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Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorijps@gmail.com

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Research Article
Immune Response of Laying Hens Exposed to 30 ppm Ammonia for 25 Weeks

[1]H. Chen, [2]F.F. Yan, [2]J.Y. Hu, [3]Yanan Wu, [4]C.M. Tucker, [4]A.R. Green and [5]H.W. Cheng

[1]College of Animal Science and Technology, Agricultural University, 071001 Hebei, China
[2]Department of Animal Sciences, Purdue University, West Lafayette, 47907 IN, USA
[3]College of Animal Science and Technology, Nanjing Agricultural University, 210095 Jiangsu, China
[4]Department of Agricultural and Biological Engineering, University of Illinois, 61820 IL, USA
[5]USDA-ARS, Livestock Behavior Research Unit, 125 Russell street, West Lafayette, 47907 IN, USA

Abstract

Background and Objective: Ammonia (NH_3) is one of the most prominent aerial pollutants inside poultry barns, affecting chicken health and well-being based on its level and exposure duration. The aim of this study was to investigate the effect of 30 ppm NH_3 on the immune response of laying hens. **Methodology:** Hy-Line W-36 hens at 18 weeks of age were randomly assigned to 4 hen cages and evenly distributed to two controlled environment chambers. Beginning at 25 weeks of age, one chamber was maintained continuously with fresh air (NH_3 < 5 ppm; control group) and the other one was injected with NH_3 and controlled at 30 ppm (NH_3 group) for 25 weeks. At 50 weeks of age, plasma concentrations of total immunoglobulins (IgA, IgG and IgM), complement factors (C_3 and C_4), albumin (ALB), Alpha-1-acid glycoprotein (AGP) and cytokines including interleukin (IL)-1β, IL-6, interferon gamma (IFN-γ) and Tumor Necrosis Factor alpha (TNF-α) as well as mRNA expressions of IL-1β, IL-6 and TNF-α in the spleens were determined (n = 16). **Results:** Hens exposed to NH_3 had a greater Heterophil/Lymphocyte (H/L) ratio ($p<0.05$) but lower plasma concentrations of IgM and C4 ($p<0.05$, respectively) than control hens. There were no differences in the concentrations of other measured parameters between NH_3 exposed hens and control hens ($p>0.05$, respectively). **Conclusion:** These findings suggested that NH_3 exposure at 30 ppm for 25 weeks increases stress status and suppresses immunity of laying hens as indicated by the changes of H/L ratio and plasma IgM and C4 concentrations.

Key words: Ammonia, stress, heterophil/lymphocyte ratio, immunity, hen

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Corresponding Author: H.W. Cheng, USDA-ARS, Livestock Behavior Research Unit, 125 Russell Street, West Lafayette, 47907 IN, United States
Tel: 765-494-8022

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Ammonia (NH_3), a colorless toxic gas, has been recognized as one of the most prominent aerial pollutants of poultry facilities, affecting health condition and well-being of farm workers and chickens[1-3]. The levels of NH_3 vary between different housing and weather conditions, NH_3 concentration has been observed as high as 120 ppm in floor pen system[4]. Excessive NH_3 adversely affects hen health and well-being, irritate respiratory tract membrane to increase susceptibility of bacterial respiratory inflammation and infection[3,5,6]; causing hepatic damage and oxidative stress[7,8]; reducing body weight gain and feed conversion rate[9,10] and decreasing production performance[11-13].

Previous studies have shown that NH_3 affects immunological function of broiler chickens, reduces specific antibody titers[14], increases disease susceptibility[9] and alters the expression and translation of pro-inflammatory cytokine genes[7,15]. Wang and Leung[16] reported that 1 day-old Arbor Acres broiler chicks exposed to 26 or 52 ppm NH_3 for 21 days decreased Newcastle virus hemagglutination inhibition antibody titers. However, few studies have examined NH_3 effects on immunity in laying hens.

Various biomarkers have been used as indicators for evaluating immunity in various animals, including chickens. Albumin (ALB), one of the major serum proteins, has been widely used as an indicator of liver function[17]. The α-1-acid glycoprotein (AGP), a typical acute phase protein, is a critical element in response to inflammation[18]. Both complement components, C3 and C4, play important roles in the complement system in tissue regeneration[19,20]. Immunoglobulin (Ig) M, IgG and IgA are three major classical immunoglobulins secreted by activated immune B-cells[21]. Under a normal condition, these antibodies provide protection for the host to against non-self-antigens[22,23]; while under a pathophysiological condition such as stress; enhanced autoantibodies can cause autoimmune diseases in humans and various animals including chickens[24-27]. In chicken immune system, similar to mammals, cytokines play important roles in a host's immunity by regulating both humoral and cell-mediated immune responses[28]. Interleukin (IL)-1 β, IL-6, interferon gamma (IFN-γ), Tumor Necrosis Factor alpha (TNF-α) are potent pro-inflammatory and immunomodulatory cytokines[29]. For example, IL-6 blockage, a recombinant humanized anti-IL-6 receptor antibody, has been used effectively in treating several autoimmune diseases in humans[30,31].

In the husbandry guidelines for U.S. egg laying flocks, it has been recommended "NH_3 level should be less than 10 ppm and not exceed 25 ppm inside poultry houses"[32]. However, the cellular mechanisms for the recommendation has not been well studied. In addition, no significant changes of immunological biomarkers in hens have been reported followed by long time exposure to NH_3 at 30 ppm for 45 weeks[33]. Previous studies have indicated that the effects of NH_3 on immunity of hens vary depending upon several factors including NH_3 exposure duration[34]. The objective of this study was to determine the effects of intermediate exposure of 25 weeks old hens to 30 ppm NH_3 on the reactions of Acute Phase Proteins (APP) and immune parameters in laying hens.

MATERIALS AND METHODS

Animals and housing conditions: The study was conducted at the Environmental Research Laboratory of the University of Illinois at Urbana-Champaign. All hens used in this experiment were housed and cared for under the protocol approved by the Animal Care and Use Committee of University of Illinois (IACUC#: 14161).

At 18 weeks of age, Hy-Line W-36 hens were randomly assigned to 4 hen cages, 82 in^2 floor space per hen and evenly distributed between two controlled environment chambers. When hens reached 25 weeks old, one chamber was maintained continuously with fresh air (<5 ppm NH_3) (control group) and the other one was injected with NH_3 and maintained at 30 ppm (NH_3 group) for 25 weeks.

Feed and water were provided with free access and the lighting schedule was gradually stepped up to 16 L: 8 D, which was achieved when the hens were at 30 weeks of age. The manure was removed from the chambers every three days.

Blood sampling: At 50 weeks of age, 16 birds were taken from the control and NH_3 chambers (n = 8 per treatment). Hens were sedated by using sodium pentobarbital through brachial vein (30 mg kg^{-1} b.wt.) and then 5 mL blood from each sampled hen was collected into an ice-cooled Ethylene-Diamine-Tetra-Acetic-acid (EDTA) tube via cardiac puncture within 2 min of removing the hen from her home cage because the capture stress to be at or near the baseline level in birds within 3 min[35,36]. The blood samples were centrifuged at 3000×g for 10 min at 4°C (Sorvall BC 3B Plus, Thermo Fisher Scientific, Waltham, MA). Plasma were aliquoted into 500 µL tubes and kept at -80°C until further analysis.

Following blood collection, hens were euthanized immediately by cervical dislocation. An approximate 1 cm^3 of spleen sample was collected from the same location of each sampled hen and immediately placed on dry ice, then transferred to -80°C until further analysis.

Heterophil/Lymphocyte (H/L) ratio: Duplicate blood smear slides per hen were made by generating a thin layer of cells using a cover glass technique[37]. The slides were stained next day with Wright's staining (Thermo Fisher Scientific Inc. Waltham, USA). One hundred leukocytes per slide (200 cells per hen), including both granular (heterophils, eosinophils and basophils) and non-granular (lymphocytes and monocytes) cells, were counted at 2000× magnification using a light microscope. The H/L ratio was calculated followed the procedure published previously[38].

Enzyme-Linked Immunosorbent Assay (ELISA): Plasma concentrations of IgA (ELISA kit from Bethyl Laboratories Inc, Catalog #E33-103), IgM (ELISA kit from Bethyl Laboratories Inc, Catalog#E33-102), IgG (ELISA kit from Bethyl Laboratories Inc, Catalog #E33-104), C3 (ELISA kit from MyBiosource, Catalog #MBS013330), C4 (ELISA kit from MyBiosource, Catalog #MBS263773), ALB (ELISA kit from MyBiosource, Catalog #MBS044483), AGP (ELISA kit from Life Diagnostics Inc, Catalog #2510-3), IL-1β (ELISA kit from Cusabio Biotech COL., LTD, Catalog # E11230Ch-96), IL-6 (ELISA kit from MyBiosource, Catalog #MBS737945), IFN-γ (Cat No. MBS 025498) and TNF-α (ELISA kit from Cusabio Biotech COL., LTD., Catalog # E11231Ch-96) were measured in duplicate according to respective manufacturer instructions. Plasma samples were diluted for the measurements of all three immunoglobulins according to the protocols; while the rest of the parameters were detected without dilution[39].

Quantitative reverse transcriptase real-time PCR: Primer pairs were designed for IL-1β, IL-6 and TNF-α genes using the Primer 3 software (primer 3.sourceforge.net) (Table 1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a house keeping gene. Splenic RNA was extracted using the total RNA extraction kit (Qiagen Inc., MD) and reverse transcribed using Taqman reverse transcription reagents (Applied Biosystems Inc., CA) as specified by the manufactures. Quantitative real-time PCR was performed using Taqman probe (Applied Biosystems Inc., CA) and an ABI StepOnePlus Real-time PCR System (Applied Biosystems Inc, CA). Briefly, a reaction solution was created by mixed 12.5 μL of universal master mix; 2.25 μL of each of optimum concentrations of primers; 1.625 μL of optimum concentration of probe; 2.5 μL of cDNA sample and 3.875 μL of water. A 25 μL of the reaction solution per well was added to a 96-well PCR plate. The reaction conditions were as follows: 50°C for 2 min, 95°C for 10 min, 40 cycles at 95°C for 15 sec, at 60°C for 1 min. The mean fold change in mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method, as described previously[40]. To assure accuracy and consistency, all samples were measured in duplicates and the standards were in triplicates[39].

Table 1: Primer and probe sequences

Genes[1]	Primers and probes	Application efficiencies (%)	Product length (bp)
IL-1β	F: TGCTGGTTTCCATCTCGTATGTAC	95	80
	R: CCCAGAGCGGCTATTCCA		
	P: AGTACAACCCCTGCTGCCCCGC(VIC/MGB)		
IL-6	F: CCCGCTTCTGACTGTGTTT	86	139
	R: GCCGGTTTTGAAGTTAATCTTT		
	P: TGTGTTTCGGAGTGCTTT(VIC/MGB)		
TNF-α	F: CCCCTACCCTGTCCCACAA	75	62
	R: ACTGCGGAGGGTTCATTCC		
	P: CTGGCCTCAGACCAG(VIC/MGB)		

[1]Gene expression reported in relative abundance to GAPDH, IL-1β: Interleukin 1 beta, IL-6: Interleukin 6, TNF-α: Tumor necrosis factor alpha, F: Forward primer, R: Reverse primer, P: Probe

Statistical analyses: Data from the randomized design were subjected to an ANOVA using MIXED model of SAS 9.3 software (SAS Institute Inc., Cary, NC). The NH_3 treatment is a fixed effect and cage was used as experiment unit. If data lacked homogenous variances, transformation was made and the data were reanalyzed. Because statistical trends were similar for both transformed and untransformed data, the untransformed results were presented. Physiological measurements were tested in duplicates with CV $\leq$15%. Tukey-Kramer was used to find the partition differences among means. Significant statistical differences were reported when $p<0.05$. The means and standard deviation were presented.

RESULTS AND DISCUSSION

Hens exposed to 30 ppm NH_3 had higher H/L ratios compared to control hens ($p<0.05$, Table 2). In addition, compared to control hens, plasma IgM and C4 concentrations were reduced in the NH_3 exposed hens, respectively ($p<0.05$, Table 3).

There were no differences in plasma concentrations of ALB, AGP, IgG, IgA, C3, IL-1β, IFN-ϒ and TNF-α between the NH_3 exposed hens and control hens, respectively ($p>0.05$, Table 2-4).

Table 2: Measurements of heterophil/lymphocyte ratio and plasma concentrations of albumin, α-1-acid glycoprotein between ammonia exposure and control groups

Groups	H/L (%)	Albumin (mg mL^{-1})	AGP (μg mL^{-1})
Ammonia	0.30 ± 0.06^{A}	9.45±3.32	169.93±71.17
Control	0.19 ± 0.05^{B}	8.80±2.45	139.17±28.17
p-value	0.001	0.533	0.285

Mean±SD (n = 8), ^{AB}Means within columns with no common superscript differ significantly at $p<0.01$, AGP: α-1-acid glycoprotein, ALB: Albumin, H/L: Heterophil/Lymphocyte ratio

Table 3: Measurements of plasma concentrations of IgM, IgG, IgA, C3, C4 between ammonia exposure and control groups

Groups	IgM (μg mL^{-1})	IgG (mg mL^{-1})	IgA (μg mL^{-1})	C_3 (μg mL^{-1})	C_4 (ng mL^{-1})
Ammonia	276.71±64.97^{A}	4.61±1.59	232.74±82.88	58.44±21.65	1731.29±250.49^{b}
Control	373.67±69.76^{B}	5.55±1.85	236.59±51.17	64.47±23.78	2104.18±297.14^{a}
p-value	0.001	0.294	0.876	0.459	0.015

Mean±SD (n = 8), ^{AB}Means within columns with no common superscript differ significantly at p<0.01, C: Complement factor, Ig: Immunoglobulin

Table 4: Measurements of plasma concentrations of IL-1β, IFN-ϒ, TNF-α between ammonia exposure and control groups

Groups	IL-1β (pg mL^{-1})	IFN-ϒ (pg mL^{-1})	TNF-α (pg mL^{-1})
Ammonia	3.08±3.87	14.94±9.99	14.78±19.11
Control	5.28±3.88	23.40±12.59	27.86±40.93
p-value	0.119	0.160	0.273

Mean±SD (n = 8), IL-1β: Interleukin-1β, IL-6: Interleukin-6, IFN-γ: Interferon gamma and TNF-α: Tumor necrosis factor alpha

Table 5: Splenic cytokines IL-1β, IL-6 and TNF-α mRNA expression between ammonia exposure and control groups

Groups	IL-1β	IL-6	TNF-α
Ammonia	0.019±0.024	0.013±0.012	0.022±0.003
Control	0.012±0.005	0.009±0.002	0.022±0.005
p-value	0.453	0.387	0.314

Mean±SD (n = 8), IL-1β: Interleukin-1β, IL-6: Interleukin-6 and TNF-α: Tumor necrosis factor alpha, Data are expressed as relative abundance of the interested genes to the house keeping genes

Splenic cytokine IL-1β, IL-6 and TNF-α mRNA expressions also did not show any difference between NH_3 exposed hens and controls, respectively (p>0.05, Table 5).

High levels of atmospheric NH_3 have been associated with reduced chicken health and well-being, decreased feed intake, growth rate, production performance and overall livability[41]. The current results further indicated that exposure to 30 ppm NH_3 for 25 weeks is an environmental stressor to laying hens, as evidenced by increased H/L ratios and suppressed immunity as evidenced by reduced plasma IgM and C4 concentrations.

In the current study, hens exposed to NH_3 had significantly high H/L ratios compared with control hens. The results may indicate that hens exposed to NH_3 were under a stress state. Ratio of H/L has been used as an indicator for monitoring stress levels and immune functions in poultry[42,43]; especially assessing long-term stimulations[44]. Similarly, Gudev *et al.*[45] reported that H/L ratios were increased in laying hens reared under high ambient NH_3 (28-84 ppm). Mcfarlane and Curtis[46] also found, H/L ratios were increased in female Hubbard×Hubbard chicks exposed to various stressors including 125 ppm NH_3. The current and previous results indicated that stress-induced changes of leukocytes are less variable[46] and consistent with the hypothesis that H/L ratio is a reliable stress indicator[42].

Plasma albumin (ALB) levels were not affected by the NH_3 exposure, 30 ppm for 25 weeks in the current study. Acute Phase Proteins (APP), as biomarkers of the acute phase response of the innate immune system, are a class of proteins whose plasma concentrations increase (positive acute phase proteins, such as AGP) or decrease (negative acute phase proteins such as albumin) in response to inflammation, infection and trauma[18,36,47]. Similar results were also found in a previous study, in which no change of plasma albumin (ALB) concentrations was observed in hens exposed to 30 ppm NH_3 for 45 weeks[33]. Wei *et al.*[7] also reported broiler chickens exposed to 70 mg kg^{-1} NH_3 for 21 days had lower ALB than those exposed to 30 mg kg^{-1} NH_3 regardless of humidity (relative humidity 35, 60 and 85%). These results suggested that the NH_3 concentrations at 30 ppm or lower may not be high enough to cause hepatocyte damage to reduce protein synthesis. The hypothesis is supported by the previous findings that reduced serum ALB concentration in the NH_3 exposed broiler chickens is due to NH_3-impaired hepatocyte functions in protein synthesis and metabolism[15,48].

In the current study, no significant differences were observed in plasma AGP concentration between NH_3 exposed hens and control hens. As a major acute phase protein of poultry, the functions of AGP include inhibition of phagocytosis, neutrophil degranulation, respiratory burst and chemotaxis as well as monocyte apoptosis[49-51]. Monitoring AGP level has been used as a useful indicator of monitoring health and welfare in poultry including laying hens[52]. Shakeri *et al.*[53] reported that broiler chickens housed at high density (0.067 m^2 bird^{-1}) had greater serum AGP concentrations than those housed at lower density (0.1 m^2 bird^{-1}). In addition, organic broiler chickens had higher blood concentrations of AGP than conventionally housed broiler chickens, due to the increased risk for inflammation and infection in organic broiler chickens[54]. However, results from current experiment may indicated that birds have some degree of adaptation to the intermediate 25 weeks NH_3 exposure, resulting in a relative stability of the protein metabolic system[55].

Regarding to immunoglobulin levels, birds exposed to 30 ppm NH_3 for 25 weeks may have adverse effects on the immune system as that plasma concentrations of IgM but not IgG and IgA were significantly reduced in NH_3 exposed hens compared to control hens. The IgG, IgM and IgA have been used as indicators to evaluate the immune status of various animals including chickens due to their important roles in

immune functions, especially in humoral immunity[56]. These results are in partial agreement with previous findings that IgG, IgA and IgM levels were significantly reduced in hens exposed to 52 ppm but not 13 and 26 ppm NH_3 for 21 days[57]. The mechanisms of suppressed IgM expression but not IgG and IgA are unclear but it may be similar to the ones proposed in humans. The IgM isotype is the majority of natural antibodies[58]. Synthesis and release of IgM are the first affected by immunosuppression than other isotypes as that serum IgM is produced firstly in a primary response[58] and IgM memory B cells have the propensity to refined adaptation upon re-stimulation before becoming plasma cells but not activated memory IgG B cells[59].

In the current study, plasma C4 but not C3 levels were reduced in hens exposed to 30 ppm NH_3 for 25 weeks, which further evidenced that intermediate NH_3 exposure may have adverse effects on the immune system of laying hens. Reduced C4 levels were correlated with decreased blood levels of IgM. The complement system, as a component of non-specific immunity, serves a critical role in preventing infections, neoplastic transformation, autoimmune diseases and restoring injured tissues following stress[60,61]. The C3 and C4, two major functional factors, are capable to covalent tagging of the foreign molecules, resulting in phagocytosis of phagocytes and or cytolysis through binding to the complement receptors[62]. These results are different from the results reported in previous study, which observed neither C3 nor C4 levels were affected in hens exposed to 30 ppm NH_3 for 45 weeks[33]. The current and previous results may suggest that hens' reaction to NH_3 stimuli is depended on the duration of exposure and hens have capability to adapt long-term NH_3 exposure, 45 weeks vs. 25 weeks. The reasons for decreasing plasma C4 levels are unclear but may be related to NH_3-induced decrease of IgM as seen in the current study. Previous studies have evidenced that IgM is more efficient to activate the complement system than IgG[56].

In the current study, the plasma levels of IL-1β, TNF-α and IFN-γ as well as splenic mRNA expressions of IL-1β, IL-6 and TNF-α were not affected by NH_3 exposure. Cytokines including IL-1β, IL-6, IFN-γ and TNF-α provide major roles in the innate immune response to inflammation and infection[63-66]. In adult chickens, the major immunological organ is the spleen[67] as well as the thymus and bursa of Fabricius when they were young[68]. Resident macrophages in the spleen release pro-inflammatory cytokines following various injuries and stimulations[69]. Partially agreed with the current findings, Wei *et al.*[7] reported that exposure to a high level of NH_3 (70 ppm) for 21 days increased the IL-1β gene expression in the spleen and suppressed the immunity of broiler chickens compared with a relatively lower level of NH_3 (30 ppm). These results were in agreement with the hypotheses that NH_3 effect is dependent on exposure concentration and duration as well as species of animals (laying hens vs. broilers) and animals' environments (cages inside chambers vs. floor pens). In addition, Timmer *et al.*[70] revealed that a long period of exposure to a low concentration of NH_3 is not to cause long-term health problems in humans. Similar to the findings in humans, the naturally produced NH_3 may not accumulate inside the chicken body. In the current study, no treatment differences in cytokine levels between NH_3 exposed hens and control hens may be also due to the relatively low NH_3 level, intermediate exposure time and or limited sample size.

CONCLUSION

In conclusion, hens exposed to NH_3 had higher H/L ratios and lower levels of plasma IgM and C4 compared to control hens. These results indicated that NH_3 at 30 ppm is an environmental stressor to hens under the current condition, causing a stress state and suppressing immunity in laying hens.

SIGNIFICANT STATEMENTS

Ammonia is one of the most prominent aerial pollutants inside poultry production facilities, affecting chicken health and well-being based on its levels and exposure durations. The study aimed at investigating the effect of 30 ppm ammonia on the immune response of laying hens. Unlike previous studies, this study evaluated the cellular mechanisms for the ammonia effect on laying hens. The data showed that ammonia exposed hens had higher heterophil to lymphocyte ratios, a stress indicator and lower plasma antibody concentrations compared to control hens. These results suggest that ammonia exposure at 30 ppm for 25 weeks increases stress state and suppresses immunity in laying hens. However, further studies could be conducted to evaluate the effect of ammonia on laying hens with different levels and exposure periods. The current findings could be used by producers and scientists for conducting further investigation and developing management guidelines for improving hen housing and well-being.

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