Effects of Cold Stress on Immune Responses and Body Weight of Chicken Lines Divergently Selected for Antibody Responses to Sheep Red Blood Cells

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ABSTRACT Effects of cold stress (CS) on the immune system of chicken lines divergently selected for high (H line) and low (L line) antibody responses to SRBC next to a randombred control (C) line were studied. Three- to four-week-old growing chicks of the three lines were feed-restricted at 80% ad libitum and subjected to CS at 10°C continuously for 7, 5, 3, 1, or 0 d prior to immunization with keyhole limpet hemocyanin (KLH). Specific and natural antibodies were measured in the three chicken lines subjected to or not subjected to various durations of CS prior to immunization. In addition to antibodies we also measured in vitro lymphocyte proliferation as a measure of cell-mediated immunity (CMI), zymosan-induced reactive oxygen intermediates (ROI) production as a measure of phagocytosis, and BW gain as a measure of production trait.

In general, significantly higher antibody responses to KLH and natural antigens were found in the H line as compared to the other two lines. Specific antibody responses to KLH were not significantly affected by CS, but an acute transient increase in natural antibody titers to ovalbumin was found in H line birds subjected to 1 d of CS, which was not found in C or L line birds. On the other hand, an acute significant increase in natural antibody titers to lipopolysaccharide (LPS) was found in C and L line birds subjected to 1 d CS but not in H line birds. Cold stress enhanced the ROI production. In addition, 7 d of CS significantly enhanced cellular immunity in vitro, but no significant line effects with respect to cellular immunity were found. BW gain was negatively affected by CS, especially when CS was applied for longer periods.

We concluded that birds responded immediately to CS with enhanced innate (phagocyte and natural antibody) immunity, irrespective of genetic background. When CS is prolonged, the cellular adaptive immune response is affected also. Although reallocation of energy was not measured, our data suggested that under limited conditions (e.g., restricted feeding) with simultaneous stress (e.g., CS), growth and cellular immunity were more sensitive than thermoregulation and humoral immunity. With respect to vital life traits, thermoregulation may have first priority followed by adaptive cellular immunity, humoral immunity, and BW gain. The relationships between immune responses and adaptation to stress are discussed.

(*Key words*: adaptation, chicken, cold stress, immune response)

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INTRODUCTION

The future of poultry husbandry is aimed at enhanced animal welfare, with minimal use of preventive medical treatments. These husbandry conditions will resemble more natural or ecological conditions. Under such farming systems, birds will undergo various physical and climatic stresses (cold, heat, and wind), infectious diseases, and social stress. Animals maintained under such future conditions must be able to cope with or adapt to much more dynamic environments than nowadays, preferably without an increase in production costs and risk of diseases. Because environmental stressors can alter the susceptibility of animals to infective agents, it is important to learn how stressors affect the immune system of food animals, the adaptive capacity of animals to respond, and

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Abbreviation Key: C = randombred control; CMI = cell-mediated immunity; CPM = counts per minute; CS = cold stress; 0CS = not subjected to CS; 1CS = subjected to 1 d CS; 3CS = subjected to 3 d CS; 5CS = subjected to 5 d CS; 7CS = subjected to 7 d CS; H = selected for high response to SRBC; KLH = keyhole limpet hemocyanin; L = selected for low response to SRBC; LPS = lipopolysaccharide; NAb = natural antibody; NBT = nitroblue tetrazolium assay; OVA = ovalbumin; ROI = reactive oxygen intermediates.

the time it takes for animals to return to homeostasis. With respect to chickens, results of studies on the effects of cold stress (CS) on antibody-mediated and cell-mediated immune (CMI) responses are not consistent. Cold stress of 7°C enhanced antibody production (Subba Rao and Glick, 1977). However, Hester et al. (1996) reported that exposure to 0°C decreased antibody responses of single-caged hens but not for hens in colony cages. Regnier et al. (1980) found little effect of acute CS on antibody titers to SRBC. Dabbert et al. (1997) found no evidence for suppressed humoral immunocompetence following experimentally induced CS in the Northern Bobwhite, *Colinus virginianus*. Whereas Svensson et al. (1998) reported that stress in the form of cold exposure has a negative impact on humoral immunocompetence in Blue tits. CMI was depressed in chickens exposed to cold (Regnier and Kelley, 1981). Recently, we found enhanced CMI in birds exposed to low temperatures (van Loon et al., 2003). It is reasonable to suggest that type and duration of CS treatments as well as the genetic make up of the birds underlie these divergent results. So far no studies have reported on the effect of duration of CS on innate and adaptive components of the immune system of genetically selected chicken lines or their adaptive capacity to CS treatments.

In the present study, two lines divergently selected for high (H line) or low (L line) antibody responses to SRBC and a nonselected chicken line originating from the same parental stock were used. These lines differ with respect to 1) specific humoral immune responses, the H line has higher antibody titers to various antigens (Parmentier et al., 1993, 1996); 2) CMI responses, the L line has higher CMI in vitro (Parmentier et al., 1993, 1994; Kreukniet et al., 1995); 3) innate humoral immune responses, the H line has higher natural antibody (Nab) levels (Parmentier et al., 2004); and 4) resistance to infectious diseases (Pinard et al., 1993). In addition, differences with respect to BW gain were found with the L line being heavier and growing faster (Mashaly et al., 2000; Parmentier et al., 1996).

In the current study, birds of all three lines were restricted-fed at 80% ad libitum with a commercial diet and were subjected to CS (10.4 ± 0.5°C) continuously during 7, 5, 3, and 1 d or no stress prior to immunization with keyhole limpet hemocyanin (KLH). The objectives of this study were fourfold. First, the effect of different durations of CS on immunity was studied. Second, effects of different durations of CS were studied on the three lines of chickens that usually respond differently when immunized under normal conditions. Third, a time during the CS that birds have adapted to or have recovered from the CS was studied. Fourth, because immune responses are costly (Lochmiller and Deerenberg, 2000), reallocation of resources between growth and immune response due to the simultaneous stress of restricted feeding and CS was studied.

We measured specific and NAb as a measure of specific and innate humoral immunity, respectively, in three chicken lines subjected to or not subjected to various durations of CS prior to immunization. In addition to antibodies we also measured in vitro lymphocyte proliferation as a measure of cell-mediated immunity (CMI), reactive oxygen intermediates (ROI) production as a measure of phagocytosis, and BW gain as a measure of production.

MATERIALS AND METHODS

The Institutional Animal Care and Use Committee of Wageningen University approved all experimental protocols.

Chickens

One hundred eighty 23-d-old growing ISA Brown (Warren) medium heavy layer hens from three lines were used in this study. The first two lines were divergently selected for 21 generations for high (H line) or low (L line) primary antibody responses at d 5 after i.m. immunization with SRBC at 35 d of age. The third line was randomly bred, originated from the same parental line, and served as the control (C) line (van der Zijpp and Nieuwland, 1986). The C line resembles the genetic pool of the original parental stock of layers (Pinard et al., 1993).

From the day of hatch, until 20 d of age and subsequently from +15 d after immunization with KLH[2] (40 d of age), chicks were fed ad libitum with a commercial diet (200 g/kg CP, 2,600 kcal/kg ME). Birds were kept according to routine procedures for layer hens in brooder cages.

During the experimental period until the first 15 d after immunization with KLH, birds were restricted-fed once a day with the same commercial diet at 80% ad libitum (160% of ME for maintenance 120 kcal·kg$^{-0.75}$/d) (Mashaly et al., 2000) for these selection lines. All birds had free access to water throughout the experiment. Lighting was as follows: 14L:10D (0400 to 1800 h). A similar lighting schedule was maintained when birds were subjected to CS. The birds were vaccinated for Marek's disease and infectious bronchitis at hatch and then infectious bursal disease on d 15 of age.

Experimental Design

At 23 d of age (experimental d –9), five groups of 12 hens of each (H, C, and L) line were randomly assigned to one of five treatment groups, that received a different duration of CS. Initially all groups were housed in one climate respiration chamber (Verstegen et al., 1987) and maintained at 24.4 ± 0.4°C with RH of 70.0 ± 0.4% (control temperature) until CS treatment. The five treatment groups were subjected to 7 d (7CS), 5 d (5CS), 3 d (3CS), and 1 d (1CS) of CS by transferring the birds to another respiration chamber that was maintained at 10.4 ± 0.5°C (RH of 76.1 ± 1.1%) or were not subjected to CS (0CS). The control group (0CS) was transported out and back

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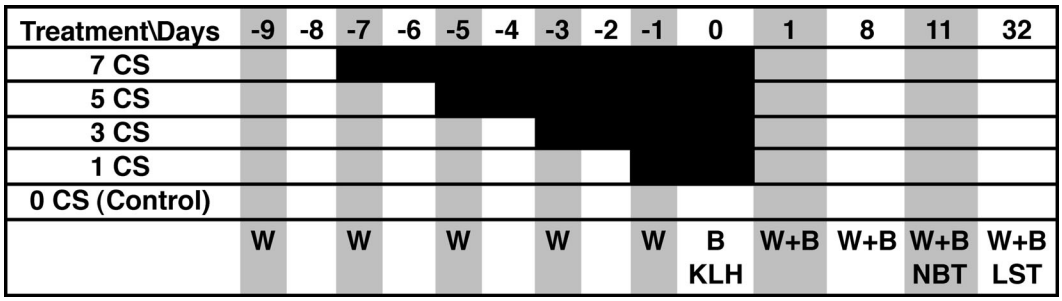

FIGURE 1. Experimental plan: Five groups of 36 hens (12 hens of high, control, and low line each) were subjected to cold stress (10°C) for 7 d (7CS), 5 d (5CS), 3 d (3CS), 1 d (1CS), or 0 d (0CS) before immunization with keyhole limpet hemocyanin (KLH) (1 mg/bird) at 33 d of age. All treatment groups were weighed (W) before, during, and after cold stress treatment. Blood samples (B) were collected on d 0, +1, +8, +11, and +32. Nitroblue tetrazolium assay (NBT) on d + 11 and lymphocyte stimulation test (LST) on d +32 were performed.

to the same chamber. Immediately after CS treatment, all birds were moved back to brooder cages and kept at 22.5 ± 2.2°C (RH of 70 ± 0.2%) until the last experimental day.

At d 0, i.e., 33 d of age, all birds were injected subcutaneously with 1 mg KLH[2] in 1 mL PBS (pH 7.2) per bird (Figure 1). Blood samples were collected from all individual birds at experimental d 0, +1, +8, +11, and +32 after immunization. Specific antibody titers to KLH were determined in plasma obtained at experimental d +1, +8, +11, and +32 after immunization. Blood samples obtained at d +11 were also used to measure ROI production during zymosan A stimulation in whole blood. In blood samples collected on d +32, lymphocyte proliferation to concanavalin A[3] (ConA) and KLH were measured. NAb titers to ovalbumin (OVA)[3] and lipopolysaccharide (LPS)[3] were determined in plasma collected on all sampling days. All birds were weighed on experimental d –9, –7, –5, –3, –1, +1, +8, +11, and +32.

Assays

ELISA. Antibodies binding to KLH, *Escherichia coli* LPS,[3] and OVA[3] were determined in individual plasma samples obtained from all birds using an indirect two-step ELISA procedure. Plates were coated with 1 μg/mL KLH,[2] 4 μg/mL OVA,[3] or 10 μg/mL LPS[3] and after subsequent washing incubated with serial twofold dilutions (KLH and OVA) or serial fourfold dilutions (LPS) of plasma. Binding of antibodies to the antigens was detected using 1:20,000 diluted rabbit anti-chicken IgG_{H+L} labeled with peroxidase[4] ($RACh/IgG_{H+L}/PO$). After being washed, tetramethylbenzidine and 0.05% H_2O_2 were added and incubated for 10 min at room temperature. The reaction was stopped by adding 2.5 *N* H_2SO_4. Extinctions were measured with a Multiskan[5] at 450 nm. The titers were expressed as the 2log values of the highest dilution giving a positive reaction. Positivity was derived from the extinction values of twofold diluted standard positive plasma present on every microtiter plate.

Lymphocyte Stimulation Test. An in vitro lymphocyte stimulation test was performed to determine effects of duration of CS on in vitro mitogen and KLH specific T-cell proliferation. Aliquots of 200 μL whole blood were diluted 1:30 in RPMI tissue culture medium and cultured during 72 h at 41°C and 5% CO_2 in a humidified atmosphere. Medium was supplemented with 2 m*M* L-glutamine, 100 μg/mL streptomycin, and 100 IU/mL penicillin in 96-well flat bottom plates and either 10 μg/mL Con A[3] or 20 μg/mL KLH.[2] The last 12 h before harvesting, cultures set up in triplicate were pulsed with 0.5 μCi methyl-3H-thymidine.[6] 3H-thymidine uptake by cultures was determined with a β-scintillation counter.

Results were expressed as mean counts per minute (CPM) in mitogen- or antigen-stimulated cultures minus CPM in unstimulated cultures (ΔCPM) and as mean stimulation indices. The stimulation indices were calculated as CPM in mitogen- or antigen-stimulated cultures per CPM in unstimulated cultures.

ROI Production Assay. The nitroblue tetrazolium assay (NBT) was used to measure ROI production by phagocytes in whole blood as described by (Kreukniet et al., 1995) with slight modifications. One hundred microliters of 1:60 diluted heparinized blood and 50 μL of 2 mg/mL NBT[3] in PBS were added to six wells of 96-well flat-bottom microtiter plates. To the first three wells, 10 μL of 1 mg/mL of phagocyte stimulator, Zymosan A[3] (prepared from *Saccharomyces cerevisiae*), in RPMI was added. To the other three wells, 10 μL of RPMI was added (non-Zymosan stimulated). Three additional wells were used as blanks and contained 100 μL of RPMI, 50 μL of 2 mg/mL NBT[3] in PBS, and 10 μL of 1 mg/mL of Zymosan A, and three wells were used as controls, containing 100 μL of 1:60 diluted heparinized blood, 10 μL of mg/mL of Zymosan A, and 50 μL of RPMI. After 1 h of incubation at room temperature, the reaction was stopped with 100 μL of 1 *N* HCl. Then each plate was centrifuged and washed two times with PBS (10 min, 850 × *g*). Finally, 150 μL dimethylsulfoxide[7] was added directly, followed by 10 μL 1 *N* KOH. Addition of dimethylsulfoxide caused a color change to the blue spectrum, which was measured

[3]Sigma Chemical Co., St. Louis, MO.
[4]Nordic, Tilburg, The Netherlands.
[5]LabSystems, Helsinki, Finland.
[6]ICN Biochemicals, Inc., Aurora, OH.
[7]Merck, Stuttgart, Germany.

with a Multiskan[5] at 690 nm. Mean absorbance of the non-Zymosan-stimulated samples per bird was subtracted from the absorbance of the stimulated samples, as an indication of the ROI production during antigen digestion.

Statistical Analysis

Differences in titers of plasma antibodies binding KLH, OVA, and LPS were analyzed by three-way ANOVA for the effect of treatment, line, time, and their interactions by using the repeated measurement procedure with the option of bird nested within treatment and line. A two-way ANOVA was performed to determine differences between treatments and lines and their interaction with respect to lymphocyte proliferation to ConA and KLH, superoxide production, and BW gain (difference between individual BW on experimental d +1 and −9). All analyses were according to SAS Institute procedures (1990). Mean differences of treatment and line were tested with Bonferroni's test.

RESULTS

Humoral Immune Responses

Specific Antibody Responses to KLH. Kinetics of the specific antibody response to KLH in chicks of all three lines subjected to five different time lapses of CS are shown in Figure 2. Highest titers were found at d +8 after immunization in all three lines in most treatment groups. Antibody responses to KLH were affected by an interaction among duration of CS, line, and time (line by treatment by time interaction; $P < 0.001$; Table 1). Thus with respect to antibody titers to KLH, the lines responded differently to duration of CS over time. There was no line-by-treatment interaction or main treatment effect.

Evaluation of the separate lines showed that on d +1 an enhancement ($P < 0.05$) of antibodies binding KLH was found in the H line subjected to 1CS, 3CS and 7CS as compared to the H line birds not subjected to CS. But there was no significant difference among titers to KLH between H line birds exposed to CS and or not exposed to CS on d +8 and +11 post-immunization. At +11 d, H line birds subjected to 3CS exhibited ($P < 0.05$) lower titers to KLH (Figure 2A) as compared to the 0CS group of the same line. The antibody response to KLH of C line birds subjected to CS is shown in Figure 2B. None of the CS treatments significantly affected antibody titers to KLH at all time points in C line birds. In Figure 2C antibody responses to KLH of L line birds subjected to CS are shown. There was no significant effect of durations of CS on antibody responses to KLH in L line birds as compared with nonstressed L line birds. However, L line birds subjected to 7CS had lower ($P < 0.05$) antibody titers to KLH at d +32.

NAb Titers to OVA. With age, a gradual increase in levels of NAb binding OVA were found in all three lines (data not shown). There was no significant effect of durations of CS (treatment effect) on OVA-binding antibodies, but an interaction among treatment, line, and time was evident (Table 1; $P < 0.001$). All three lines differed significantly (line effect; Table 1; $P < 0.001$) from each other in OVA-binding antibody titers. The H line had the highest titers to OVA, followed by the C line, and then the L line. There was also no significant interaction between treatment and line. Cold stress induced a significant ($P < 0.05$) acute increase in OVA-binding antibody titers in the H line when subjected to 1CS only as compared to the H line birds not subjected to CS. When prolonged, CS did not affect titers to OVA. In the C and L line birds, no significant effects of CS on titers to OVA were found at any time.

NAb Titers to LPS. Plasma antibodies binding *E. coli* LPS were determined in the same plasma samples in which KLH-binding antibodies were determined. With age, low but gradually increasing levels of NAb-binding LPS were found in all three lines (data not shown). An effect (Table 1; $P < 0.05$) of durations of CS (treatment effect) on the level of antibodies binding LPS was found. In the H line, titers to LPS were not affected by CS (Table 1). In C line birds subjected to 1CS ($P < 0.05$) higher levels of antibodies binding LPS were found compared with C line birds subjected to 0CS. Except for 7CS, all CS treatments induced a significant ($P < 0.05$) increase in the levels of antibodies binding LPS in the plasma of L line birds compared with the L line birds subjected to 0CS. As was true for KLH and OVA, titers of antibodies binding LPS were affected by interaction of time by line by treatment.

In Vitro Lymphocyte Proliferation. Stimulation indices of in vitro lymphocyte proliferation to ConA were affected by the duration of CS before immunization ($P < 0.05$; Table 2). Seven-day CS ($P < 0.05$) enhanced in vitro lymphocyte proliferation to ConA as compared to 0CS. Higher, but not significantly different lymphocyte proliferation, was also found in 5CS, 3CS, and 1CS birds.

Specific in vitro lymphocyte proliferation to KLH at d +32 after immunization with KLH was not significantly affected by duration of CS, genetic background of birds, or interaction between line and treatment (Table 2).

ROI Production. Blood samples from all birds collected on d +11 after immunization with KLH were analyzed for ROI production as a measure of phagocytosis. CS enhanced ($P < 0.05$) ROI production of all three lines as compared to the non-CS group (Table 3). There was no significant difference in ROI production among the three lines. However, there was a significant interaction between CS treatment and line. In the H line, an increase ($P < 0.05$) in ROI production was found in birds subjected to 1CS and 5CS as compared to the H line birds subjected to 0CS. Only when subjected to 3CS, higher ROI ($P < 0.05$) was found in C line birds as compared to C line birds not subjected to CS, whereas longer or shorter duration of 3CS did not significantly affect ROI production in the C line birds. The L line birds had enhanced ($P < 0.05$) ROI production when subjected to all CS, irrespective of the duration as compared to the L line birds subjected to 0CS. The magnitude of enhancement was, however, less in

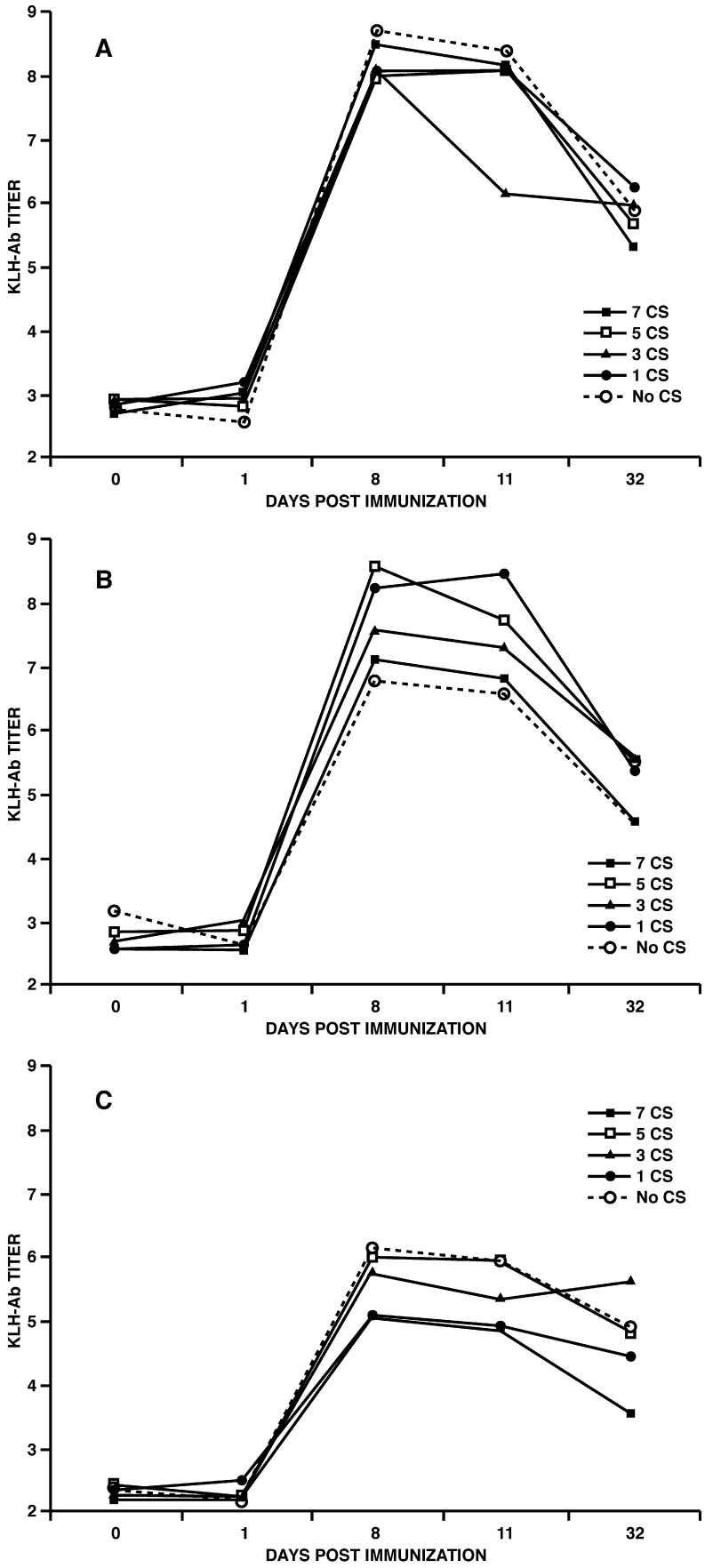

FIGURE 2. Antibody titers (2log values of the highest dilution giving a positive reaction) to keyhole limpet hemocyanin (KLH) of high line (A), control line (B) and low line (C) birds subjected to cold stress (10°C) for 7 d (■), 5 d (□), 3 d (▲), 1 d (●) or no cold stress (○) before immunization with KLH at 33 d of age.

TABLE 1. Average total plasma antibody titers[1,2] from high (H), control (C), and low (L) line hens[3] subjected to one of the five durations of cold stress treatment before immunization with keyhole limpet hemocyanin (KLH) during the complete experimental period

		Antigen		
Line	Treatment[4]	KLH	OVA	LPS
H	7CS	5.55[2]	2.66[a]	2.91
	5CS	5.50	2.58[a]	3.30
	3CS	5.22	2.79[a,b]	3.36
	1CS	5.68	3.20[b]	3.53
	0CS	5.65	2.63[a]	3.32
C	7CS	4.72	2.56	3.56[ab]
	5CS	5.46	2.24	3.55[ab]
	3CS	5.24	2.72	3.83[ab]
	1CS	5.46	2.41	3.92[a]
	0CS	4.74	2.45	3.13[b]
L	7CS	3.57	1.65[a]	3.18[ab]
	5CS	4.69	2.24[b]	3.49[a]
	3CS	4.25	2.07[ab]	3.40[a]
	1CS	3.86	2.06[ab]	3.71[a]
	0CS	4.28	1.95[ab]	2.57[b]
SEM		0.30	0.21	0.27
Main effect				
Treatment		NS	NS	*
Line		***	***	NS
		H > C > L	H > C > L	
Treatment × line		NS	NS	NS
Time		***	***	***
Time × treatment		*	*	***
Time × line		***	*	***
Time × treatment × line		***	***	*

[a,b]Means within treatment and line group with no common superscript differ significantly ($P < 0.05$).

[1]Values are least square means ± SEM of the complete experimental period.

[2]Titers are expressed as the 2log values of the highest dilution giving a positive reaction. Positivity was derived from the extinction values of twofold diluted standard positive plasma present on every microtiter plate.

[3]Twelve hens per group (treatment, line).

[4]7CS = birds subjected to cold for 7 d before immunization; 5CS = birds subjected to cold for 5 d before immunization; 3CS = birds subjected to cold for 3 d before immunization; 1CS = birds subjected to cold for 1 d before immunization; and 0CS = birds were kept in a type 2 respiration cell maintained at 24°C continuously (control).

*$P < 0.05$; ***$P < 0.001$.

those L line birds subjected to a longer duration of CS (7CS).

BW Gain. Subjecting chicks to CS for different durations before immunization resulted in reduced BW gain ($P < 0.001$; Table 4). BW gain of the birds was inversely related with the duration of CS. BW of the CS birds was not significantly different from the 0CS birds (Table 4). There were no significant line effects or interactions between treatment and line. There was a time effect and a time-by-treatment interaction with respect to BW ($P < 0.001$, Table 4). There was no significant interaction among time, line, and CS treatment.

DISCUSSION

The future of animal husbandry is aimed to optimize production and enhance animal welfare by minimal use of preventive medical treatments and more naturally based husbandry conditions. Under such farming systems, e.g., free-range systems, animals will face pressure from different pathogens and simultaneously undergo various kinds of physical, climatic, and social stresses. A robust animal under such stressful conditions must be able to cope with or adapt to these challenges without an increase in production costs or risks of diseases. In this respect, we propose to define robustness as the capacity of an individual to respond properly to a challenging factor under stress conditions. In addition, the robust animal should maintain or return to a response equal to that of an individual that is similarly challenged but not under stress conditions. It is expected that the genetic background of an individual is an important factor for the adaptation response. Variations in strain, type of CS, and type of immune parameter measured may underlie the inconsistent results of the effects of CS on immune responses of poultry (Subba Rao and Glick, 1977; Hester et al., 1996).

The present study was conducted to study the effects of divergent selection of chicken lines for an immune parameter (primary antibody responses to SRBC) on the adaptive response to CS, and to measure robustness as defined above. We measured innate and specific immunity and BW gain in young growing layer chicks subjected to various durations of CS. In addition, chicks were re-

TABLE 2. Simulation indices of in vitro lymphocyte proliferation of whole blood from high (H), control (C) and low (L) line hens[1] subjected to one of the five durations of cold stress treatment before immunization with keyhole limpet hemocyanin (KLH)

Line	Treatment[2]	ConA[3]	KLH
H	7CS	109.2[a,4]	2.3
	5CS	81.0[ab]	1.4
	3CS	54.2[b]	1.5
	1CS	65.9[ab]	1.5
	0CS	48.6[b]	1.7
C	7CS	128.9[a]	1.6
	5CS	54.5[bc]	1.4
	3CS	98.4[ab]	1.8
	1CS	73.1[bd]	1.8
	0CS	43.4[cd]	2.3
L	7CS	85.7	1.6
	5CS	90.7	2.0
	3CS	43.8	1.5
	1CS	80.1	2.0
	0CS	55.8	1.8
SEM		19.4	0.3
Main effect			
Treatment		*	NS
Line		NS	NS
Treatment × line		NS	NS

[a–d]Means within treatment and line group with no common superscript differ significantly ($P < 0.05$).

[1]Twelve hens per group (treatment, line).

[2]7CS = birds subjected to cold for 7 d before immunization; 5CS = birds subjected to cold for 5 d before immunization; 3CS = birds subjected to cold for 3 d before immunization; and 1CS = birds subjected to cold for 1 d before immunization; and 0CS = birds were kept in a type 2 respiration cell maintained at 24°C continuously (control).

[3]Concanavalin A.

[4]Values are least square means of stimulation index ± SEM.

*$P < 0.05$.

TABLE 3. Reactive oxygen intermediates (ROI) production[1] during Zymosan A stimulation in the whole blood samples sampled on d +11 after immunization with keyhole limpet hemocyanin (KLH) from high (H), control (C) and low (L) line hens[2] subjected to one of the five durations of cold stress treatment before immunization with KLH

Line	Treatment[3]	ROI production[1]
H	7CS	0.08[abc]
	5CS	0.17[a]
	3CS	0.01[b]
	1CS	0.15[a]
	0CS	0.02[b]
C	7CS	0.06[a]
	5CS	0.06[a]
	3CS	0.41[b]
	1CS	0.08[a]
	0CS	0.05[a]
L	7CS	0.05[a]
	5CS	0.23[b]
	3CS	0.10[a]
	1CS	0.22[b]
	0CS	–0.07[c]
SEM		0.03
Main effect		
Treatment		***
Line		NS
Treatment × line		***

[a–c]Means within treatment and line group with no common superscript differ significantly ($P < 0.05$).

[1]Values are least square means ± SEM of the mean absorbance of the nonstimulated samples subtracted from the absorbance of the stimulated samples.

[2]Twelve hens per group (treatment, line).

[3]7CS = birds subjected to cold for 7 d before immunization; 5CS = birds subjected to cold for 5 d before immunization; 3CS = birds subjected to cold for 3 d before immunization; 1CS = birds subjected to cold for 1 d before immunization; and 0CS = birds were kept in type 2 respiration cell maintained at 24°C continuously (control).

***$P < 0.001$.

stricted-fed to force the birds to set priorities between vital processes (Lochmiller and Deerenberg, 2000), in this case thermoregulation, various immune responses, and growth.

We used layer chicken lines divergently selected for 21 generations for high and low antibody responses to SRBC. These lines differ with respect to various immune responses and BW gain when measured under normal husbandry temperatures.

The first objective of this experiment was to determine effects of duration of CS on parameters of specific and innate immunity. In general, the specific antibody response to KLH was hardly affected by the CS treatments. In both H and L lines, antibody titers to KLH were lower in birds that were subjected to CS, whereas in the C line, higher titers were found in cold-stressed birds. These effects were, however, not significant. Similarly, also the specific CMI to KLH was not affected by the CS treatments. On the other hand, CS affected parameters of innate immunity, e.g., phagocytosis and NAb levels, respectively, with both being enhanced, especially after 1CS. A comparison of the results of this study with a previous study (van Loon et al., In press) in which chickens were kept for a prolonged period under cold environmental free-range conditions indicates that only a short duration of CS affected ROI production. In other words, a chronic exposure to low temperature may not significantly affect the cellular part of the innate immunity.

Similarly, mitogen responses to Con A, which combines both features of T-cell responses and innate (macrophage derived IL-1) immunity, were enhanced in all birds that were cold stressed. Chickens subjected to the CS continuously for 7CS showed higher ($P < 0.05$) nonspecific cellular immunity to ConA response when compared with the control group (Table 2). This finding is in agreement with a previous study (van Loon et al., In press) and also suggests that the innate instead of the specific immune system is more sensitive to CS. Opposite to the specific antibody response, the mitogenic response was enhanced the most in the H line but little in the L line. The enhanced mitogen responses to ConA in vitro were measured at d 32 post CS treatment. It remains to be established in future studies whether this enhancement reflects the stress effect or is due to an enhanced rebound response to an earlier decreased mitogen responses to ConA during or shortly after CS.

Our second purpose was to indicate the involvement of the genetic background of the birds in the response to the CS treatments. The current results showed little or no

TABLE 4. Average BW[1] and BW gain[2] from high (H), control (C) and low (L) line hens[3] subjected to one of the five durations of cold stress treatment before immunization with keyhole limpet hemocyanin (KLH)

Line	Treatment[4]	BW (g)	BW gain (g)
H	7CS	276.8	49.4[a]
	5CS	293.3	64.6[c]
	3CS	297.7	75.2[bc]
	1CS	302.2	89.4[b]
	0CS	292.9	89.3[b]
C	7CS	275.7	41.8[a]
	5CS	291.0	64.1[b]
	3CS	307.0	70.4[bc]
	1CS	309.3	83.8[cd]
	0CS	308.6	93.3[d]
L	7CS	276.4	39.2[a]
	5CS	286.9	55.4[ab]
	3CS	307.1	65.9[b]
	1CS	310.4	83.3[c]
	0CS	298.4	100.3[d]
SEM		18.5	5.71
Main effect			
Treatment		NS	***
Line		NS	NS
Treatment × line		NS	NS
Time		***	
Time × treatment		***	
Time × line		NS	
Time × treatment × line		NS	

[a–d]Means within treatment and line group with no common superscript differ significantly ($P < 0.05$).

[1]Values are least square means ± SEM of the complete experimental period.

[2]Body weight at 34 d of age minus body weight at 24 d of age.

[3]Twelve hens per group (treatment, line).

[4]7CS = birds subjected to cold for 7 d before immunization; 5CS = birds subjected to cold for 5 d before immunization; 3CS = birds subjected to cold for 3 d before immunization; and 1CS = birds subjected to cold for 1 d before immunization. 0CS = birds were kept in a type 2 respiration cell maintained at 24°C continuously (control).

***$P < 0.001$.

treatment by line interactions, indicating that the three lines responded similarly to the CS treatments, with the exception of phagocytosis. Only in this assay, difference in response of the three lines to the CS treatments was found. Together these results indicate that divergent selection might have resulted in such a fixation of genes that little variation is left to respond differently to various CS treatments in the H and L lines. Effects of CS were most pronounced in the immune responses to which the lines had not been selected for (specific antibodies in the L line and CMI in the H line), whereas most variation was still found in the C line. The latter suggests that the lack of difference in responses to CS were not due to lack of variation within the original founder line. However, our results suggest that divergent selection for immune response genes did not affect CS responses. Future analyses of gene expression during stress in the current lines, therefore, probably requires identification of different quantitative trait loci than those that are currently developed for measuring immune responses within these lines.

Our third objective was to find a possible time point that the birds of the three lines have adapted to the CS environment; in other words that the birds respond similarly to the birds not exposed to CS during antigenic challenge. As discussed above, specific antibodies to KLH were not significantly affected in the three lines with any CS treatment. This result indicates that the durations of CS treatments to the current lines, at this age, and with the level of feed restriction were not severe enough to prevent the birds from all three lines to cope with an antigen-specific antibody response compared to the non-stressed birds. Determination of NAb was included in the current study because 1) NAb next to complement form an important part of humoral innate immunity and the first line of defense, 2) they may determine subsequent specific immunity, and 3) with respect to protein content, they constitute a very large part of the antibody repertoire and antigen-binding globulines (Jerne, 1979; Ochsenbein et al., 1999). With respect to the innate antibodies, only 1CS treatment enhanced NAb levels in the C and L lines, but the birds of these lines showed an adaptive response to the longer CS treatments. All the CS treatments resulted in enhanced mitogenic (CMI) responses that could still be measured at 32 d after ending the CS treatments. With the exception of the L line, the other two lines did not show any adaptive response to the CS treatments in terms of mitogen responses to ConA.

Our fourth objective was to force the animals to set priorities between vital processes, e.g., thermoregulation, specific and innate immune responses, and BW gain. It was expected that birds might adapt to the cold condition with for instance energy reallocation away from growth toward thermoregulation. BW gain was considered to be a more appropriate measure rather than the BW because treatment groups were not balanced by weight at the beginning of observation. In the current experiment we did not find significant line differences in BW gain as reported earlier when the current lines were fed ad libitum (Parmentier et al., 1996; Mashaly et al., 2000). Restriction in the available resources (feed) in the present experiment may be the possible reason for not finding the line differences in terms of BW gain. Birds subjected to CS for more than 2 d (7, 5, and 3 d) had significantly lower BW gain than the control group. This finding seems logical because birds subjected to CS may have to spend more energy on thermoregulation.

Previously we found little effect of humoral immune responses on energy metabolism in growing chicks, and we speculated that combinations of simultaneous climatic stress and restricted feeding may force birds to choose among vital processes with greatest benefits (Parmentier et al., 2002). Furthermore we speculated that innate and cellular immunity as opposed to humoral immunity might be energy demanding as was also proposed by others (Klasing, 1998). Although we did not measure energy reallocation within the birds, the lack of CS effects on specific humoral immunity, but the enhancing effects on CMI, and the decreasing effects on BW gain under feed limited conditions can be interpreted in two ways as follows. First, it suggests that after thermoregulation, innate and CMI have higher priority than humoral immu-

nity and BW gain, respectively. However, it cannot be excluded that the specific humoral immune response to KLH in the CS birds was maintained at the expense of growth. Thus, studies with different restricted feed regimens should reveal reallocation between immune responses, thermo-regulation and growth.

In conclusion, different duration of CS did not affect adaptive humoral immunity. Short duration of CS enhanced phagocyte activity, and innate humoral immunity (LPS- and OVA-binding antibodies). Longer duration of CS was associated with significantly higher in vitro cellular immunity and lower BW gain. This finding suggests that birds respond immediately to CS with enhanced innate (cellular and humoral) immunity to the nonsteady-state situation, irrespective of their genetic background. Our data suggest that thermoregulation has first priority followed by adaptive cellular immunity, humoral immunity, and growth. Additional studies are, however, needed to further clarify adaptation to stressful conditions and priority setting for vital life process under stressful conditions.

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