

Proper cold stimulation starting at an earlier age can enhance immunity and improve adaptability to cold stress in broilers

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ABSTRACT The effects of long-term cold stimulation on the immune function of ileum and adaptability to cold stress in broilers were examined. A total of 360 Arbor Acres broilers was divided into 3 groups and four replicates per group. C (control) was reared in normal thermal environment. C-3 and C-12 (treatments) were kept in cold condition of 3 or 12°C lower than the temperature of C from days 8 to 42. At day 42, all the groups were exposed to an acute cold stress challenge, designated as S, S-3, and S-12. The mRNA levels of immune molecules and heat shock proteins as well as oxidative stress-related indicators in ileum tissues, and immunoglobulins contents in serum were examined at 14, 42, and 43 d of age. The C-3 regimen had no ad-

verse effect on production performance, whereas the C-12 regimen reduced the production performance relative to C ($P < 0.05$). At day 42, C-3 had higher levels of immune indexes ($P < 0.05$), whereas C-12 had lower levels than C ($P < 0.05$). No differences in levels of oxidative stress-related indicators were found between C and C-3 at day 42 ($P > 0.05$). S-3 had higher levels of immune indexes and lower levels of oxidative stress-related indicators ($P < 0.05$), as compared to S and S-12. The results suggest that 34 d of cold stimulation at 3°C lower than the normal temperature had no adverse impacts on production performance but enhanced the immunity of ileum and adaptability to acute cold challenge in broilers.

Key words: cold stimulation, broiler ileum, immunity, performance, adaptability

2020 Poultry Science 99:129–141
<http://dx.doi.org/10.3382/ps/pez570>

INTRODUCTION

Cold stress can impose adverse impacts on the health and productivity of poultry. Cold stress normally increases feed intake to maintain thermal homeostasis (Mendes et al., 1997) and leads to lower daily gain of broilers (Hangalapura et al., 2003). In addition, cold stress also can induce diseases such as ascites and increase mortality (Shlosberg et al., 1996). “Acclimation” refers to physiological changes of an organism, which enhances tolerance to stressful components of the environment (Bernabucci et al., 2010). For example, broiler chicks of 3 to 4 d old became more tolerant to low temperature environment after adapting to a cold environment of 15°C for several hours (Shinder et al., 2002). Acclimation to high temperatures in a short-term enhanced resistance of broilers to heat stress, and acclimated broilers had significantly lower fatality caused by heat stress (May et al., 1987). The degree of gastric

mucosal injury of rats that had adapted to chronic mild restraint stress for 2 to 10 d was remarkably lower than that of the control counterparts when they were simultaneously exposed to cold-restraint stress (Wallace et al., 1983). It is generally believed that cold acclimation can improve cold tolerance, reduce cold damage, and accelerate temperature recovery of the body in cold environments (Morabito et al., 2014).

Intestinal immune function is closely related to the health of body. Immunoglobulin is an important component of the immune system and plays an important role in the process of immune regulation and mucosal defense (Zhao et al., 2013). Antibacterial peptides are essential peptides for maintaining intestinal barrier function and immune homeostasis (Otte and Vordenbäumen, 2011), and they can effectively fight against a variety of pathogens (Brogden et al., 2003). Many clinical studies have proven that low levels of immune molecules (such as immunoglobulins and antimicrobial peptides) are closely related to occurrence of various intestinal diseases. For instance, mRNA levels of immunoglobulins (including IgA, IgM, and IgY) were found to decrease in ileum of broilers with colitis (Simon et al., 2016). The lack of secretory IgA in

Received March 8, 2019.

Accepted September 28, 2019.

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intestine leads to gastrointestinal mucositis and necrotizing colitis (Corthésy, 2013). Impaired production of IgA and defensins can cause inflammatory bowel diseases such as Crohn's disease (Otte and Vordenbäumen, 2011). In contrast, high levels of antimicrobial peptides in animals can enhance disease resistance. For example, chickens with higher levels of antimicrobial peptide expression in intestine showed higher resistance to *Salmonella enteritis* (Sunkara et al., 2011). Inducible nitric oxide synthase (iNOS) is abundantly expressed under injuries and pathological conditions. iNOS can continuously release large amounts of nitric oxide (NO) and damage the intestinal mucosa. NO participates in immune responses of the organism by negative feedback regulation of cytokines (Karpuzoglu and Ahmed, 2006). For instance, IFN- γ is an effective potent inducer of NO, while high levels of NO in turn down-regulates the content of IFN- γ (Taylor-Robinson, 1997). Studies have shown that heat shock proteins (HSPs) are some highly conserved molecular chaperones that participate in immune regulation of the body by triggering T cell regulatory functions and inhibiting the expression of cytokines such as IL-1 β , IL-6, and TNF- α (Srivastava, 2002), playing an important role in maintaining intestinal homeostasis (Rakoff-Nahoum et al., 2004). As typical injury markers, HSPs are present in low levels under normal physiological state and can be produced in large quantities under various stressful conditions to protect cells from damages (Zhao et al., 2016). Heat stress can damage intestinal epithelial cells and result in intestinal immunosuppression (Abdelqader et al., 2017). The research of Zhao et al. (2013) showed that cold stress caused changes in intestinal immune function and morphological damages in broilers. The study of Zhang et al. (2011) indicated that cold stress increased NO content in duodenum of chicks and caused intestinal hemorrhage and ulceration.

Cold stress causes decreased growth performance and immune function in chickens. However, appropriate cold stimulation did not negatively affect growth performance such as slaughter weight and feed conversion (Deaton et al., 1996), and improved the survival rate of broilers to some extent (Shinder et al., 2002). Recent results reported by Su et al. (2018) suggested that long-term mild cold stimulation on 8-day-old broilers could effectively alleviate high expression levels of pro-inflammatory cytokines, Th1/Th2 imbalance and stress injuries in NF- κ B inflammatory pathway induced by cold stress in broiler ileum tissues.

Therefore, this study based on the results of previous research by Su et al. (2018) involving broilers. We observed production performance as well as levels of immune parameters, HSPs and oxidative stress indexes in serum or ileum tissues of the broiler with or without cold stimulation of 34 d, before and after their exposure to an acute cold stress. The aim of the present study was to further explore the effects of long-term cold stimulation on the immunity of ileum and adaptability to cold stress in broilers.

MATERIALS AND METHODS

Animals and Experimental Design

All procedures described in this study have been approved by the Institutional Animal Care and Use Committee of Northeast Agricultural University. The chick model of cold stimulation has been described in detail in our previous publication (Su et al., 2018). Briefly, 360 1-day-old female Arbor Acres (AA) broiler chicks were randomly divided into the Group I (C), Group II (C-3), and Group III (C-12), each with 4 replicates (30 broiler chicks per replicate). Broilers were reared in 3 climatic chambers, respectively. The stocking density was 10 birds/m². The birds in C were kept in a normal thermal environment as the control group from days 1 to 42, i.e., 34°C from days 1 to 3, 33°C from days 4 to 7, and the temperature was reduced gradually by 1°C every 2 d from day 8 (07:00 h) till it dropped to 20°C at 32 d of age and maintained at 20°C until day 42 (07:00 h). Air temperature of C-3 and C-12 was same as in C from days 1 to 7. From day 8 (07:00 h) onward, the temperature in C-3 and C-12 was, respectively, 3 and 12°C lower than that of C. Therefore, the temperature in C-3 and C-12 dropped to 17°C on day 32 (07:00 h) and day 14 (07:00 h), respectively, and maintained at 17°C till day 42 (07:00 h). All broilers were exposed to acute cold stress (at 7°C for 24 h.) starting from 07:00 h on day 42. The experimental thermal conditions are shown in Figure 1. After the acute cold stress, C, C-3, and C-12 were designated as S (C + acute cold stress), S-3 (C-3 + acute cold stress), and S-12 (C-12 + acute cold stress), respectively. During the experiment, relative humidity was maintained in the range of 55 to 65%. Photoperiod followed the schedule of 23L:1D from days 1 to 3 and 16L:8D from days 4 to 43. Commercial broiler diets were fed to the birds, including starter diet (metabolizable energy of 12.1 MJ/kg and crude protein of 21.0% for 1 to 3 wk of age) and finisher diet (metabolizable energy of 12.6 MJ/kg and crude protein of 19.0% for 4 to 6 wk of age). The birds had free access to water and feed.

All broilers in each replicate were weighed at the first day and end of each week. Daily feed consumption of each replicate was obtained by measuring the amount of feed added and residual feed each day. Average daily feed intake (ADFI), average daily weight gain (ADWG), and feed conversion ratio (FCR) of each replicate for the experiment period were calculated. The birds were checked every day for their healthy conditions. Dead birds were recorded and diagnosed as ascites-related mortality when those with accumulation of abdominal or pericardial fluid. Two birds were randomly euthanized by cervical dislocation from each replicate per group at 07:00 h on days 14, 42, and 43, and their middle ileum tissues were rapidly excised and stored at -80°C until further evaluation. At the same time, blood samples were collected and put into tubes. These blood samples were kept for 30 min at 37°C for

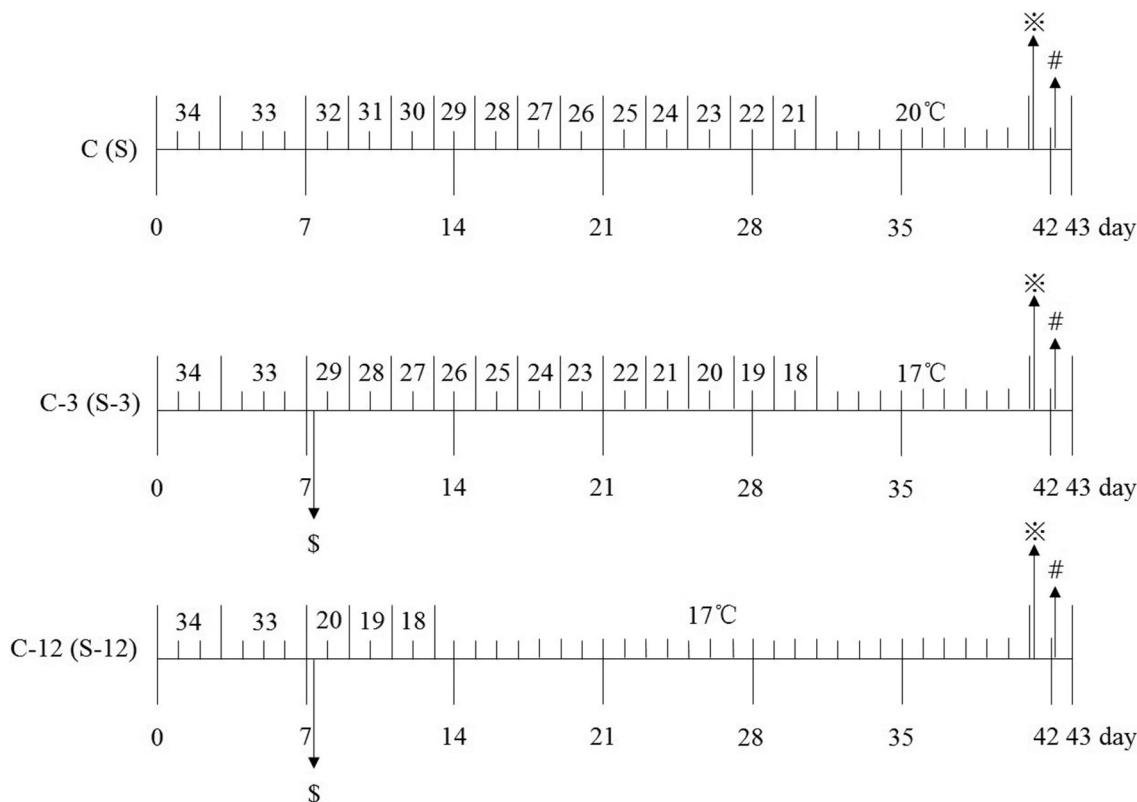


Figure 1. Experimental thermal conditions used in this study. “\$” represents that cold stimulation starting from 07:00 h on day 8. “*” represents that acute cold stress starting from 07:00 h on day 42. “#” represents that acute cold stress finishing on 07:00 h on day 43.

clotting and then rapidly centrifuged at 3000 r/min for 10 min. The prepared serum was stored at -40°C for subsequent ELISA analysis.

NO Content and iNOS Activity

NO content (type: A012) and iNOS activity (type: A014-1) were detected using the commercially available kits (Jian Cheng Bioengineering Institute, Nanjing, China) after the ileum tissues were homogenized. All steps were strictly in accordance with the manufacturer’s instructions.

Quantitative Real-Time PCR

RNAiso Plus (Takara, China) was used to extract total RNA from each ileum tissue and the procedure was carried out in strict accordance with the manufacturer’s instructions. Complementary DNA (cDNA) was reverse transcribed using RR047 kit (Takara, China) according to the manufacturer’s protocol. Specific primers were designed and synthesized by the Sangon Biotech Co., Ltd (Shanghai, China). The primer sequences are shown in Table 1. We conducted qRT-PCR using the Agilent Mx3000P Detection System (Agilent Technologies, USA) and Fast Universal SYBR Green Master kit (Roche, Basel, Switzerland), according to the manufacturer’s instructions. The house-keeping gene β -actin was used as the internal reference. The relative abun-

dance of mRNA was calculated according to the $2^{-\Delta\Delta\text{Ct}}$ method (Pfaffl, 2001).

Western Blot Analysis

We detected the protein levels of HSP60, HSP70 and HSP90 in ileum tissues, as described previously with no modification (Su et al., 2019). The dilution ratio of primary antibodies in our experiment is as follows: HSP60 (1:1400), HSP70 and HSP90 (1:500), β -actin (1:1000, Santa Cruz, USA) and HRP-conjugated secondary antibodies against rabbit IgG (1:1000, Santa Cruz, USA). The Laboratory of Veterinary Internal Medicine in Northeast Agricultural University provided the antibodies of HSPs.

ELISA

We used commercially available ELISA kits (Xinle, Shanghai, China) to detect the levels of IgA, IgM and IgG in serum of each group. All steps were strictly in accordance with the manufacturer’s instructions.

Statistical Analysis

The data were analyzed using IBM SPSS Statistics software (version 21.0, USA). Data normal distribution was examined by the Kolmogorov-Smirnov procedure.

Table 1. Sequence of primers used in experiment.

Gene	Serial number	Forward Primer (5'-3')	Reverse primer (5'-3')	Size of the products (bp)
β -actin	NM_205518.1	CCGCTCTATGAAGGCTACGC	CTCTCGGCTGTGGTGGTGAA	128
IgA	S40610	GTCACCGTCACCTGGACTACA	ACCGATGGTCTCCTTCACATC	192
IgM	X01613.1	GCATCAGCGTCACCGAAAGC	TCCGCACTCCATCCTTTCG	98
IgY (IgG)	X07174.1	ATCACGTCAAGGGATGCCCG	ACCAGGCACCTCAGTTTGG	118
AvBD1	NM_204993.1	CCCAGGCTCTAGGAAGGAAG	TGAGCATTTCCCACTGATGA	98
AvBD6	NM_001001193.1	TTCCAGCCCTATTCATGCTT	CCTGTTCCCTCACACAGCAAG	124
AvBD7	NM_001001194.1	TTTTCCAGGGATCTGTGCAAGG	GAATGGAGTTGGAGTGCCAGA	160
AvBD9	NM_001001611.2	TACAGCCAAGAAGACGCTGA	CATTTTCAGCTTCCCACCAC	119
AvBD10	NM_001001609.2	AGACCCACTTTTCCCTGACA	GGAATCTTGGCACAGCAGTT	132
AvBD12	NM_001001607.2	CCAGACAGCTGTAACCACGA	GTGGGAGTTGGTGACAGAGG	125
HSP27	NM_205290.1	ACACGAGGAGAAAACAGGATGAG	ACTGATGGCTGGCTTGG	158
HSP40	NM_001199325.1	GGGCATTCAACAGCATAGA	TTCACATCCCCAAGTTTAGG	151
HSP60	NM_001012916.1	AGCCAAAGGGCAGAAATG	TACAGCAACAACCTGAAGACC	208
HSP70	NM_001006685.1	CGGGCAAGTTTGACCTAA	TTGGCTCCCACCCTATCTCT	250
HSP90	NM_001109785.1	TCCTGTCTGGCTTTAGTTT	AGGTGGCATCTCCTCGGT	143

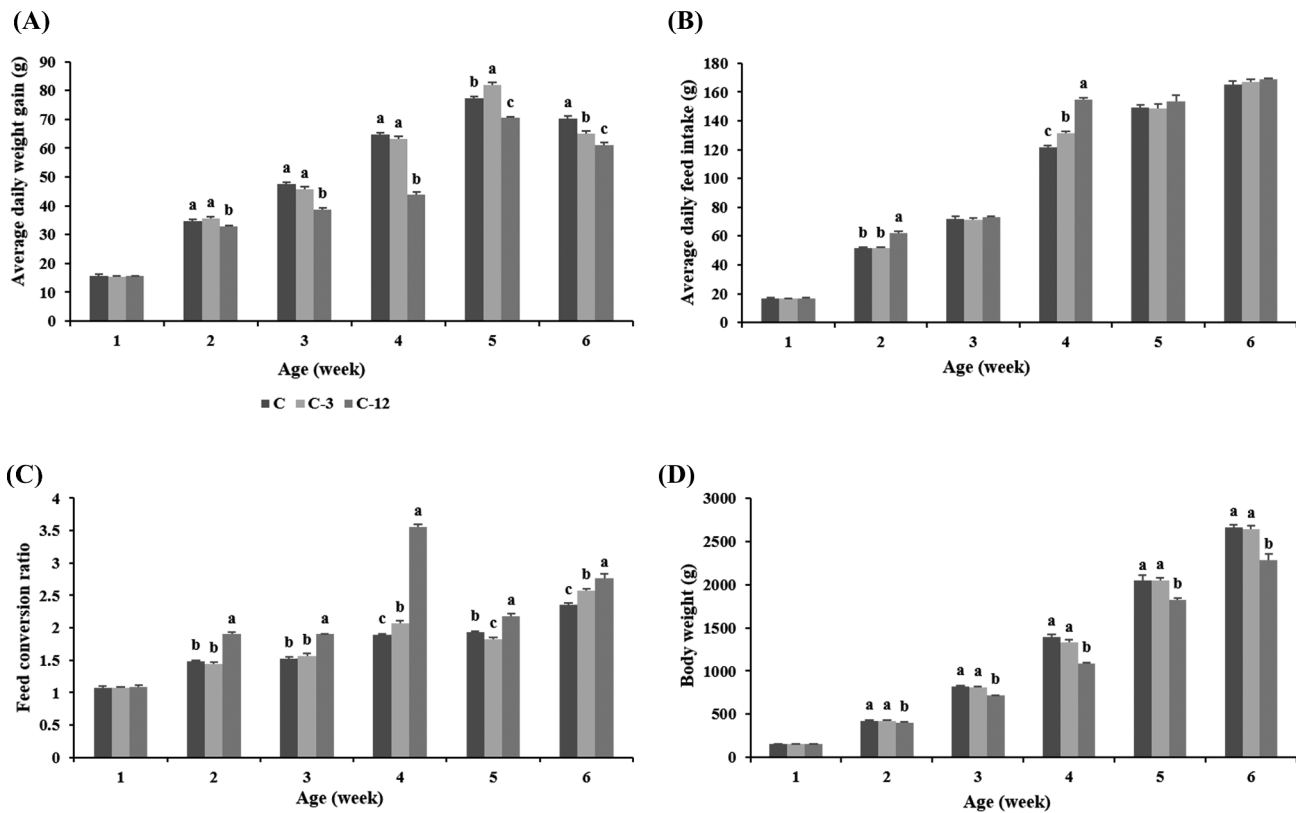


Figure 2. Effects of long-term cold stimulation from 8 to 42 d of age on growth performance of AA broilers. Average daily weight gain (A), average daily feed intake (B), feed conversion ratio (C), and body weight (D). Data are presented as mean \pm SE. Different superscript letters for a performance response indicate significant difference ($P < 0.05$), whereas with no superscripts or the same superscripts mean no significant difference ($P > 0.05$).

Variables of different groups were compared by one-way ANOVA and Duncan's multiple comparison tests. Indicators including NO content and iNOS activity in ileum as well as levels of immunoglobulins in serum were analyzed by inter-group differences at the same time points and intra-group differences at the different time points, respectively. Mortality data were analyzed using χ^2 analyses. Data are expressed as mean \pm standard error. The differences were considered significant at probability values of <0.05 .

RESULTS

Changes in Production Performance

As shown in Figure 2A, ADWG did not differ between C and C-3 during 1 to 4 wk ($P > 0.05$), and ADWG was significantly lower in C compared to C-3 in the 5th wk ($P < 0.05$), however it was significantly higher in C compared to C-3 in the 6th wk ($P < 0.05$). No significant difference in ADWG was observed

Table 2. Effects of long-term cold stimulation from 8 to 42 d of age on growth performance of AA broilers (mean ± SE).

Performance	C	C-3	C-12	P value
ADWG (g/d)	51.67 ^a ± 0.90 ¹	51.21 ^a ± 0.98	43.69 ^b ± 0.49	0.001
ADFI (g/d)	96.00 ^b ± 1.09 ¹	97.69 ^b ± 1.14	104.83 ^a ± 0.92	0.002
FCR	1.86 ^b ± 0.01 ¹	1.91 ^b ± 0.01	2.40 ^a ± 0.01	0.001
BW (kg)	2.66 ^a ± 0.05 ²	2.64 ^a ± 0.06	2.29 ^b ± 0.09	0.008

Note: Different superscript letters for a performance response indicate significant difference ($P < 0.05$). ADWG = average daily weight gain; ADFI = average daily feed intake; FCR = feed conversion ratio; BW = body weight.

¹n = 4 (based on replicate, a total of 4 replicates).

²n = 48 (4 replicates × 12 broilers/replicate).

between C and C-12 in the 1st wk ($P > 0.05$), however ADWG was significantly higher in C compared to C-12 during 2 to 6 wk ($P < 0.05$). As shown in Figure 2B, in the 1st, 3rd, 5th, and 6th wk, there was no significant difference in ADFI among the three groups ($P > 0.05$). At the 2nd wk of age, ADFI did not differ between C and C-3 ($P > 0.05$), and both of which were significantly lower than C-12 ($P < 0.05$). At the 4th wk of age, ADFI in C was significantly lower than C-3 and C-12 ($P < 0.05$), and C-3 was significantly lower compared to C-12 ($P < 0.05$). As shown in Figure 2C, at the 1st wk of age, there was no significant difference among the three groups in FCR ($P > 0.05$). No significant difference in FCR was observed between C and C-3 during 2 to 3 wk ($P > 0.05$). FCR was significantly lower in C compared to C-3 in the 4th and 6th wk ($P < 0.05$), however, FCR in C was significantly higher than that of C-3 at the 5th wk of age ($P < 0.05$). FCR in C was significantly lower than in C-12 during 2 to 6 wk ($P < 0.05$). As shown in Figure 2D, at the 1st wk of age, there was no significant difference among the three groups in BW ($P > 0.05$). BW did not differ between C and C-3 during 2 to 6 wk ($P > 0.05$), and both of which were significantly higher than C-12 ($P < 0.05$). Effects of long-term cold stimulation on production performance of AA broilers during 1 to 6 wk are shown in Table 2. Cold stimulation temperature had significant effects on ADWG, ADFI, FCR, and BW as of day 42 ($P < 0.05$). The results showed no significant differences in ADWG, ADFI, FCR, and BW were

observed between C and C-3 ($P > 0.05$). ADWG and BW in C and C-3 were significantly higher than in C-12 ($P < 0.05$). ADFI and FCR in C and C-3 was significantly lower than in C-12 ($P < 0.05$). Weekly mortality of each group during the whole cold stimulation period is shown in Table 3. Cold stimulation temperature did not affect mortality of each group during 2 to 4 wk ($P > 0.05$), however, it significantly affected mortality during 5 to 6 wk ($P < 0.05$). Mortality in C-12 was significantly higher than in C and C-3 at the 5th and 6th wk of age ($P < 0.05$). Mortality in the whole cold stimulation period was significantly affected by temperature treatment ($P = 0.04$). Although that of C-3 was the lowest, it did not differ with C ($P = 0.34$). Mortality in C-3 was significantly lower than in C-12 ($P = 0.04$), and there was no difference between C and C-12 ($P = 0.41$).

NO Content and iNOS Activity

The changes in NO content and iNOS activity in broiler ileum tissues are shown in Table 4. At days 14 and 42, no difference in NO content was observed between C and C-3 ($P > 0.05$), and significantly increased NO content was observed in C-12 compared with C and C-3 ($P < 0.05$). Compared with day 42, NO content in each group increased significantly after the acute cold stress ($P < 0.05$), but NO content in S-3 was the lowest. At days 14 and 42, iNOS activities in C and C-3 were remarkably lower than that of C-12 ($P < 0.05$) and no differences were observed between C and C-3 ($P > 0.05$). After the acute cold stress, iNOS activities in S and S-3 increased significantly compared to C(day 42) and C-3(day 42) ($P < 0.05$), and there was no difference in iNOS activity between S-12 and C-12(day 42) ($P > 0.05$). However, significantly decreased iNOS activity was observed in S-3 compared with S and S-12 ($P < 0.05$).

Expression Levels of Immunoglobulin mRNA

The changes in expression levels of immunoglobulin mRNAs in broiler ileum tissues are shown in Figure 3.

Table 3. Effects of long-term cold stimulation from 8 to 42 d of age on weekly mortality of AA broilers during the whole cold stimulation period (mean ± SE).

Group	Mortality (%)	2 wk	3 wk	4 wk	5 wk	6 wk	2 to 6 wk
C	Other than ascites	0.88 (1/114)	2.02 (2/99)	1.15 (1/87)	0 (0/79)	0 (0/70)	3.51 (4/114)
	Ascites	0 (0/114)	0 (0/99)	0 (0/87)	0 (0/79)	0 (0/70)	0 (0/114)
	Total	0.88	2.02	1.15	0	0	3.51
C-3	Other than ascites	0 (0/119)	0.94 (1/106)	0 (0/85)	0 (0/75)	0 (0/65)	0.84 (1/119)
	Ascites	0 (0/119)	0 (0/106)	0 (0/85)	0 (0/75)	0 (0/65)	0 (0/119)
	Total	0	0.94	0	0	0	0.84
C-12	Other than ascites	0.85 (1/118)	0 (0/102)	0 (0/79)	0 (0/69)	0 (0/57)	0.85 (1/118)
	Ascites	0 (0/118)	0 (0/102)	1.27 (1/79)	4.35 (3/69)	5.26 (3/57)	5.93 (7/118)
	Total	0.85	0	1.27	4.35	5.26	6.78
P value		0.55	0.32	0.76	0.03	0.03	0.04

Note: Numbers in the parentheses indicate number of dead bird/total number of birds.

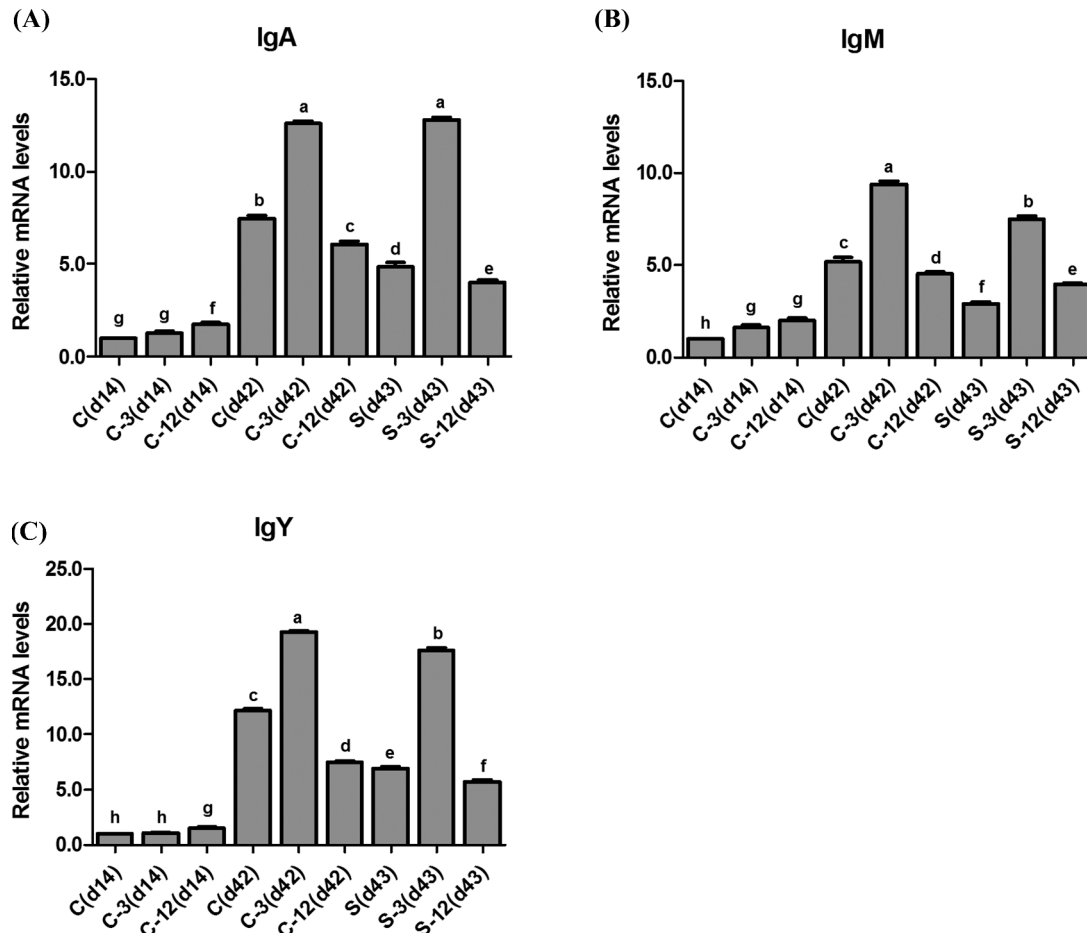
Table 4. Effects of the acute cold stress on NO content and iNOS activity in ileum of the AA broilers with or without long-term cold stimulation (mean \pm SE).

	Age (day)	C (S)	C-3 (S-3)	C-12 (S-12)
NO ($\mu\text{mol/gprot}$)	14	1.60 ^{bz} \pm 0.09	1.68 ^{bz} \pm 0.06	2.14 ^{az} \pm 0.05
	42	2.36 ^{by} \pm 0.05	2.28 ^{by} \pm 0.08	3.70 ^{ay} \pm 0.08
	43	4.07 ^{ax} \pm 0.04	2.96 ^{bx} \pm 0.09	4.22 ^{ax} \pm 0.08
iNOS (U/mgprot)	14	1.46 ^{bx} \pm 0.06	1.55 ^{bx} \pm 0.07	1.88 ^{ax} \pm 0.06
	42	0.55 ^{bz} \pm 0.05	0.62 ^{bz} \pm 0.04	1.36 ^{ay} \pm 0.04
	43	1.21 ^{by} \pm 0.05	1.01 ^{cy} \pm 0.03	1.50 ^{ay} \pm 0.05

Note: a, b and c indicate the significant difference among the 3 groups at the same time ($P < 0.05$); x, y and z indicate the significant difference of each group at different time points ($P < 0.05$). Different superscripts in the same line or the same column mean significant difference ($P < 0.05$), whereas with no superscripts or the same superscripts mean no significant difference ($P > 0.05$).

At day 14, IgA and IgY mRNA levels in C and C-3 did not differ ($P > 0.05$), and significantly increased mRNA levels of IgA and IgY were observed in C-12 compared with C and C-3 ($P < 0.05$). Significantly increased IgM levels were observed in C-3 and C-12 compared with C ($P < 0.05$). At day 42, significantly increased levels of immunoglobulin (including IgA, IgM, and IgY) mRNAs were observed in C-3 compared with C ($P < 0.05$), and the mRNA levels of immunoglobulins (including IgA, IgM, and IgY) in C-12 were significantly lower than

those of C ($P < 0.05$). Following the acute cold stress, significantly decreased IgA mRNA levels in S and S-12 were found compared with C (day 42) and C-12(day 42) ($P < 0.05$), respectively; and no difference was found between S-3 and C-3(day 42) ($P > 0.05$). The expression levels of IgM and IgY mRNAs in each group were down-regulated after the acute cold stress ($P < 0.05$). However, significantly increased expression levels of immunoglobulin mRNAs were observed in S-3 compared with S and S-12 ($P < 0.05$).

**Figure 3.** Messenger RNA levels of immunoglobulins in ileum of the AA broilers before and after the acute cold stress. Different superscripts represent significant difference ($P < 0.05$), whereas the same superscripts represent no significant difference ($P > 0.05$).

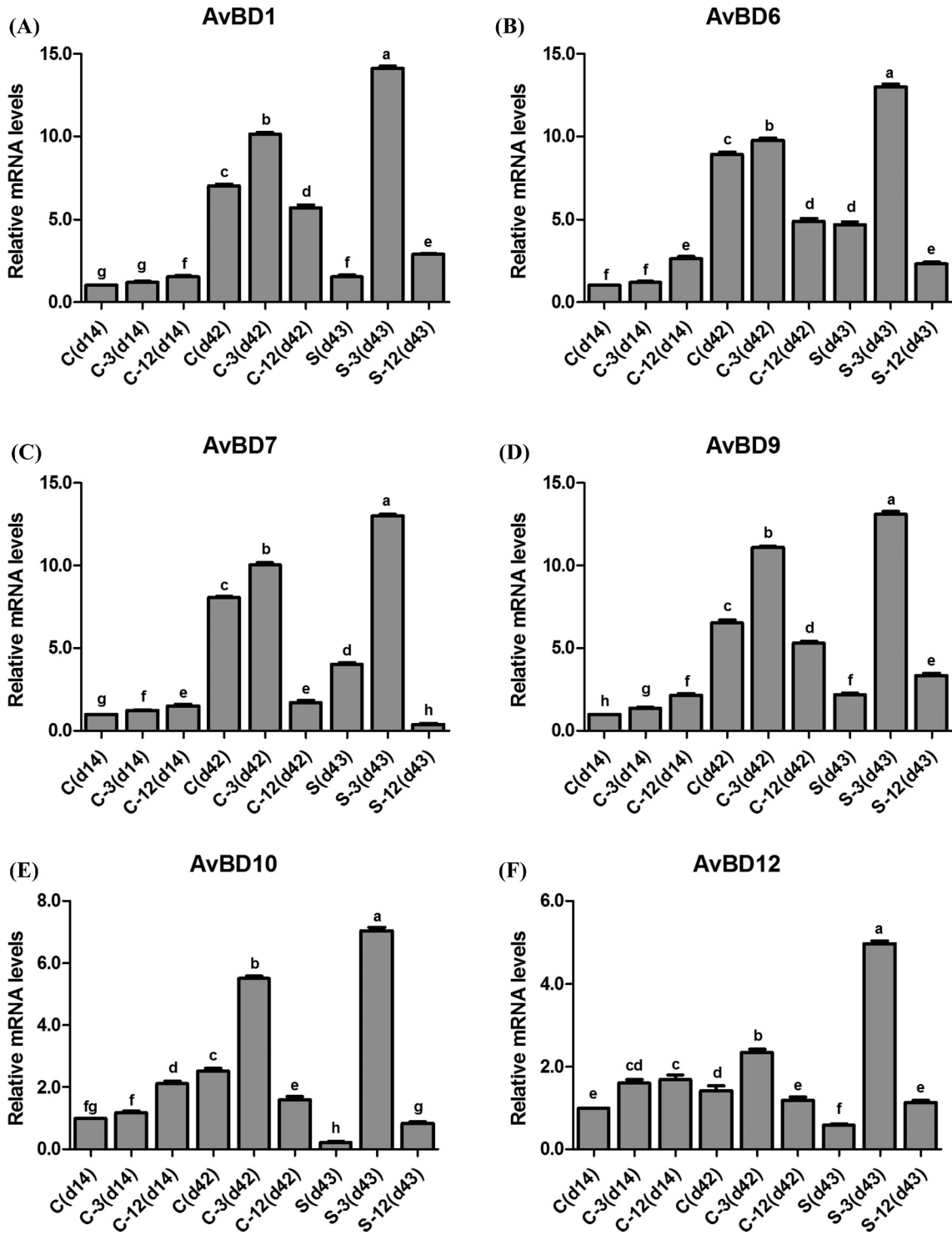


Figure 4. Messenger RNA levels of antimicrobial peptides in ileum of the AA broilers before and after the acute cold stress. Different superscripts represent significant difference ($P < 0.05$), whereas the same superscripts represent no significant difference ($P > 0.05$).

Expression Levels of Antimicrobial Peptide mRNA

The changes in mRNA levels of antimicrobial peptides in broiler ileum tissues are shown in Figure 4. At day 14, mRNA levels of AvBD1, AvBD6 and AvBD10 in C and C-3 did not differ ($P > 0.05$), and these levels in C-12 were significantly increased compared with

C and C-3 ($P < 0.05$). Significantly increased levels of AvBD7, AvBD9, and AvBD12 were observed in C-3 and C-12 compared with C ($P < 0.05$). At day 42, significantly increased mRNA levels of all antimicrobial peptides were observed in C-3 compared with C ($P < 0.05$), and significantly increased mRNA levels of all antimicrobial peptides were observed in C compared with C-12 ($P < 0.05$). After the acute cold stress

at day 43, significantly decreased mRNA levels of all antimicrobial peptides were observed in S compared with C(day 42) ($P < 0.05$). The mRNA levels of all antimicrobial peptides except AvBD12 in S-12 had significant decreases compared with C-12(day 42) ($P < 0.05$). Levels of all antimicrobial peptide mRNAs in S-3 up-regulated significantly compared to C-3(day 42) ($P < 0.05$). Significantly increased mRNA levels of all antimicrobial peptides were observed in S-3 compared with S and S-12 ($P < 0.05$).

Expression Levels of HSP mRNA

The changes in mRNA levels of HSPs in broiler ileum tissues are presented in Figure 5. At days 14 and 42, mRNA levels of all HSPs in C and C-3 did not differ ($P > 0.05$), and significantly increased levels of these HSPs were found in C-12 compared with C and C-3 ($P < 0.05$). Following the acute cold stress, significantly increased mRNA levels of all HSPs were observed in S compared with C (day 42) ($P < 0.05$). All the other HSPs mRNA levels except HSP60 in S-3 and C-3(day 42) did not differ ($P > 0.05$), and HSP60 level in S-3 was increased remarkably compared to C-3(day 42) ($P < 0.05$). All the other HSPs expression levels except HSP40 in S-12 were not different from those in C-12(day 42) ($P > 0.05$), and significantly increased HSP40 level was observed in S-12 compared with C-12(day 42) ($P < 0.05$). S-3 had significantly lower mRNA levels of HSPs compared to S and S-12 ($P < 0.05$).

Expression Levels of HSP Protein

The changes in protein levels of HSPs (including HSP60, HSP70, and HSP90) in broiler ileum tissues are shown in Figure 6. At day 42, no difference was observed in HSP60 level between C and C-3 ($P > 0.05$), and significantly increased HSP60 level was found in C-12 compared with C and C-3 ($P < 0.05$). Significantly decreased protein levels of HSP70 and HSP90 were found in C compared with C-3 and C-12 ($P < 0.05$). Following the acute cold stress, compared with C (day 42), S had significant increasing protein levels of all HSPs ($P < 0.05$). S-3 had significant increasing HSP60 protein expression level ($P < 0.05$), with decreasing HSP70 ($P < 0.05$) and HSP90 ($P > 0.05$) protein expression levels. S-12 had increasing protein levels of HSP60 ($P > 0.05$), HSP70 and HSP90 ($P < 0.05$). Significantly decreased protein levels of HSP60 and HSP90 were observed in S-3 compared with S and S-12 ($P < 0.05$).

Contents of Immunoglobulin in Serum

The changes in immunoglobulin contents in serum of broilers are presented in Table 5. At day 14, no significant difference in IgA level was detected between C and C-3 ($P > 0.05$), and significantly increased IgA level was observed in C-12 compared with C and C-3 ($P < 0.05$).

The IgM and IgG contents in serum of C were significantly lower than in C-3 and C-12 ($P < 0.05$), and significantly decreased these contents were observed in C-3 compared with C-12 ($P < 0.05$). At day 42, the contents of all the 3 immunoglobulins in serum of C were significantly lower than in C-3 ($P < 0.05$), and remarkably decreased levels of immunoglobulins were witnessed in C-12 compared with C ($P < 0.05$). Following the acute cold stress, S had significantly lower immunoglobulins levels in serum compared to C (day 42) ($P < 0.05$). Compared with C-3(day 42), S-3 had significantly lower contents of IgM and IgG ($P < 0.05$), with basically constant IgA level ($P > 0.05$). IgM level in S-12 and C-12(day 42) did not differ ($P > 0.05$), and S-12 had significant decreasing contents of IgA and IgG compared with C-12(day 42) ($P < 0.05$). S-3 had significant increasing contents of all the three immunoglobulins compared with S and S-12 ($P < 0.05$).

DISCUSSION

Stress can impose adverse impacts on the health and productivity of poultry. However, small amounts of seemingly harmful or stressful factors may be conducive to enhancing health (Shevchuk and Radoja, 2007). For example, moderate cold exposure may have no adverse effect on productivity but positive impact on immune and antioxidant function of chickens. The research of Cândido et al. (2016) showed that body weight and feed conversion rate of chicks grown in mild cold stress environment (27°C in the first week, 24°C in the second week, and 21°C in the third week) from 1 to 21 d of age had no difference from those under the normal temperature conditions. A study of Hangalapura et al. (2003) has demonstrated that cell-mediated immune function of chicken was improved after exposure to cold environment for 7 d. Li et al. (2017a) pointed out that intermittent cold stimulation improved the total antioxidant capacity and the activity of glutathione peroxidase in serum of broilers. The present results in this research indicated that cold stimulation condition of 12°C below the normal thermal environment (NT-12°C) resulted in cold stress response that reduced production performance and immune function of the broilers. Whereas, cold stimulation condition of 3°C below the normal thermal environment (NT-3°C) proved to be relatively mild for the broilers, which not only had no adverse effects on their production performance but enhanced their intestinal immune function and improved adaptability to cold stress. These benefits were evidenced by the similar ADWG, ADFI, BW, and FCR (in C-3) and lower mortality (albeit the relatively low number of birds involved in the study) as compared to the normal temperature (C) at day 42. At the same time, S-3 had remarkably higher levels of immune molecules (immunoglobulins and antimicrobial peptides) and lower levels of NO and HSPs, as well as iNOS activity after the acute cold stress.

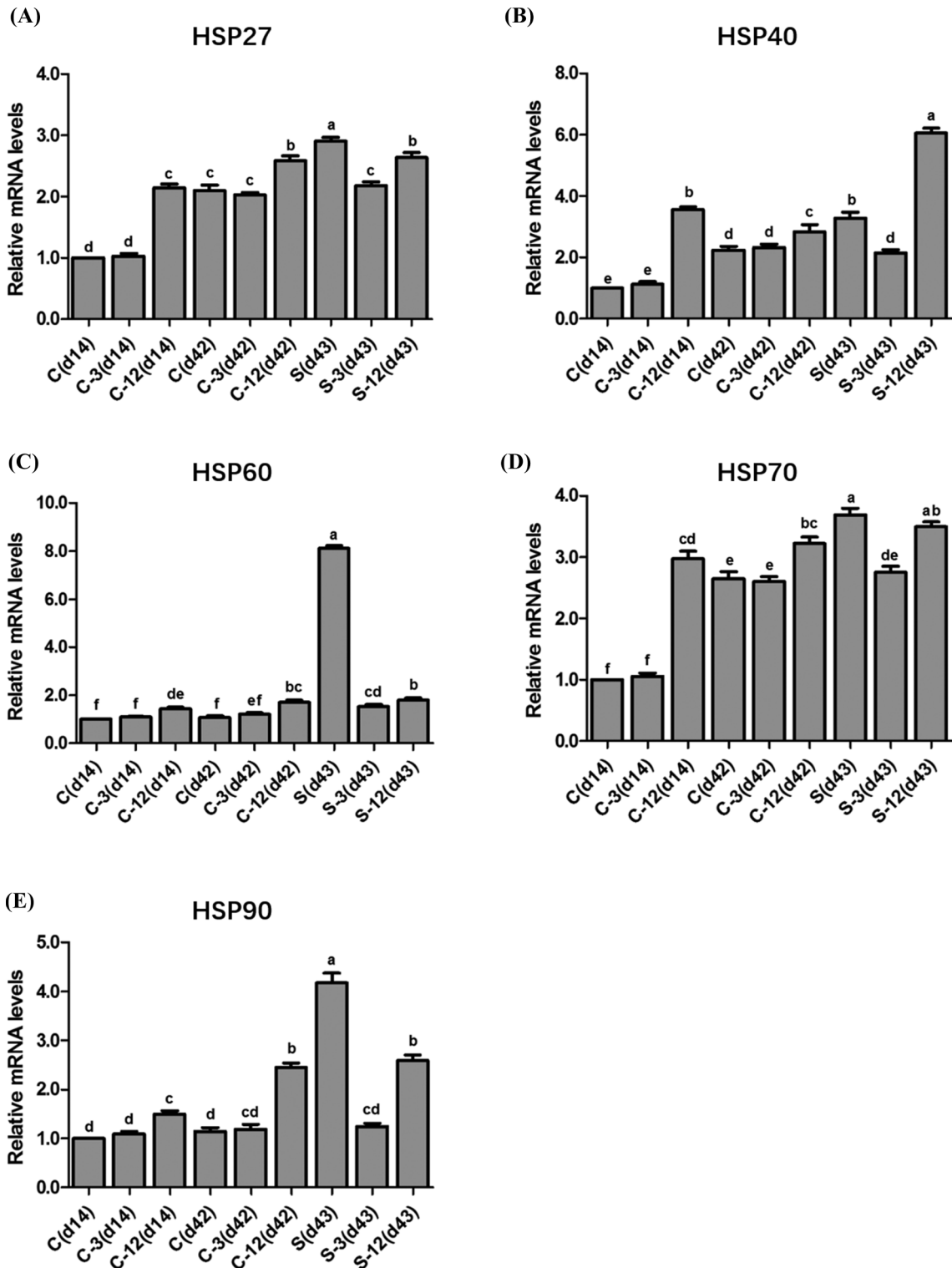


Figure 5. Messenger RNA levels of HSPs in ileum of the AA broilers before and after the acute cold stress. Different superscripts represent significant difference ($P < 0.05$), whereas the same superscripts represent no significant difference ($P > 0.05$).

Cold stress can reduce productivity of chickens. Mendes et al. (1997) pointed out that cold stress environment of 15.5°C significantly increased feed intake, FCR and mortality of broilers aged 3 to 6 wk, and significantly reduced their body weight at 42 d of age. Renwick and Washburn (1982) showed that compared with broilers raised at 32.2°C brooding temperature,

those raised at 26.7°C brooding temperature had increased FCR and mortality as well as decreased body weight during the early stage of growth. In our study, cold stimulation of TN-12°C (C-12) markedly reduced the ADWG and BW as of day 42, and significantly increased ADFI and FCR as of day 42, as well as mortality aged 5 to 6 wk, which was consistent with the

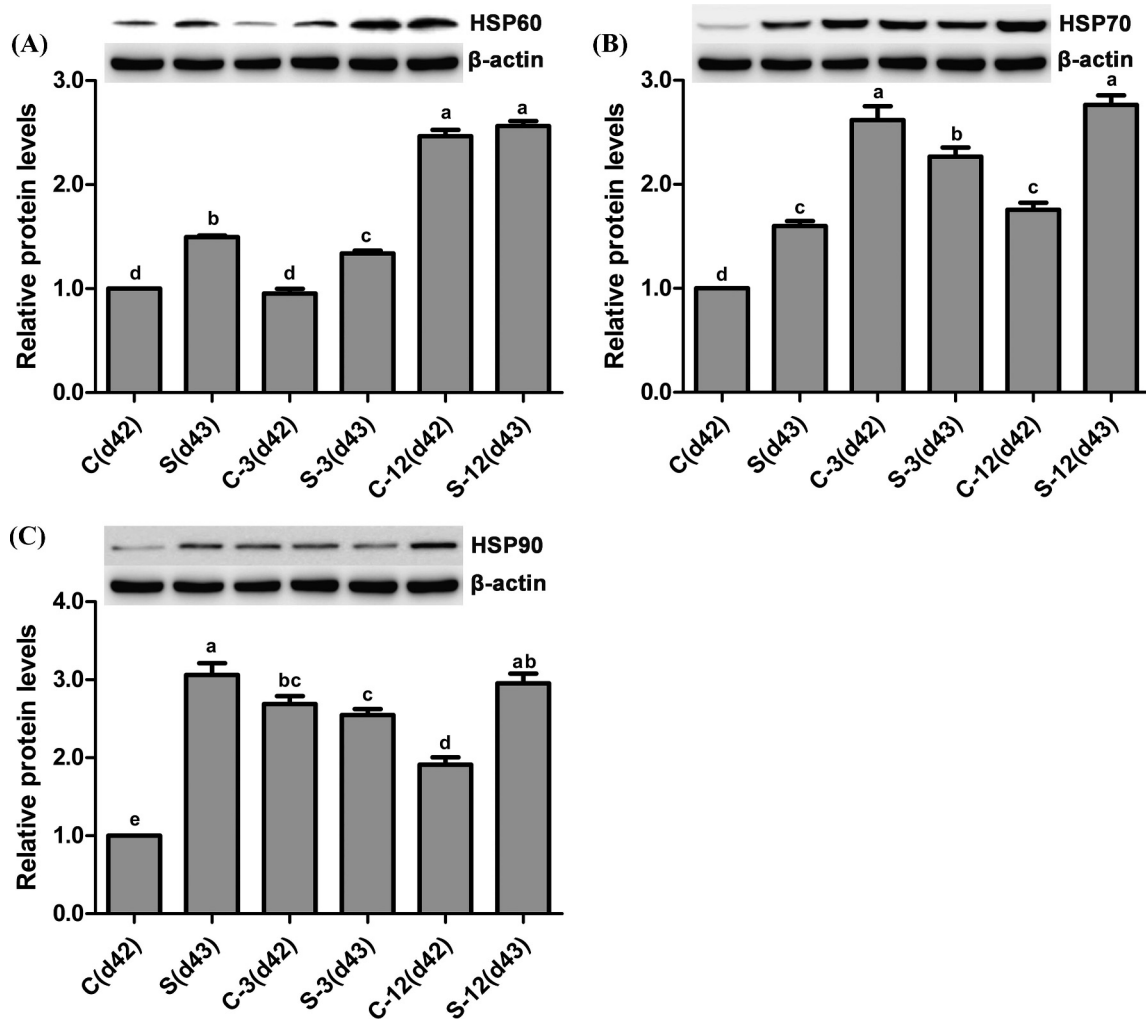


Figure 6. Protein levels of HSP60, HSP70, and HSP90 in ileum of the AA broilers before and after the acute cold stress. Different superscripts represent significant difference ($P < 0.05$), whereas the same superscripts represent no significant difference ($P > 0.05$).

previous results. This outcome may suggest that cold condition of TN-12°C investigated in this study caused cold stress in broilers, hence led to their decreased production performance. However, cold condition of TN-3°C (C-3) did not have any adverse impacts on the production performance. The research of Nguyen et al. (2015) showed that chronic mild cold environment did not affect feed intake of broiler chicks. Harris et al. (1975) reported that when the initial brooding temperature was 30.8°C, body weight gain, feed intake and FCR of broiler chicks were not different from 35°C groups. Shinder et al. (2002) found that broilers exposed to short-term repeated cold stress during the early growth period had significantly increased livability rate when they were subsequently reared in a low temperature environment compared to the control group. The results of the current study agreed with those of Harris et al. (1975), Nguyen et al. (2015), and Shinder et al. (2002). This indicated that cold condition of TN-3°C was mild for chicks, within their adaptable temperature range. The trend of higher livability rate for broilers in C-3 indicates that cold condition of TN-3°C

may activate immune system of the broilers to some extent.

Immunoglobulins and antimicrobial peptides are important components of the immune system and play an important role in maintaining intestinal immune function and immune homeostasis (Otte and Vordenbäumen, 2011). The contents of sIgA and IgM were reduced by cold stress (Ring et al., 2000; Choi et al., 2017). In the current experiment, IgA, IgM, and IgY contents were significantly reduced when the broilers were reared in the cold condition of TN-12°C, in line with the findings found by Ring et al. (2000) and Choi et al. (2017). It suggested that broilers could not adapt to the cold condition of TN-12°C. Consequently, the immune function of C-12 was inhibited and the contents of immunoglobulins were reduced. Whereas, cold condition of TN-3°C significantly increased mRNA levels of all the immunoglobulins in C-3 (day 42). Rao and Glick (1977) showed that cold exposures of 30 min for 2 or 4 times significantly increased IgM antibody production of chickens. Ours results agreed with the results of Rao and Glick (1977). It suggested that certain low

Table 5. Effects of the acute cold stress on levels of immunoglobulins in serum of the AA broilers with or without long-term cold stimulation (mean ± SE).

	Age (day)	C (S)	C-3 (S-3)	C-12 (S-12)
IgA (μg/ml)	14	3.69 ^{by} ± 0.09	3.95 ^{by} ± 0.07	4.57 ^{ax} ± 0.13
	42	4.43 ^{bx} ± 0.12	4.95 ^{ax} ± 0.13	4.01 ^{cy} ± 0.13
	43	3.41 ^{by} ± 0.06	4.70 ^{ax} ± 0.13	3.06 ^{cz} ± 0.12
IgM (μg/ml)	14	2.41 ^{cy} ± 0.07	3.55 ^{bz} ± 0.07	3.93 ^{ax} ± 0.07
	42	3.44 ^{bx} ± 0.08	4.71 ^{ax} ± 0.09	2.85 ^{cy} ± 0.09
	43	2.67 ^{by} ± 0.14	3.93 ^{ay} ± 0.10	2.59 ^{by} ± 0.14
IgG (mg/ml)	14	7.33 ^{cy} ± 0.15	7.81 ^{bz} ± 0.09	8.47 ^{ax} ± 0.10
	42	8.71 ^{bx} ± 0.09	9.62 ^{ax} ± 0.12	7.00 ^{cy} ± 0.09
	43	6.98 ^{bz} ± 0.08	8.78 ^{ay} ± 0.11	5.74 ^{cz} ± 0.09

Note: a, b and c indicate the significant difference among the three groups at the same time ($P < 0.05$); x, y and z indicate the significant difference of each group at different time points ($P < 0.05$). Different superscripts in the same line or the same column mean significant difference ($P < 0.05$), whereas with no superscripts or the same superscripts mean no significant difference ($P > 0.05$).

temperature environment could mediate immunoglobulins to regulate immune function (Carr et al., 1992), and suitable cold stimulation, as is the case with C-3 of this study, can activate immune system to a certain extent.

Antibacterial peptides are important elements in the first line of defense against pathogens in animals (Lee et al., 2014). As an important part of innate immunity, antibacterial peptides have a variety of biological activities, such as antibacterial, anti-inflammatory, and antioxidant activities (Malan et al., 2016). Li et al. (2017b) pointed out that cold exposure induced mRNA levels of antimicrobial peptide genes in insects. He et al. (2014) suggested that cold adaptation enhanced antimicrobial peptide mRNA levels of tilapia. To date, effects of low temperature environment on antimicrobial peptides expression in chicken ileum have rarely been reported. The results of this experiment showed that C-3 (day 42) had significant increasing mRNA levels of antimicrobial peptides compared with C (day 42) after 34 d of cold stimulation training, agreed with the findings found by Li et al. (2017b) and He et al. (2014). This proved that cold condition of TN-3°C as the source of a mild stressor activated the innate immune system of chickens to a certain extent. So, C-3 (day 42) had relatively higher antibacterial peptides mRNA levels, while higher mRNA levels of antibacterial peptides help to enhance the ability to resist to cold stress. Therefore, S-3 had significantly increased antimicrobial peptide mRNA levels compared with S and S-12 after the acute cold stress.

NO is one kind of important messenger molecule, and its overproduction can induce the occurrence of oxidative stress (Chi et al., 2017). iNOS can aggravate intestinal tissue damage by producing NO which induced oxidative stress (Barocelli et al., 2006). In addition, production of NO and iNOS in large quantities can lead to inflammatory reactions in various tissues of chicken (Shao et al., 2018). Researches have proven that oxidative stress can cause abnormal levels of immunoglobulins (Nandakumar et al., 2008) and reduce the immune function (Srivastava et al., 2017). Gao et al. (2014)

found that oxidative stress reduced IgA and IgG levels. High levels of HSPs are observed in the process of oxidative stress. HSPs can be produced in large quantities under a variety of pathological and stress conditions (Yildirim et al., 2018). The research of Kaushik and Kaur (2003) indicated that chronic cold stress caused oxidative stress in different tissues of rats. The results of Zhang et al. (2011) indicated that chronic cold exposure of 12°C raised NO contents and iNOS mRNA levels in duodenum of broilers, triggering oxidative stress, and impairing intestinal tissues. Zhao et al. (2014) reported that cold stress at 12°C could induce oxidative stress of broilers, which increased mRNA levels of HSPs and reduced the immune function. The outcomes of this study are agreed with those reported by Kaushik and Kaur (2003), Zhang et al. (2011), and Zhao et al. (2014). The cold stimulation condition of TN-12°C (C-12) resulted in cold stress in broilers, causing intestinal oxidative stress, thereby reducing the immune function. However, the cold condition of TN-3°C (C-3) did not lead to oxidative stress, which indicated that broilers could easily adapt to this kind of cold condition. At the same time, such low temperature environment enhanced the resistance of broilers to cold stress. Specifically, the NO content, iNOS activity and levels of HSPs in S-3 were the lowest after the acute cold stress, which may be related to C-3 with high levels of antimicrobial peptides after cold stimulation. Because antimicrobial peptides can effectively scavenge NO and protect cells from oxidative damage (Malan et al., 2016). We detected mRNA levels of HSPs in broiler ileum tissues and verified by examining the protein levels of HSPs in ileum tissues. The results indicated that mRNA levels and protein levels were not identical. This may be because many factors affect the transcription and translation process, and the final synthesized proteins are not determined solely by RNA. Other factors, such as half-life of proteins and rate of synthesis, also affected the protein level that we detected. However, from the overall results of HSPs, the levels of HSPs in cold stimulation group of TN-3°C were remarkably lower than those of other groups after the acute cold stress. Wei et al. (2018) found that compared

with the untrained broilers, those cold-acclimated for 34 d had lower mRNA levels of HSPs (HSP27, HSP70, and HSP90) when they were exposed to cold stress at the same time. Our results in this research agreed with those found by Wei et al. (2018), indicating that suitable cold stimulation could alleviate the potential harm resulting from cold stress by reducing HSPs levels and oxidative stress.

CONCLUSION

The current results of this experiment indicated that a cold stimulation condition of 12°C below the normal thermal environment caused cold stress responses in broilers. However, a cold stimulation condition of 3°C below the normal thermal environment did not have any adverse effects on production performance of the broilers. After a 34-d cold stimulation, the broilers may have established cold acclimation. Cold acclimation could enhance the immunity of ileum to some extent and improve the adaptability of the broilers to subsequent acute cold stress challenge.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (Grant No. 31772647).

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