ± 1.9^{a}	22.6 ± 8.3	13.1 ± 3.7	22.7 ± 2.1
	–	+	–	3.1 ± 0.3^{ab}	20.6 ± 2.7	15.1 ± 1.8	24.1 ± 1.5
	+	–	–	16.2 ± 3.2^{b}	13.2 ± 3.8	40.5 ± 7.4	18.5 ± 3.1
	+	+	–	6.4 ± 2.4^{ab}	17.6 ± 5.9	32.6 ± 8.0	16.9 ± 3.4
10-14 dpc	–	–	+	7.3 ± 2.2^{ab}	14.1 ± 3.7	19.3 ± 3.8	19.0 ± 2.8
	–	+	+	7.3 ± 1.3^{a}	11.5 ± 2.0	23.2 ± 3.3	20.0 ± 4.8
	+	–	+	4.1 ± 1.3^{ab}	19.7 ± 4.8	20.8 ± 4.2	19.9 ± 3.9
	+	+	+	4.2 ± 1.0^{ab}	10.6 ± 3.9	19.6 ± 5.2	21.1 ± 3.5
	–	–	–	1.3 ± 0.4^{b}	22.1 ± 3.5	13.3 ± 2.2	20.0 ± 5.1

c) Experiment 3

Day	Treatment			% Leukocytes (Mean ± SEM)	% MHC I/II^{lo} (of leukocytes)	% MHC I/II^{hi} (of leukocytes)	% IgM Expression (Mean ± SEM)
	Vaccine	Ammonia	Challenge				
10-14 dpv	–	–	–	1.2 ± 0.1^{a}	26.1 ± 2.9^{ac}	5.4 ± 0.5^{a}	11.5 ± 0.7^{a}
	–	+	–	1.3 ± 0.1^{a}	26.7 ± 2.1^{a}	5.8 ± 0.4^{a}	12.2 ± 0.6^{a}
	+	–	–	11.5 ± 1.1^{b}	15.6 ± 1.6^{b}	32.4 ± 1.8^{b}	22.1 ± 2.1^{ab}
	+	+	–	9.1 ± 1.0^{b}	16.5 ± 1.7^{bc}	29.6 ± 1.9^{b}	22.3 ± 1.2^{b}
5 dpc	–	–	+	17.3 ± 3.0	7.2 ± 1.4	69.5 ± 6.0	54.8 ± 5.6
	–	+	+	24.4 ± 4.9	5.3 ± 0.4	67.6 ± 6.0	53.0 ± 7.4
	+	–	+	4.6 ± 1.0	10.7 ± 1.0	43.5 ± 5.5	18.9 ± 0.8
	+	+	+	5.1 ± 0.9	7.7 ± 1.5	51.3 ± 4.6	28.7 ± 3.7
	–	–	–	1.3 ± 0.2	24.2 ± 2.8	12.1 ± 1.2	19.7 ± 1.1
10-14 dpc	–	–	+	7.2 ± 0.9	8.0 ± 0.7	28.2 ± 5.8^{ab}	29.0 ± 4.7
	–	+	+	8.8 ± 1.3	6.1 ± 0.8	35.4 ± 2.5^{a}	23.7 ± 1.7
	+	–	+	6.1 ± 1.2	6.2 ± 0.9	33.5 ± 3.4^{ab}	19.2 ± 2.2
	+	+	+	4.4 ± 1.4	5.9 ± 0.9	26.1 ± 3.3^{ab}	19.1 ± 1.1
	–	–	–	1.5 ± 0.9	7.6 ± 1.3	17.3 ± 2.1^{b}	24.8 ± 2.6

[a,b,c]Different letters indicate significant differences at $p < 0.05$.
* SEM = standard error of the mean.
† dpv = days post-vaccination.
‡ dpc = days post-challenge.

proportion of the MHC I^{+}/II^{hi} cells in the HG consists of B lymphocytes. Therefore, it would follow that the shift in predominance from MHC I^{+}/II^{lo} cells to MHC I^{+}/II^{hi} cells following IBV infection represents, at least in part, macrophage activation plus colonization and clonal expansion of mature B lymphocytes.

In Experiments 2 and 3, the percent of IgM^{+} cells in the HG was initially greater among vaccinated birds at 10–14 dpv, and by 24–28 dpv the percent among non-vaccinated birds had increased to the level of vaccinated birds. The small sample size collected may account for the lack of consistency in the percent of IgM^{+} cells between Experiment 1 and Experiments 2 and 3. Since IgM^{+} cells measured in this study were not necessarily IBV-specific, we speculate that the increase in IgM^{+} cells in non-vaccinated birds was a normal age-related process of colonization and clonal expansion in the HG in response to other immunogens driving IgM responses. This is not surprising, since the majority of lymphoid cells in the HG of young birds express IgM (Albini et al., 1974). Non-vaccinated birds challenged at 28 DOA experienced a peak percent of IgM^{+} lymphocytes at 5 dpc, coinciding with the peak of virus RNA load, and a decline to pre-challenge levels by 10–14 dpc, implying the cessation of IBV-induced IgM^{+} B cell clonal expansion and the initiation of class switch and subsequent loss of IgM expression. These data suggest that IgM responses are rapid and transient, which agree with previous work in which IBV-specific IgM in sera were first detected five days after IBV exposure (De Wit et al., 1998), reaching

Table 5
Percent $CD4^+$ and $CD8^+$ lymphocytes and CD4:CD8 ratio in Harderian gland post-vaccination and post-challenge.

a) Experiment 1

Day	Treatment			% $CD4^+$ (Mean ± SEM*)	% $CD8^+$ (Mean ± SEM)	CD4:CD8 Ratio† (Mean ± SEM)
	Vaccine	Ammonia	Challenge			
10-14 dpv‡	–	–	–	17.7 ± 1.7	14.6 ± 2.7[a]	1.5 ± 0.4
	+	–	–	17.3 ± 3.9	22.2 ± 1.2[ab]	0.8 ± 0.2
	+	+	–	16.8 ± 2.9	24.6 ± 2.1[b]	0.7 ± 0.1
24-28 dpv	–	–	–	24.9 ± 1.5[a]	11.8 ± 0.6	2.2 ± 0.2[a]
(Pre-Challenge)	+	–	–	16.9 ± 1.7[b]	20.4 ± 2.8	0.9 ± 0.1[b]
	+	+	–	16.9 ± 1.3[b]	15.3 ± 2.7	1.3 ± 0.3[ab]
10-14 dpc§	–	–	+	20.5 ± 1.8	20.7 ± 2.9	1.1 ± 0.2
	+	–	+	16.5 ± 1.4	16.6 ± 1.4	1.0 ± 0.1
	+	+	+	17.1 ± 2.1	13.2 ± 1.7	1.5 ± 0.4

b) Experiment 2

Day	Treatment			% $CD4^+$ (Mean ± SEM)	% $CD8^+$ (Mean ± SEM)	CD4:CD8 Ratio (Mean ± SEM)
	Vaccine	Ammonia	Challenge			
10-14 dpv	–	–	–	7.4 ± 0.7[a]	5.5 ± 0.4[a]	1.4 ± 0.1
	–	+	–	7.1 ± 1.9[a]	6.0 ± 1.0[a]	1.2 ± 0.3
	+	–	–	13.5 ± 2.2[ab]	19.5 ± 4.2[ab]	0.8 ± 0.1
	+	+	–	18.9 ± 2.8[b]	23.1 ± 2.0[b]	0.8 ± 0.1
24-28 dpv	–	–	–	16.0 ± 1.8	11.0 ± 1.7	1.5 ± 0.1
(Pre-Challenge)	–	+	–	20.3 ± 2.3	11.2 ± 1.8	2.0 ± 0.4
	+	–	–	14.9 ± 1.6	11.3 ± 2.7	1.6 ± 0.3
	+	+	–	13.3 ± 2.5	11.5 ± 2.4	1.2 ± 0.1
10-14 dpc	–	–	+	17.9 ± 2.4	19.1 ± 3.0	1.0 ± 0.2
	–	+	+	22.2 ± 2.3	21.5 ± 3.6	1.2 ± 0.2
	+	–	+	14.2 ± 0.8	15.1 ± 0.9	1.0 ± 0.1
	+	+	+	13.5 ± 1.4	11.4 ± 1.1	1.2 ± 0.1
	–	–	–	14.5 ± 2.6	14.7 ± 2.3	1.1 ± 0.2

c) Experiment 3

Day	Treatment			% $CD4^+$ (Mean ± SEM)	% $CD8^+$ (Mean ± SEM)	CD4:CD8 Ratio (Mean ± SEM)
	Vaccine	Ammonia	Challenge			
10-14 dpv	–	–	–	12.9 ± 1.2	15.8 ± 1.5[a]	0.9 ± 0.1
	–	+	–	12.3 ± 1.1	15.3 ± 1.4[a]	0.9 ± 0.1
	+	–	–	16.1 ± 1.1	24.7 ± 1.6[b]	0.7 ± 0.1
	+	+	–	14.8 ± 1.3	24.3 ± 1.1[b]	0.6 ± 0.1
5 dpc	–	–	+	12.0 ± 4.7	7.7 ± 1.9	1.5 ± 0.4
	–	+	+	7.9 ± 0.8	13.0 ± 4.4	0.7 ± 0.2
	+	–	+	10.4 ± 0.8	16.7 ± 2.2	0.6 ± 0.1
	+	+	+	11.9 ± 1.2	10.0 ± 1.9	1.3 ± 0.3
	–	–	–	19.3 ± 0.9	8.1 ± 0.4	2.4 ± 0.2
10-14 dpc	–	–	+	19.3 ± 3.9	20.3 ± 1.7[ab]	0.8 ± 0.2
	–	+	+	21.9 ± 0.9	22.1 ± 2.0[a]	1.1 ± 0.1
	+	–	+	18.3 ± 1.5	16.9 ± 1.8[ab]	1.0 ± 0.1
	+	+	+	17.4 ± 0.9	16.9 ± 2.3[ab]	0.9 ± 0.1
	–	–	–	17.8 ± 1.0	12.1 ± 1.6[b]	1.8 ± 0.2

[a,b]Different letters indicate significant differences at $p < 0.05$.
* SEM = standard error of the mean.
† CD4:CD8 Ratio = ratio of the percent of $CD4^+$ cells to the percent of $CD8^+$ cells.
‡ dpv = days post-vaccination.
§ dpc = days post-challenge.

peak titers at 8 days and waning by 24 days (da Silva Martins et al., 1990). In contrast, vaccinated, challenged birds in our study showed no increase in the percent of IgM^+ cells in the HG, which would seem to imply that vaccinated birds effectively neutralized the virus and that the IgM^+ cells were not activated.

The percent of $CD4^+$ and $CD8^+$ lymphocytes in the HG was initially greater among vaccinated birds at 10–14 dpv, and the lower CD4:CD8 ratio indicated that vaccinated birds experienced predominantly a $CD8^+$ T cell response following vaccination. These findings confirm previous research describing cytotoxic T cells as the major cells involved in primary exposure to IBV (Seo and Collisson, 1997). These differences largely became nonsignificant by 24–28 dpv, and the CD4:CD8 ratio in vaccinated birds increased as the percent of $CD8^+$ cells decreased, which corresponded with viral clearance and the resolution of infection. The importance of the cytotoxic T cell response following primary IBV exposure was further confirmed when non-vaccinated birds challenged at 28 DOA exhibited a strong $CD8^+$ cell response, with a simultaneous decrease in CD4:CD8 ratio, at 10–14 dpc. Among vaccinated birds, no clear shift in the T cell populations post-challenge was detected, which implies that the HG T cell may have a minor role in the immune response to secondary IBV exposure or the level of humoral immunity from vaccinated birds was robust enough to

minimize mucosal penetration and mediate clearance.

5. Conclusion

Young commercial broilers exposed to ambient NH_3 produced from used litter during a study mimicking a typical grow-out period responded well to both post-hatch IBV vaccine and a 28-day IBV challenge when compared to non-vaccinated birds in no-NH_3 control groups. Furthermore, ambient NH_3 did not impact vaccine or challenge virus replication and clearance, ciliostasis, or tracheal histopathology scores. In addition, ambient NH_3 also did not affect host immune response to the IBV vaccine or challenge. The only notable adverse observation was that airsacculitis was associated with non-vaccinated, ambient NH_3-exposed birds that were challenged with IBV, however some non-vaccinated, IBV-challenged birds in the no-NH_3 control group also had airsacculitis.

The IBV vaccine mobilized leukocytes with increased MHC I^+/II^{hi}, IgM^+, and $CD8^+$ expression in the HG, and serum IgG antibody titers remained elevated but comparable to non-vaccinated titers through 14 DOA. This observed IBV epitope-specific humoral immune profile pattern, measured in all bird groups, was likely due to a robust protective maternal immunity. Following IBV challenge, although all bird groups responded, non-vaccinated birds at 5 dpc had the greatest measurable increase in activated leukocytes denoted by increased MHC I^+/MHC II^{hi} and IgM^+ expression with mucosal and serum immunoglobulin levels and $CD8^+$ expression peaking at 10–14 dpc; the time period in which most of the birds had cleared the virus. It is also worth mentioning that the IBV challenge strain in the HG was cleared more quickly than the vaccine strain. Furthermore, the minimal immune activation in the HG of vaccinated, challenged birds indicates that the challenge virus was efficiently neutralized before mucosal penetration and viral replication.

Declaration of Competing Interest

None.

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