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Ammonia concentration and relative humidity in poultry houses affect the immune response of broilers

F.X. Wei[1,2], X.F. Hu[3], B. Xu[2], M.H. Zhang[1], S.Y. Li[2], Q.Y. Sun[2] and P. Lin[2]

[1]State Key Laboratory of Animal Nutrition, Institute of Animal Sciences, The Chinese Academy of Agricultural Sciences, Beijing, China
[2]Institute of Animal Husbandry and Veterinary Science, Henan Academy of Agricultural Sciences, Zhengzhou, China
[3]Henan Key Lab of Animal Immunology, Henan Academy of Agricultural Science, Henan, Zhengzhou, China

Corresponding authors: M.H. Zhang / S.Y. Li
E-mail: zmh66@126.com / lsy9617@aliyun.com

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ABSTRACT. To investigate the effect of ammonia (NH_3) and humidity on the immune response of broilers, broilers were exposed to 30 or 70 mg/kg atmospheric NH_3 for 21 days. Additionally, birds were exposed to 35, 60, and 85% relative humidity (RH). The relative weights of lymphoid organs, serum total protein, serum globulin, serum albumin, serum lysozyme, proliferation index of peripheral blood lymphocytes, and splenic cytokine gene expression were determined. Exposure to 70 mg/kg NH_3 decreased the relative weight of the spleen during the experimental period, serum lysozyme concentration in the first and second weeks, and serum globulin concentration in the third week. The proliferation of peripheral blood lymphocytes was reduced. High levels of NH_3 caused increase in *IL-1β* gene expression in the experimental period and *IL-4* gene expression in the first week. Birds exposed to

85% RH had lower thymus and bursa of Fabricius weights in the third week and serum lysozyme concentration in the first week; *IL-1β* and *IL-4* expressions were higher in the second and third weeks and first and second weeks, respectively, than in birds exposed to 60% RH. *IL-4* expression was lower during the first week, and *IL-1β* expression was higher during the second week with 35% RH than with 60% RH. In conclusion, high NH_3 level in the poultry house suppressed the immune response of broiler chickens. Neither high nor low RH benefited the immune response of broilers. Furthermore, there was an interactive effect between NH_3 and RH on the immune response of broilers.

Key words: Broiler; Ammonia concentration; Relative humidity; Immune response

INTRODUCTION

During their lifetime, broilers encounter a number of stressors, such as the temperature (Quinteiro-Filho et al., 2012), relative humidity (Yahav et al., 1995), stocking density (Simitzis et al., 2012), immunological challenge (Takahashi et al., 2000), handling (Zulkifli et al., 2000), and airborne contaminants (Do et al., 2005). Of the airborne contaminants, ammonia (NH_3) is considered to be the most harmful gas released from broiler litter (Carlie, 1984) and is considered detrimental in the modern poultry house (Beker et al., 2004; Miles et al., 2004). Exposure to an excessive amount of NH_3 decreases the growth performance of animals (Beker et al., 2004; Miles et al., 2004) and compromises productivity (Deaton et al., 1982; Miles et al., 2004). Additionally, it results in tracheal and pulmonary lesions (Beker et al., 2004), impairs meat and egg quality (Benton and Brake, 2000), and increases animal mortality and morbidity (Beker et al., 2004; Miles, 2004). NH_3 in poultry houses also affects immunological function, reducing specific antibody titers (Caveny et al., 1981) and increasing disease susceptibility (Beker et al., 2004). Moreover, the effects of NH_3 and other simultaneous stressors are generally additive (McFarlane et al., 1989).

Research has shown that stress delays lymphoid organ development and alters the expression and translation of inflammatory cytokine genes (Zhang et al., 2011). However, there is little documentation on the effects of NH_3 exposure or/and relative humidity (RH), two common stressors, on the development of the immune system of broiler chickens. Therefore, we suggest that NH_3 exposure may delay the development of lymphoid organs and change the gene expression of inflammatory cytokines, and that these effects may show an interaction with the RH in the broiler house. We tested the effect of the level of atmospheric NH_3 at different levels of RH on the immune system of broiler chickens.

MATERIAL AND METHODS

Bird husbandry and treatment

Two hundred and eighty-eight 21-day-old Arbor Acres male broiler chickens with close body weights were allotted into 36 equal pens (8 birds/pen) so that the differences in the birds' mean weight for each pen was insignificant. The birds were allotted into 6 equal

groups of 6 pens with each pen acting as a replicate unit. The basal diet contained 19.5% crude protein, 0.90% calcium, 0.40% available P, and 3.00 Mcal/kg metabolizable energy on a calculated basis. Birds were kept in programmable artificial climate chambers, with a computer-controlled environment (variability-temperature ±1.0°C, RH ±2.5% and NH_3 ±1 mg/kg). Six treatment chambers, holding 6 pens each containing 8 birds, were employed.

The experiment consisted of a 2 x 3-factorial design with 2 between-subject factors. The poultry houses contain 2 levels of NH_3 concentration, 30 and 70 mg/kg, and 3 levels of RH, 35, 60, and 85% RH. Thus, there were 6 groups: 30 mg/kg NH_3 + 35% RH; 30 mg/kg NH_3 + 60% RH; 30 mg/kg NH_3 + 85% RH; 70 mg/kg NH_3 + 35% RH; 70 mg/kg NH_3 + 60% RH; and 70 mg/kg NH_3 + 85% RH. Anhydrous NH_3 was metered continuously in the chambers and maintained at 30 or 70 mg/kg NH_3, which was determined daily along with the humidity. During the study, ambient temperature was maintained at 26°C. All birds were allowed to consume mashed feed and tap water *ad libitum*, and 24 h of artificial light was supplied. The experimental period was 21 days, covering 22 to 42 days of age. The experiment was carried out at the State Key Laboratory of Animal Nutrition in Beijing.

Sampling

At 28, 35, and 42 days of age, 6 healthy birds in each group (1 bird/pen) were chosen randomly and weighed. Blood samples were withdrawn from a wing vein and centrifuged at 3000 *g* for 10 min at 4°C, and the serum was stored at -80°C for further assay. A second blood sample was collected in a 5-mL heparinized tube to determine the peripheral blood lymphocyte proliferation. Then, these birds were slaughtered, and the thymus, bursa of Fabricius (BF), and spleen were collected, and their wet weights and relative weights were recorded. The spleens were washed with 0.1% diethylpyrocarbonate water, wrapped in sterile and RNase-free foil, rapidly frozen in liquid nitrogen, and stored at -80°C for further analysis.

Analysis

The relative weights of the lymphoid organs were expressed as lymphoid organ wet weight (g) divided by body weight (kg).

Serum albumin was determined using a kit (Jiancheng, Nanjing, China). Twenty microliters of serum sample was added to 5 mL bromocresol green colorimetry solution. Ten minutes later, the optical density (OD) of the solutions was measured at 628 nm. Serum total protein was assayed with a kit (Jiancheng, Nanjing, China). Fifty microliters serum, diluted 1:50 with saline, was added to 3 mL Coomassie brilliant blue solution. The ODs of the mixtures were read at 595 nm after 10 min. Serum globulin was calculated as total protein minus albumin. Serum lysozyme activity was detected using a kit (Jiancheng). Five milligrams bacterium powder dissolved in 1 mL bacterium diluent was carefully homogenized and then diluted to 20 mL in the same diluent. After incubation for 15 min at 37°C followed by 3 min at 0°C, the turbidity at 560 nm was determined.

A 3-[4,5-dimethylthiazol]-2,5-diphenyltetrazolium bromide (MTT) assay was used to determine the peripheral blood lymphocyte proliferation response. The heparinized blood samples were added to isotonic lymphocyte separation medium (density = 1.077; HaoYang, Tianjin, China). Lymphocytes were isolated after a 30-min centrifugation at 1006 *g* at 4°C. The lymphocyte fraction was collected from the interface and washed 3 times with RPMI

1640 (Invitrogen, Grand Island, NY, USA) incomplete culture medium. Lymphocytes were then resuspended in 2 mL RPMI 1640 complete culture medium supplemented with 5% (v/v) fetal calf serum, 0.5% penicillin (final concentration, 100 U/mL), 0.5% streptomycin (final concentration, 100 µg/mL), and 1% N-(2-hydroxyethyl)-piperazine-N-2-ethane-sulfonic acid (HEPES, final concentration, 24 mM; Amresco 0511, Amresco, Cleveland, OH, USA). Live cells were counted by trypan blue dye exclusion, and the density of the cells was adjusted to 1 x 10^7 cells/mL culture medium.

One hundred microliters cell suspension, and the lymphocyte mitogen concanavalin A (Con A; Sigma, St. Louis, MO, USA) (final concentration 45 µg/mL) or lipopolysaccharide (LPS; Sigma) (final concentration 25 µg/mL) was added to a 96-well microtiter plate (Costar 3599, Corning, NY, USA). Cells were then incubated (MCO-18AIC CO_2 incubator; Sanyo, Tokyo, Japan) at 37°C with 5% CO_2. Sixty-eight hours later, 15 µL 5 mg/mL MTT was added to each well, followed by a further 4-h incubation. Subsequently, 100 µL 10% sodium dodecyl sulfate dissolved in 0.04 M HCl solution was added to each well to lyse the cells and solubilize the MTT crystals; finally, the plates were read using an automated ELISA reader (model 550 Microplate Reader, Bio-Rad Pacific, Hong Kong, China) at 570 nm.

The relative abundance of *IL-1β* and *IL-4* mRNAs was determined by quantitative real-time polymerase chain reaction (PCR). The frozen spleen was mashed in a sterile mortar, and the powder was used for total RNA extraction employing a kit (Takara, Dalian, China). The integrity of the RNA was verified by the OD absorption ratio $2.0 > OD_{260}/OD_{280} > 1.9$ and further by electrophoresis on a 1.0% (w/v) ethidium bromide agarose formaldehyde gel.

Reverse transcription was carried out using a kit (Takara). First-strand cDNAs were synthesized from 1 µg total RNA using oligo (dT) as primers in the presence of MML-V reverse transcriptase for 5 min at 20°C, 60 min at 42°C, and 5 min at 70°C.

PCRs were performed in a volume of 20 µL containing 10 µL 2X SYBR Green PCR Master Mix containing Taq DNA polymerase (Applied Biosystems, Foster City, CA, USA), 2 µL cDNA product, reaction buffer, dNTPs, 1 µL forward primer, and 1 µL reverse primer. The primer sequences used for the cytokine genes and the housekeeping gene β-actin are shown in Table 1. A standard curve was made as described by Hu et al. (2010). With the determined cycle threshold value and the standard curve, the relative original concentration of target gene and β-actin was obtained. The target gene relative abundance was expressed as the relative original copy of target gene/the relative original copy of β-actin. All PCR analyses were performed in triplicate.

Table 1. Gene-specific oligonucleotide primer pairs used for quantitative real-time polymerase chain reaction.

Name	Oligo	Primer sequence	Predicted size (bp)	GenBank accession No.
IL-1β	Forward primer	5'-GGGACTTTGCTGACAGCGACCTG-3'	128	NM_204524.1
	Reverse primer	5'-GTCGAAGGACTGTGAGCGGGTGT-3'		
IL-4	Forward primer	5'-GGAGAGGTTTCCTGCGTCAAGAT-3'	183	NC_006100
	Reverse primer	5'-TTCAGGAGCTGACGCATGTTGAG-3'		

Statistical analysis

Data were analyzed by analysis of variance (ANOVA) with NH_3 and RH in the poultry house as main effects and interactions using the generalized linear model procedure (SPSS 13.0 software for Windows, SPSS Inc., Chicago, IL, USA). One-way ANOVA was also em-

ployed to analyze the differences between the 6 groups. Differences between mean values were considered to be statistically significant at $P < 0.05$.

RESULTS

Effect of NH_3 and RH on lymphoid organs of broilers

As shown in Table 2, exposure to high NH_3 concentrations produced a borderline decrease in the relative weight of the spleen on 28 ($P = 0.065$), 35 ($P = 0.066$), and 42 days ($P = 0.053$). The relative weights of the thymus and BF were unaffected by the NH_3 concentration over the period of the experiment.

Table 2. Relative weight of lymphoid organs of broilers (N = 6).

Treatment		First week			Second week			Third week		
		Thymus	Spleen	BF	Thymus	Spleen	BF	Thymus	Spleen	BF
30 mg/kg + 35%		0.45	0.12	0.25	0.55	0.11	0.24	0.43^{ab}	0.10^{ab}	0.16^{ab}
30 mg/kg + 60%		0.47	0.11	0.20	0.52	0.12	0.21	0.47^{a}	0.12^{a}	0.17^{a}
30 mg/kg + 85%		0.44	0.10	0.23	0.47	0.12	0.22	0.36^{ab}	0.10^{ab}	0.15^{ab}
70 mg/kg + 35%		0.40	0.10	0.20	0.44	0.10	0.21	0.43^{ab}	0.09^{ab}	0.16^{ab}
70 mg/kg + 60%		0.44	0.08	0.22	0.50	0.10	0.21	0.42^{ab}	0.11^{ab}	0.16^{ab}
70 mg/kg + 85%		0.42	0.09	0.22	0.41	0.10	0.16	0.34^{b}	0.09^{b}	0.13^{b}
SE		0.015	0.004	0.009	0.022	0.005	0.009	0.016	0.004	0.005
Ammonia	30 mg/kg	0.45	0.11	0.23	0.51	0.12	0.22	0.42	0.11	0.16
	70 mg/kg	0.42	0.09	0.21	0.45	0.10	0.19	0.40	0.09	0.15
Humidity	35%	0.42	0.11	0.23	0.49	0.10	0.22	0.43^{a}	0.10	0.16^{ab}
	60%	0.45	0.10	0.21	0.51	0.11	0.21	0.45^{a}	0.11	0.17^{b}
	85%	0.43	0.10	0.23	0.44	0.11	0.19	0.35^{b}	0.09	0.14^{a}
	Ammonia level	0.275	0.065	0.438	0.182	0.066	0.126	0.591	0.053	0.439
P value	Humidity	0.723	0.276	0.552	0.388	0.865	0.322	0.040	0.142	0.080
	Interaction	0.901	0.799	0.314	0.694	0.970	0.444	0.781	0.883	0.818

BF, bursa of Fabricius. Different superscript letters in the same column indicate significant differences ($P < 0.05$).

The RH had no significant effect on the lymphoid organ relative weight in the first and second weeks ($P > 0.05$). Birds exposed to 85% RH for 3 weeks had lower relative weights of thymus ($P < 0.05$) and BF ($P = 0.080$) compared to those exposed to 60% RH, whereas changes in the spleen were slight and insignificant ($P > 0.05$) on the third week. Birds exposed to 70 mg/kg NH_3 + 85% RH had lower relative weights of thymus, spleen, and BF compared with those in 30 mg/kg NH_3 + 60% RH by the third week ($P < 0.05$).

Effect of NH_3 and RH on immunological measures of broilers

The results in Table 3 show that NH_3 and RH had no effect on serum total protein and albumin ($P > 0.05$). Moreover, RH had no effect on globulin ($P > 0.05$). In the first and second weeks, NH_3 had no effect on globulin ($P > 0.05$), but in the third week, birds that were exposed to 70 mg/kg NH_3 had a lower globulin concentration than those exposed to 30 mg/kg NH_3 ($P < 0.05$). Birds in 70 mg/kg NH_3 + 35% RH had lower albumin than those in 30 mg/kg NH_3 + 60% RH ($P < 0.05$) during the first week. Birds in 70 mg/kg NH_3 + 85% RH had lower albumin than those in 30 mg/kg NH_3 + 60% RH ($P < 0.05$) during the third week. Birds in 70 mg/kg NH_3 + 35% RH and 70 mg/kg NH_3 + 85% RH had lower albumin than those in 30 mg/

kg NH_3 + 60% RH ($P < 0.05$) during the third week. Birds exposed to 70 mg/kg NH_3 had a lower lysozyme concentration than those exposed to 30 mg/kg NH_3 ($P < 0.05$) during the first and second weeks. Birds exposed to 85% RH had a lower lysozyme concentration than those exposed to 35% RH or 60% RH ($P < 0.05$) during the first week. Birds in the 30 mg/kg NH_3 + 60% RH group had a higher lysozyme concentration than those in the 70 mg/kg NH_3 + 35% RH or 70 mg/kg NH_3 + 85% RH groups ($P < 0.05$) during the first week.

Table 3. Serum total protein (TP), albumin, globulin, and lysozyme concentrations of broilers (N = 6).

Treatment		First week				Second week				Third week			
		Albumin (mg/mL)	TP (mg/mL)	Globulin (mg/mL)	Lysozyme (μg/mL)	Albumin (mg/mL)	TP (mg/mL)	Globulin (mg/mL)	Lysozyme (μg/mL)	Albumin (mg/mL)	TP (mg/mL)	Globulin (mg/mL)	Lysozyme (μg/mL)
30 mg/kg + 35%		12.98^{ab}	29.13	16.15	4.42^{a}	17.47	38.11	20.65	2.94	22.39	31.94^{ab}	9.44^{b}	2.76
30 mg/kg + 60%		14.55^{b}	31.42	16.87	4.37^{a}	17.40	37.61	20.21	2.59	25.61	35.18^{a}	9.57^{b}	2.60
30 mg/kg + 85%		13.54^{ab}	30.31	16.76	3.46^{b}	15.35	35.93	20.58	2.99	24.30	30.86^{ab}	9.05^{b}	2.21
70 mg/kg + 35%		12.43^{a}	28.49	15.68	3.25^{b}	17.85	32.48	17.62	2.49	25.01	30.85^{ab}	5.84^{a}	2.35
70 mg/kg + 60%		13.26^{ab}	29.34	16.08	2.98^{bc}	16.67	35.53	18.85	2.39	25.02	32.07^{ab}	7.05^{ab}	2.30
70 mg/kg + 85%		13.91^{ab}	28.67	14.76	2.52^{c}	18.96	35.20	16.24	2.32	22.50	28.83^{b}	6.32^{a}	2.29
SE		0.263	0.581	0.481	0.083	0.873	0.848	0.909	0.057	0.679	0.734	0.342	0.077
Ammonia	30 mg/kg	13.69	30.28	16.59	4.08	16.74	37.22	20.48	2.84	24.01	32.54	9.35	2.52
	70 mg/kg	13.20	28.86	15.50	2.92	17.83	34.40	17.57	2.40	24.18	30.58	6.40	2.31
Humidity	35%	12.70	28.84	15.93	3.83^{b}	17.66	35.30	19.27	2.72	23.70	31.34	7.47	2.55
	60%	13.90	30.38	16.48	3.67^{b}	17.04	36.57	19.53	2.49	25.29	33.48	8.20	2.45
	85%	13.73	29.49	15.76	2.99^{a}	17.16	35.57	18.41	2.66	23.40	29.84	7.56	2.25
P value	Ammonia	0.357	0.222	0.268	<0.001	0.538	0.107	0.121	<0.001	0.953	0.169	<0.001	0.183
	Humidity	0.151	0.553	0.812	0.001	0.953	0.813	0.874	0.247	0.484	0.123	0.676	0.276
	Interaction	0.446	0.878	0.791	0.548	0.579	0.484	0.793	0.249	0.390	0.859	0.790	0.406

Different superscript letters in the same column indicate significant differences ($P < 0.05$).

As shown in Table 4, RH did not significantly influence the proliferation of peripheral blood lymphocyte in response to Con A or LPS ($P > 0.05$). It appeared that the proliferation index was significantly linked to NH_3 concentration (Table 4), but humidity showed no consistent interaction. The effect of exposure to high levels of NH_3 remained constant over the 3 weeks of the experiment.

Table 4. Proliferation of peripheral blood lymphocytes of broilers [stimulus index (SI); N = 6].

Treatment		First week		Second week		Third week	
		SI (Con A)	SI (LPS)	SI (Con A)	SI (LPS)	SI (Con A)	SI (LPS)
30 mg/kg + 35%		0.99	0.98^{b}	1.03	1.08	1.02^{b}	0.98
30 mg/kg + 60%		1.00	0.98^{b}	1.02	1.05	1.01^{b}	1.00
30 mg/kg + 85%		0.99	0.96^{ab}	1.00	1.01	1.01^{b}	0.95
70 mg/kg + 35%		0.94	0.90^{a}	0.93	0.96	0.90^{a}	0.91
70 mg/kg + 60%		0.96	0.93^{ab}	0.98	0.98	0.95^{ab}	0.94
70 mg/kg + 85%		0.93	0.92^{ab}	0.91	0.95	0.90^{a}	0.92
SE		0.009	0.009	0.019	0.018	0.012	0.012
Ammonia	30 mg/kg	0.99	0.97	1.02	1.04	1.01	0.98
	70 mg/kg	0.95	0.91	0.94	0.96	0.92	0.92
Humidity	35%	0.97	0.94	0.98	1.02	0.96	0.95
	60%	0.98	0.95	1.00	1.01	0.98	0.97
	85%	0.96	0.94	0.95	0.98	0.95	0.93
	Ammonia	0.011	0.001	0.038	0.025	<0.001	0.026
P value	Humidity	0.637	0.658	0.624	0.641	0.601	0.486
	Interaction	0.960	0.693	0.801	0.714	0.629	0.786

Con A = concanavalin A; LPS = lipopolysaccharide. Different superscript letters in the same column indicate significant differences ($P < 0.05$).

Effect of NH_3 and RH on cytokine gene expression of broilers

The relative mRNA abundance of *IL-1β* and *IL-4* in spleen of broilers is shown in Table 5. The results indicate that both NH_3 concentration and RH have effects on the expression of cytokine mRNA in the spleen of birds. Birds exposed to 70 mg/kg NH_3 had a higher mRNA abundance of IL-1β in the first, second, and third weeks than those exposed to 30 mg/kg NH_3 ($P < 0.05$). The relative mRNA abundance of IL-4 also had the same change as *IL-1β* in the first week ($P < 0.05$). In the second week, birds treated with 60% RH had a lower mRNA abundance of *IL-1β* than those treated with 35 or 85% RH ($P < 0.05$). In the third week, birds treated with 35% RH had a higher mRNA abundance of IL-1β than those treated with 85% RH ($P < 0.05$). In the first week, birds treated with 60% RH had a lower mRNA abundance of IL-4 than those treated with 85% RH and a higher abundance than those treated with 35% RH ($P < 0.05$). In the second week, birds treated with 85% RH had a higher mRNA abundance of IL-4 than those treated with 35 and 60% RH ($P < 0.05$). In the first week, birds in the 30 mg/kg NH_3 + 60% RH group had a lower mRNA abundance of *IL-1β* and *IL-4* than those in the 70 mg/kg NH_3 + 35% RH and 70 mg/kg NH_3 + 85% RH groups ($P < 0.05$), as well as *IL-1β* in the second week ($P < 0.05$). In the third week, birds in the 30 mg/kg NH_3 + 60% RH group had a lower mRNA abundance of *IL-1β* than those in the 70 mg/kg NH_3 + 85% RH group ($P < 0.05$).

Table 5. Relative mRNA abundance of cytokines in spleen of broilers.

Treatment		First week		Second week		Third week	
		IL-1β	*IL-4*	*IL-1β*	*IL-4*	*IL-1β*	*IL-4*
30 mg/kg + 35%		0.03^{a}	0.27^{a}	0.37^{a}	2.57	0.91^{a}	0.04
30 mg/kg + 60%		0.03^{a}	0.60^{a}	0.36^{a}	2.76	1.42^{ab}	0.05
30 mg/kg + 85%		0.06^{b}	0.75^{ab}	0.66^{a}	3.28	1.78^{abc}	0.06
70 mg/kg + 35%		0.07^{b}	1.21^{b}	1.15^{b}	2.15	2.07^{bc}	0.04
70 mg/kg + 60%		0.06^{b}	2.16^{c}	0.69^{a}	2.66	2.69^{cd}	0.08
70 mg/kg + 85%		0.06^{b}	2.54^{c}	1.29^{b}	3.75	3.23^{d}	0.04
SE		0.003	0.071	0.043	0.150	0.130	0.004
Ammonia	30 mg/kg	0.04	0.55	0.46	2.87	1.37	0.05
	70 mg/kg	0.06	1.98	1.05	2.85	2.66	0.05
Humidity	35%	0.05	0.75^{a}	0.76^{a}	2.36^{a}	1.49^{a}	0.04
	60%	0.04	1.38^{b}	0.53^{b}	2.71^{a}	2.05^{ab}	0.06
	85%	0.06	1.65^{c}	0.98^{a}	3.51^{b}	2.50^{b}	0.05
P value	Ammonia	<0.01	<0.01	<0.01	0.956	<0.01	0.770
	Humidity	0.167	<0.01	<0.01	0.016	0.018	0.104
	Interaction	0.065	0.067	0.130	0.490	0.908	0.420

Different superscript letters in the same column indicate significant differences ($P < 0.05$).

DISCUSSION

It was reported that stress resulted in lymphoid organ (thymus, spleen, and BF) atrophy (Selye, 1998). Some of these effects could only be partly reproduced in this study of broiler chickens, in which 70 mg/kg NH_3 in a poultry house decreased the relative weights of the spleen, but we did not find differences in the relative weights of the thymus and BF between the 70 and 30 mg/kg NH_3 groups during the first, second, and third weeks of NH_3 exposure. Moreover, in this study, RH had no effect on the relative weight of the spleen; 85% RH decreased the relative weights of the thymus and BF in the third week of exposure. Previous reports also had inconsistent results about the effect of stress on the thymus, spleen, and BF

(Quinteiro-Filho et al., 2010, 2012; Li et al., 2012). Quinteiro-Filho et al. (2010) showed that long-term (7 days) heat stress decreased the relative weights of the thymus, spleen, and BF, and Quinteiro-Filho et al. (2012) also found that acute (10 h) heat stress had no effect on the relative weights of the same lymphoid organs. However, mycotoxin-induced stress increased the relative weight of the thymus and BF Li et al. (2012). These different results may be due to the type of stressor, its duration and frequency, and the temporal relationship between the stress application and the immune system evaluation (Avitsur et al., 2003; Bartolomucci et al., 2005; Queiroz et al., 2008). In this study, we also found that a high level of NH_3 and high RH had an additive effect on the relative weight of the thymus, spleen, and BF of broiler chickens after long-term exposure (3 weeks).

In this study, a high level of NH_3 decreased the serum globulin concentration on the third week, which is partly in agreement with the results of previous research (Javed et al., 1995; Stanley et al., 2004; Gao et al., 2009; Andretta et al., 2012; Li et al., 2012). Moreover, in this study, NH_3 and RH had an additive detrimental effect on albumin, total protein, and globulin. Lysozyme is mainly secreted by phagocytes and is a nonspecific immune effector. In this study, a high level of NH_3 decreased serum lysozyme in the first and second weeks, and 85% RH had a similar effect in the first week. NH_3 and RH also had an additive detrimental effect on lysozyme. The decreased lysozyme levels in stressed birds suggest that phagocytes were delayed with the stressor of NH_3 and RH. Therefore, stresses delay the nonspecific immunity of birds at some level. The lowered serum albumin, total protein, globulin, and lysozyme concentration may be due to damage in hepatocytes and impaired functions, such as amino acid transport and protein synthesis, in stressed broilers (Meissonnier et al., 2005; Faixová et al., 2007).

Antigen- or nonspecific mitogen-induced proliferation of peripheral blood lymphocytes is a good indicator of the initiation of an immune response, which ultimately determines host resistance to disease. Antigen-specific blastogenic assays and lymphocyte proliferation tests provoked by nonspecific mitogens have been used extensively to assess the immunocompetence of poultry (Baxi and Oberoi, 1999; Vickery et al., 1999). In this study, a high level of NH_3 decreased the proliferation of peripheral blood lymphocytes. Moreover, NH_3 and RH in a poultry house had an additive detrimental effect on the proliferation of lymphocytes. It was reported that other types of stressors also decreased the proliferation of lymphocytes (Rafai et al., 2000; Swamy et al., 2004; Long et al., 2011). In chickens, it is well known that stress status is associated with increased plasma corticosterone concentrations because corticosterone is the end product of the hypothalamic-pituitary-adrenal (HPA) axis. It has also been established that corticosterone is responsible for many quantitative and qualitative changes in immune function (Shini et al., 2010). Corticosterone inhibits several immune system functions in various species, including lymphocyte proliferation and immunoglobulin production (Munck et al., 1984).

Stress influences the immune response of humans and animals (Lawrence and Kim, 2000; Padgett and Glaser, 2003). Stress activates the HPA axis and the sympathetic-adrenal-medullary axis, resulting in chronic production of glucocorticoid hormones and catecholamines, which, in turn, alters the gene expression of a variety of cytokines by a complicated physiological process (Padgett and Glaser, 2003). In this study, we also found that a high level of NH_3 or/and RH in the poultry house increased the expression of the *IL-1β* and *IL-4* genes. It was reported that other types of stressors also could increase the serum level of *IL-1β* (Dugué et al., 1993; Mekaouche et al., 1994) and the expression of the *IL-1β* (Bhalla et al., 2002; Shini et al., 2010; Li et al., 2012) and *IL-4* genes (Wu et al., 2001). Stress stimulated the HPA to

release corticosterone, and the elevated corticosterone in blood upregulated the expression of cytokine genes in lymphocytes (Shini et al., 2010).

In conclusion, a high level of NH_3 and unsuitable ambient RH in a poultry house delayed lymphoid organ development and the proliferation of peripheral blood lymphocytes, and it decreased the blood lysozyme activities and increased the expression of cytokine genes, such as *IL-1β* and *IL-4*, of broiler chickens. Moreover, NH_3 and RH had an additive detrimental effect on these parameters.

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