Effect of Single or Combined Climatic and Hygienic Stress on Natural and Specific Humoral Immune Competence in Four Layer Lines

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ABSTRACT Effects of long-term climatic stress (heat exposure), short-term hygienic stress [lipopolysaccharide (LPS)], or a combination of both challenges on the immune competence of 4 layer lines was investigated. The lines were earlier characterized for natural humoral immune competence and survival rate. Eighty hens per line were randomly divided over 2 identical climate chambers and exposed to a constant high temperature (32°C) or a control temperature (21°C) for 23 d. Half of the hens housed in each chamber were i.v. injected with LPS at d 1 after the start of the heat stress period. Within each of the treatment groups, half of the hens were s.c. immunized with human serum albumin (HuSA) at d 2 after the start of the heat stress period to measure specific antibody (Ab) titers to HuSA. The effect of heat, LPS, or a combined challenge on specific Ab titers to HuSA, natural Ab titers to keyhole limpet hemocyanin or HuSA (in hens that were not immunized with HuSA), and activity of the classical and alternative complement pathways were investigated. Heat stress enhanced specific and natural immune responses. Administration of LPS enhanced natural immune responses but decreased specific immune responses. The lack of interaction between heat stress and LPS administration, except for natural Ab titers to HuSA, suggest that these were 2 independent stressors. The lines had a similar response pattern but differed in the response level. Neither natural humoral immune competence nor survival rate, for which the lines had been characterized, was indicative of the specific and natural immune response to different stressors. Lipopolysaccharide and heat stress initiated sequential responses over time, with an earlier effect of short-term LPS exposure (within the first and second week) and a later effect of long-term heat exposure (within the second and third week). These data suggest that LPS and heat stress affect the natural and specific immune competence of laying hens.

Key words: heat stress, lipopolysaccharide, natural antibody, specific antibody, hemolytic complement activity

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INTRODUCTION

Genetic background, environmental conditions, and their interactions influence the immunological responsiveness of animals (Gross and Siegel, 1988). Coping with environmental changes is often associated with some degree of immune suppression or immune enhancement, depending on the type, duration, and intensity of the stressor. It has been suggested that chronic stress is associated with immune-suppressive effects, whereas acute stress is associated with immune-enhancing effects (Dhabhar and Viswanathan, 2005).

Several studies have been conducted evaluating the effects of high temperature (heat stress) on the immune responses of chickens. The heterophil to lymphocyte ratio has been used as a sensitive indicator of stress, including heat stress, among chicken populations (Gross and Siegel, 1983; Mashaly et al., 2004). Heat exposure resulted in an increased heterophil to lymphocyte ratio (McFarlane and Curtis, 1989; Mashaly et al., 2004), which indicates a relationship between heat stress and nonspecific immune reactive cells (heterophil cells; Mahmoud and Yaseen, 2005). To our knowledge, this relation is not found for other parts of the nonspecific immune system, for instance, natural antibodies (**Ab**) and complement activity. We assume that these humoral parts of the nonspecific immune system will also be affected by heat stress. Hangalapura et al. (2003, 2004) observed that the immune system of chickens exposed to cold stress responded immediately with enhanced levels of natural Ab. Furthermore, heat stress affects the specific immune response. Thaxton et al. (1968) were the first to demonstrate that a high environmental temperature (41 to 45°C) affected the specific humoral immune response in young chickens. The decrease of specific Ab by exposure to heat stress was later also reported in broilers (Zulkifli et al., 2000) and laying hens (Mashaly et al., 2004). The results of heat stress on specific immune responses, however, were not consistent. Heller et al.

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(1979) found significantly increased Ab titers following heat exposure, whereas Donker et al. (1990) found that heat exposure did not affect Ab titers. Differences in immune responsiveness to heat stress may depend on the duration and intensity of the heat exposure (Kelley, 1985), breed of chicken (Regnier et al., 1980), or presence of other stressors experienced at the same time.

Inhalation of environmental gram-negative bacteria (in particular their endotoxin) is a major poultry health problem (Zucker et al., 2000), because these bacteria are ubiquitous in the environment of poultry (Chapman et al., 2005). Endotoxins may affect the type and magnitude of the immune responses in poultry, which is of major importance for vaccination and health strategies (Maldonado et al., 2005). Administration of endotoxin (lipopolysaccharide, **LPS**) to chickens involves immune stimulation, such as release of interleukin-1 (Klasing and Peng, 1987) and tumor necrosis factor (Gehad et al., 2002), expression of Toll-like receptors 2 and 4 (Eicher and Cheng, 2003), and modulation of Ab responses (Gross and Siegel, 1975; Parmentier et al., 1998, 2004b; Maldonado et al., 2005). These studies with LPS have been performed mostly on young poultry (6 d through 6 wk) in broilers or laying pullets. To our knowledge, the effect of microbial challenges, mimicked by LPS, on the innate and specific humoral immunity of adult laying hens has not been studied. Furthermore, the effects and interactions of a combined heat stress and microbial challenge on humoral immunity, in the form of natural Ab and complement activity, have never been studied in poultry to our knowledge. It is, however, conceivable that interactions between heat exposure and microbial challenges commonly occur in modern poultry farming.

In a previous study (Star et al., 2007), differences in natural humoral immunity were investigated in 12 purebred layer lines. For the present experiment, 4 of the 12 lines were selected based on high or low natural immune competence and a high or low survival rate. These lines were exposed to the following environmental stressors: heat (climatic stress), LPS (hygienic stress), or a combined exposure to heat and LPS. We hypothesized that chickens are able to cope with single environmental stressors, but that problems in coping ability occur when chickens are exposed to combined environmental stressors. Because the lines were selected for natural humoral immune competence and survival rate, the present paper will focus on effects of single exposure to high temperature or LPS administration, or combined exposure to both stressors on natural and specific humoral immune competence in these 4 layer lines. Natural humoral immune competence was studied in the form of natural Ab binding to keyhole limpet hemocyanin (**KLH**) or human serum albumin (**HuSA**), and activity of the classical (**CPW**) and alternative complement pathway (**APW**). Immunization with HuSA was done to study specific humoral immune competence.

MATERIALS AND METHODS

Chickens, Housing, and Feed

Four purebred layer lines (Hendrix Genetics, Boxmeer, the Netherlands) were used: 3 White Leghorn lines (WA, WB, and WF), and 1 Rhode Island Red line (B1). These lines were characterized by a low or high survival rate and low or high natural humoral immune competence as determined in a previous study (Star et al., 2007). Line B1 was characterized by a low survival rate and high natural humoral immune competence, line WA was characterized by a high survival rate and low natural humoral immune competence, line WB was characterized by a low survival rate and low natural humoral immune competence, and line WF was characterized by a high survival rate and high natural humoral immune competence.

At 22 wk of age, 80 hens per line (320 in total) were transported from a housing facility at Hendrix Genetics to 2 identical climate respiration chambers at Wageningen University. In each climate chamber, 40 hens per line were individually housed in battery cages (45 cm height × 40 cm depth × 24 cm width). The lines were randomly divided over the cages. Hens were fed a standard commercial phase 1 diet (15.9% CP, 3.9% crude fiber, and 11.8 MJ of ME/kg). At 22 wk of age, hens were kept under a 13L:11D light scheme. In the following 2 wk, the light period was increased by 1 h. At the start of the experimental period (at 24 wk of age), hens were kept under a 15L:9D light scheme until the end of the experimental period (27 wk of age). Hens received routine vaccinations to Marek's disease (d 1), Newcastle disease (wk 2, 6, 12, 15), infectious bronchitis (d 1, wk 2, 10, 12, 15), infectious bursal disease (wk 3, 15), fowl pox (wk 15), and avian encephalomyelitis (wk 15). Beak trimming was not performed.

Experimental Design

After an adaptation period of 12 d (temperature maintained at 21°C), hens in the first climate chamber were exposed to acute heat stress. Within approximately 1 h, the temperature in this chamber was increased from 21 to 32°C, and was maintained at 32°C for the following 23 d. In the second chamber, the (control) temperature was maintained at 21°C. At d 1 after the start of the heat stress period, half of the hens in the heat treatment and half of the control hens were i.v. injected with 1 mg/kg of BW of *Escherichia coli* LPS (serotype O55:B5, Sigma Chemical Co., St. Louis, MO). The remaining hens received a placebo treatment of PBS. At d 2 after the start of the heat stress period, half of the hens from each experimental group (4 treatment groups × 4 lines × 10 hens) were s.c. immunized with HuSA (Sigma Chemical Co.) to measure the specific Ab response of the hens. Hens that were not immunized with HuSA received a PBS placebo treatment. An overview of the experimental design is given in Table 1.

Table 1. Experimental design[1]

Item	Treatment group	Temperature (°C)	HuSA[2]	Birds (n)
21°C + PBS	Control natural antibody titer (NAb)	21	–	40
	Control specific antibody titer (SpAb)	21	+	40
21°C + LPS	Effect of LPS on NAb	21	–	40
	Effect of LPS on SpAb	21	+	40
32°C + PBS	Effect of heat on NAb	32	–	40
	Effect of heat on SpAb	32	+	40
32°C + LPS	Effect of heat and LPS on NAb	32	–	40
	Effect of heat and LPS on SpAb	32	+	40

[1]There were 4 treatment groups, which were exposed to a temperature of 21 or 32°C for 23 d, and were i.v. injected with *Escherichia coli* lipopolysaccharide (LPS) or PBS at d 1 after the start of the heat stress period. Within these 4 treatment groups, half of the hens were s.c. immunized with human serum albumin (HuSA; control with PBS) at d 2 after the start of the heat stress period. Within each treatment group, 4 genetically different purebred layer lines, characterized by natural humoral immune competence and survival rate, were equally represented. Line B1 was characterized by a low survival rate and high natural humoral immune competence, line WA was characterized by a high survival rate and low natural humoral immune competence, line WB was characterized by a low survival rate and low natural humoral immune competence, and line WF was characterized by a high survival rate and high natural humoral immune competence.

[2]+ = hens immunized with HuSA; – = hens not immunized with HuSA.

Immune Parameters

Blood samples were collected from the wing vein of all 320 individual hens at d 5 prior to the start of heat stress and at d 2, 5, 8, 15, and 22 after the start of heat stress. After sampling, blood was centrifuged and serum was stored at –20°C until further processing.

Humoral Immune Response to HuSA and KLH. Antibody titers to HuSA and KLH were determined in individual samples by an indirect ELISA procedure at d –5, 2, 5, 8, 15, and 22 after the start of the heat stress period. Flat-bottomed 96-well medium-binding ELISA plates were coated with 4 μg/mL of HuSA or 1 μg/mL of KLH (MP Biomedicals Inc., Aurora, OH). After subsequent washing, the plates were incubated with serum (diluted 1:60, 1:360, 1:2,160, and 1:12,960 for HuSA, and diluted 1:30, 1:90, 1:270, and 1:810 for KLH). Binding of the Ab to HuSA and KLH antigen was visualized by using a 1:20,000 diluted rabbit antichicken IgG_{H+L} labeled with peroxidase (RACh/IgG_{H+L}/PO, Nordic, Tilburg, the Netherlands). After washing, substrate (tetramethylbenzidine and 0.05% H_2O_2) was added, and 10 min later, the reaction was stopped with 2.5 *N* H_2SO_4. Extinctions were measured in a microplate reader (Multiskan, Labsystems, Helsinki, Finland) at a wavelength of 450 nm. Levels (titers) were expressed as $\log_2$ values of the dilutions that gave extinction closest to 50% of E_{max}, where E_{max} represents the highest mean extinction of a standard positive serum present on each flat-bottomed ELISA plate.

Hemolytic Complement Assay. Classical complement pathway and APW were determined in individual samples collected at d –5, 2, 5, 8, 15, and 22 after the start of the heat stress period. The hemolytic complement assay was performed according to the method described by Demey et al. (1993). Briefly, appropriate buffers were prepared. The buffer solution for CPW was prepared by adding $MgCl_2$ (1 mmol/L) and $CaCl_2$ (0.15 mmol/L) to veronal-buffered saline. The buffer solution for APW was prepared by adding $MgCl_2$ (5 mmol/L) and ethylene glycol tetraacetate (16 mmol/L) to veronal-buffered saline.

The assay was performed in flat-bottomed 96-well microtiter plates. Sera were diluted serially in the appropriate buffers and incubated with sensitized (ref. no. 72202, Haemolysin, bioMérieux, Marcy l'Étoile, France) sheep erythrocytes or bovine erythrocytes prepared by standard methods and used as a 1% cell suspension to measure CPW or APW, respectively. During 1.5 h of incubation, the plates were shaken every 30 min. The results (the amount of light scattering by erythrocytes upon lysis) were read in a microplate reader (Multiskan, Labsystems) at a wavelength of 690 nm. Readings were transformed by a log-log equation (Von Krogh, 1916), and the hemolytic titer was expressed as the titer that lyses 50% of the red blood cells (CH50 U/mL).

Statistical Analysis

Differences in titers of natural and specific Ab binding to HuSA, natural Ab binding to KLH, and activity of CPW and APW were analyzed by a 4-way ANOVA for the effect of line, temperature, LPS administration, time, and their interactions by a repeated measurement procedure using a "hen nested within line, temperature, and LPS administration" option. The effect of HuSA immunization was not included in the statistical model, because this immunization only affects the HuSA titer. Therefore, we choose to analyze hens immunized with HuSA separately from hens not immunized with HuSA. In this way, we were able to test whether temperature and LPS administration were of influence on the levels of specific Ab binding to HuSA in immunized hens, and whether these treatments were of influence on the levels of natural Ab binding to HuSA in nonimmunized hens.

Mean differences among lines and treatments were tested with Bonferroni's test. The PROC MIXED of SAS

Table 2. Effect of temperature, *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on the average specific and natural antibody (Ab) titer[1] to human serum albumin (HuSA)[2] and natural Ab titer to keyhole limpet hemocyanin (KLH)[3] in 4 purebred layer lines characterized by natural humoral immune competence and survival rate

Line	Treatment group	Specific Ab[4] (HuSA)	Natural Ab	
			HuSA[4]	KLH[4]
B1	21°C + PBS	7.10	3.52	3.46
	21°C + LPS	6.27	4.30	4.51
	32°C + PBS	7.19	3.40	3.92
	32°C + LPS	6.77	3.30	4.47
WA	21°C + PBS	7.16	2.95	2.41
	21°C + LPS	6.03	3.31	2.74
	32°C + PBS	7.28	3.36	2.78
	32°C + LPS	6.57	2.80	3.60
WB	21°C + PBS	6.43	3.26	3.43
	21°C + LPS	6.19	3.34	3.68
	32°C + PBS	6.03	3.52	3.61
	32°C + LPS	5.84	3.04	3.72
WF	21°C + PBS	6.80	3.18	4.05
	21°C + LPS	6.12	3.26	4.44
	32°C + PBS	6.99	3.36	3.95
	32°C + LPS	6.61	2.98	4.90
	SEM	0.28	0.32	0.26
Effect	Line (L)	**	NS	***
		B1, WA ≥ WF ≥ WB		WF ≥ B1 ≥ WB > WA
	Temperature (T)	NS	NS	*
				32°C > 21°C
	LPS administration (A)	***	NS	***
		PBS > LPS		LPS > PBS
	Time (Ti)	***	***	***
	L × T	NS	NS	NS
	L × A	NS	NS	NS
	L × Ti	***	NS	**
	T × A	NS	*	NS
	T × Ti	***	*	**
	A × Ti	***	NS	***
	L × T × A	NS	NS	NS
	L × T × Ti	**	NS	NS
	L × A × Ti	NS	NS	NS
	T × A × Ti	NS	NS	NS
	L × T × A × Ti	NS	NS	NS

[1]Titers were expressed as $\log_2$ values of the dilutions that gave extinction closest to E_{max}, where E_{max} represents the highest mean extinction of standard positive serum presented on each flat-bottomed ELISA plate.

[2]Specific Ab titers to HuSA were measured in hens immunized with HuSA (n = 159), and natural Ab titers to HuSA were measured in hens not immunized with HuSA (n = 158).

[3]Natural Ab titers to KLH were measured in all hens (n = 317), irrespective of HuSA immunization.

[4]Values are least squares means of Ab titers determined in serum samples collected at d –5, 2, 5, 8, 15, and 22 after the start of the heat stress period. Half of the hens of the heat stress and control groups were injected i.v. with 1 mg/kg of BW of LPS at d 1 after the start of the heat stress period. Half of the LPS- and PBS-treated hens were s.c. immunized with 1 mg of HuSA at d 2 after the start of the heat stress period. The data presented in the table were analyzed by 4-way ANOVA for the effects of line, temperature, LPS administration, time, and their interactions by a repeated measurement procedure with a hen nested within line, temperature, and LPS administration option.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$.

was used for statistical analysis (SAS Institute, 2004). Effects were considered significant at $P < 0.05$.

RESULTS

Within 1 d after LPS administration, 3 hens died (1 of line B1 and 2 of line WA). These hens were exposed to the combined challenge of LPS and heat. All other hens survived the experimental period.

Ab Responses to HuSA and KLH

Results of the effects of single or combined heat exposure and LPS administration on specific and natural Ab binding to HuSA, and on natural Ab binding to KLH in the 4 layer lines are shown in Table 2.

Specific Ab Response to HuSA. Levels of specific Ab binding to HuSA were increased at d 8 and 15 after the start of the heat stress period (d 6 and 13 after HuSA immunization), and were still increased at d 22 after the start of the heat stress period, although the levels were lower than at d 8 and 15.

The total specific Ab response to HuSA was affected by LPS administration ($P < 0.0001$); at d 8, 15, and 22 after the start of the heat stress period, LPS-treated hens had a lower level of specific Ab to HuSA than PBS-treated hens (LPS × time interaction; $P < 0.0001$).

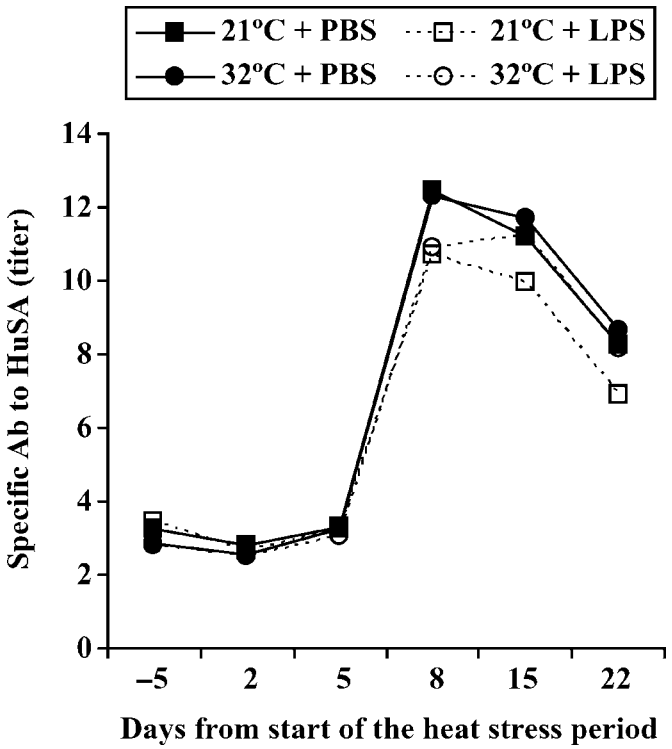

Figure 1. Effect of heat exposure, administration of *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on the specific antibody (Ab) response to human serum albumin (HuSA) of laying hens (least squares mean). Heat exposure was maintained for 23 d, with the start of the heat stress period at d 0. Lipopolysaccharide was i.v. injected at d 1 and HuSA was s.c. injected at d 2. Within each treatment group (n = 40 hens per treatment group, except for treatment group 32°C + LPS, where n = 39), 4 genetically different purebred layer lines, characterized by natural humoral immune competence and survival rate, were equally represented.

The total specific Ab response to HuSA was affected by a line × heat × time interaction ($P < 0.0001$). Heat exposure enhanced the specific Ab response to HuSA at d 15 and 22 after the start of heat stress, except for line WB, in which heat-stressed hens had a lower specific Ab response to HuSA than hens from line WB exposed to the control temperature (Table 2). Furthermore, line WB had the highest Ab titers to HuSA at d 8 after the start of the heat stress period, and Ab titers were already decreased at d 15 and were further decreased at d 22, whereas the other lines showed similar Ab titers at d 8 and 15, and were decreased at d 22 (data not shown). The effects of heat stress and LPS administration over time are shown in Figure 1.

Natural Ab Response to HuSA. The total natural Ab response to HuSA during the observation period was affected by a heat × LPS interaction ($P < 0.05$; Table 2). Single heat exposure or LPS administration increased natural Ab to HuSA, whereas combined exposure to heat and LPS decreased natural Ab binding to HuSA compared with the control group. Furthermore, the total natural Ab response to HuSA during the observation period was affected by a heat × time interaction ($P < 0.05$; Table 2); at d 5 prior to the start of heat stress, hens in the chamber prepared for heat stress had significantly lower natural Ab binding to HuSA than hens in the chamber prepared for the control temperature. The effects of heat stress and LPS administration over time are shown in Figure 2A. No line effects or interactions with line were found.

Natural Ab Response to KLH. Levels of natural Ab binding to KLH in the sera of hens treated with LPS increased at d 5 after the start of the heat stress period (d 4 after LPS administration), were highest at d 8 after the start of the heat stress period, and were decreased to the baseline level at d 22 after the start of the heat stress period.

During the complete observation period, the natural Ab response to KLH was affected by heat ($P < 0.05$), by LPS ($P < 0.0001$), and by line ($P < 0.0001$; Table 2). Each of the main effects had an interaction with time [heat × time ($P < 0.01$), LPS × time ($P < 0.0001$), line × time ($P < 0.01$)]. The effects of the treatments over time are shown in Figure 2B. Heat increased the natural Ab response to KLH at d 8 and 15 after the start of the heat stress period, whereas LPS increased the natural Ab response to KLH at d 5, 8, and 15. The line × time interaction suggests that the lines had different response patterns (data not shown). Line WF had the highest levels of natural Ab binding to KLH during the complete observation period, whereas line WA had the lowest level of natural Ab binding to KLH (Table 2).

Hemolytic Complement Activity

Results of the effects of single or combined heat exposure and LPS administration on CPW and APW in the 4 layer lines are shown in Table 3.

CPW. Activity of CPW was enhanced by LPS administration at d 5 and 8 after the start of the heat stress period (LPS × time interaction; $P < 0.01$), and by heat exposure at d 22 after the start of the heat stress period (heat × time interaction; $P < 0.0001$). Effects of the treatments over time are shown in Figure 3A.

There was a line × time interaction ($P < 0.01$); differences between the lines were found only at d 5 prior to the start of the heat stress period, where line WA had higher CPW activity than line WB (data not shown). No interactions between treatments and lines were found.

APW. Activity of APW was enhanced by LPS administration at d 2 and 8 (LPS × time interaction; $P < 0.01$). Activity of APW was decreased by heat exposure at d 5 after the start of the heat stress period, but was increased by heat exposure at d 8, 15, and 22 after the start of the heat stress period (heat × time interaction; $P < 0.0001$). Effects of the treatments over time are shown in Figure 3B.

Line differences were found at each sample moment. Prior to the start of the heat stress period (d –5), line WA had the highest APW activity and line B1 the lowest. At d 2, 5, 8, 15, and 22 after the start of the heat stress period, line WF had the highest APW activity and line WA the lowest APW activity [line × time interaction; $P < 0.0001$ (data not shown)]. Furthermore, there was a

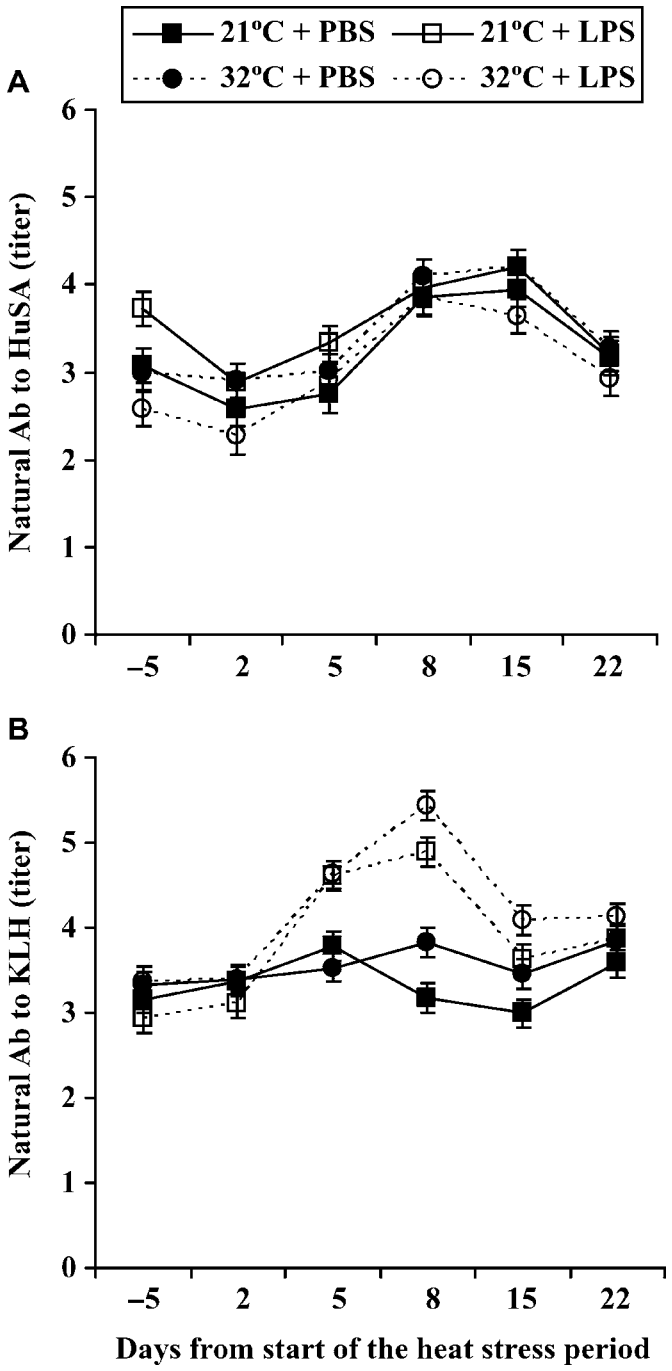

Figure 2. Effect of heat exposure, administration of *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on natural antibody (Ab) response to (A) human serum albumin (HuSA) or (B) keyhole limpet hemocyanin (KLH) of laying hens (least squares mean ± SE). Heat exposure was maintained for 23 d, with the start of the heat stress period at d 0. Lipopolysaccharide was i.v. injected at d 1. Within each treatment group (n = 40 hens per treatment group for natural Ab to HuSA, except for treatment group 32°C + LPS, where n = 38; n = 80 hens per treatment group for natural Ab to KLH, except for treatment group 32°C + LPS, where n = 77), 4 genetically different purebred layer lines, characterized by natural humoral immune competence and survival rate, were equally represented.

line × heat × time interaction ($P < 0.01$), indicating that lines had different APW activity in response to heat stress over time. Most lines showed a comparable pattern. The only line that differed in the response pattern was line WA. Where lines B1, WB, and WF showed an increase in APW activity from d 8 to 15 when exposed to heat, line WA showed a decrease in APW activity when exposed to heat. Irrespective of temperature, line WA showed an increase in APW activity from d 15 to 22, whereas lines B1, WB, and WF showed a decrease (data not shown).

DISCUSSION

In the present study, the effects of single or combined environmental stressors on humoral immune competence in 4 layer lines were investigated. Natural Ab to KLH or HuSA as well as CPW and APW were measured to study the natural humoral immune competence. Immunization with HuSA was done to study the specific humoral immune competence. Exposure to a high temperature for 23 d and single administration of the endotoxin LPS were used as environmental stressors. The administered dose of LPS (i.v. 1 mg/kg of BW) was based on previously reported effects of LPS on poultry (Klasing et al., 1987; Parmentier et al., 2004b; Maldonado et al., 2005) to ensure that the amounts given would result in measurable effects that could be distinguished from the effects originating from the intestinal microbiota within poultry kept under normal (LPS-rich) housing conditions. The protein HuSA was used to avoid possible interference with earlier obligatory vaccinations of poultry, and the administered dose (s.c. 1 mg) was reported earlier by Maldonado et al. (2005).

In the present study, heat stress and LPS administration acted as 2 independent stressors. This was illustrated by the lack of interaction between heat stress and LPS administration (except for natural Ab binding to HuSA), and by interactions over time. The response to LPS administration was more pronounced in the first part of the experimental period, whereas the response to heat was more pronounced in the second part of the experimental period. In this respect, it is noteworthy that poultry become refractory to repeated LPS administrations (Korver et al., 1998; Parmentier et al., 2006). Although the present data suggest that single LPS administration was more acute and that the effect of long-term heat stress was more extended, the data do not suggest that recovery from LPS had priority over recovery from heat exposure. Furthermore, our data do not confirm the findings by Dhabhar and Viswanathan (2005), who suggested immune-suppressive effects of chronic stress and immune-enhancing effects of acute stress.

The 4 purebred lines that were used in this study were characterized in a previous experiment (Star et al., 2007) by high or low natural immune competence and a high or low survival rate. Lines B1 and WF were selected for high natural immune competence, whereas lines WA and WB were selected for low natural immune competence. Ranking of the lines for natural Ab binding to KLH and activity of CPW and APW (data not shown) in the present study was consistent with the previous

Table 3. Effect of temperature, *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on average classical complement pathway (CPW)[1] and alternative complement pathway (APW)[1] activity in 4 purebred layer lines characterized by natural humoral immune competence and survival rate

Line	Treatment group	CPW[2]	APW[2]
B1	21°C + PBS	331	101
	21°C + LPS	314	95
	32°C + PBS	382	109
	32°C + LPS	335	116
WA	21°C + PBS	296	93
	21°C + LPS	307	89
	32°C + PBS	322	93
	32°C + LPS	357	101
WB	21°C + PBS	271	87
	21°C + LPS	306	104
	32°C + PBS	300	88
	32°C + LPS	338	107
WF	21°C + PBS	300	124
	21°C + LPS	328	133
	32°C + PBS	350	114
	32°C + LPS	341	121
	SEM	27	9
Effects	Line (L)	NS	*** WF > B1, WA, WB
	Temperature (T)	* 32°C > 21°C	NS
	LPS administration (A)	NS	NS
	Time (Ti)	***	***
	L × T	NS	NS
	L × A	NS	NS
	L × Ti	**	***
	T × A	NS	NS
	T × Ti	***	***
	A × Ti	**	***
	L × T × A	NS	NS
	L × T × Ti	NS	**
	L × A × Ti	NS	NS
	T × A × Ti	NS	NS
	L × T × A × Ti	NS	NS

[1]CPW and APW activity were measured in all hens (n = 317), irrespective of HuSA immunization.

[2]Values are least squares means of complement activity determined in serum samples collected at d –5, 2, 5, 8, 15, and 22 after the start of the heat stress period. Half of the hens of the heat stress and control groups were injected i.v. with 1 mg/kg of BW of LPS at d 1 after the start of the heat stress period. Half of the LPS- and PBS-treated hens were s.c. immunized with 1 mg of HuSA at d 2 after the start of the heat stress period. The data presented in the table were analyzed by 4-way ANOVA for the effects of line, temperature, LPS administration, time, and their interactions by a repeated measurement procedure with a hen nested within line, temperature, and LPS administration option.

*$P < 0.05$; **$P < 0.01$; ***$P < 0.0001$.

study. The absence of significant interactions between line and heat stress (except for specific Ab directed to HuSA) or LPS administration on natural and specific humoral immune responses suggests that, regardless of genotype, hens responded similarly to the environmental stressors. However, the lines differed in their response level and reacted differently over time, indicating the absence of a relationship between genotype and environment. Because the lines were characterized by high or low natural humoral immune competence and a high or low survival rate, we expected that the lines would react differently to environmental stressors. However, the characteristics of the lines were not indicative of the specific and natural immune responses to different stressors.

According to the definition of natural Ab (i.e., that there is no intentional or controllable challenge with the antigen leading to the formation of Ab), KLH and HuSA were chosen to estimate levels of natural Ab. Keyhole limpet hemocyanin is a "classical" antigen used to measure natural Ab. Keyhole limpet hemocyanin is an exoantigen that the chickens most probably had not encountered before nor would encounter during their lives. Half of the hens were not immunized with HuSA; therefore, we used these hens to measure natural Ab to HuSA. In the present study, natural Ab to KLH or HuSA were found in all chickens, irrespective of LPS administration, as previously found by Parmentier et al. (2004a) and Star et al. (2007). To some extent, single administration of LPS enhanced natural Ab titers to HuSA and more pronounced natural Ab titers to KLH. Enhancement of LPS Ab titers after immunization with KLH was reported earlier (Hangalapura et al., 2003, 2005; Parmentier et al., 2004b). Lipopolysaccharide administration also enhanced CPW and APW. Administration of LPS in blood activates APW activity almost immediately (Blatteis,

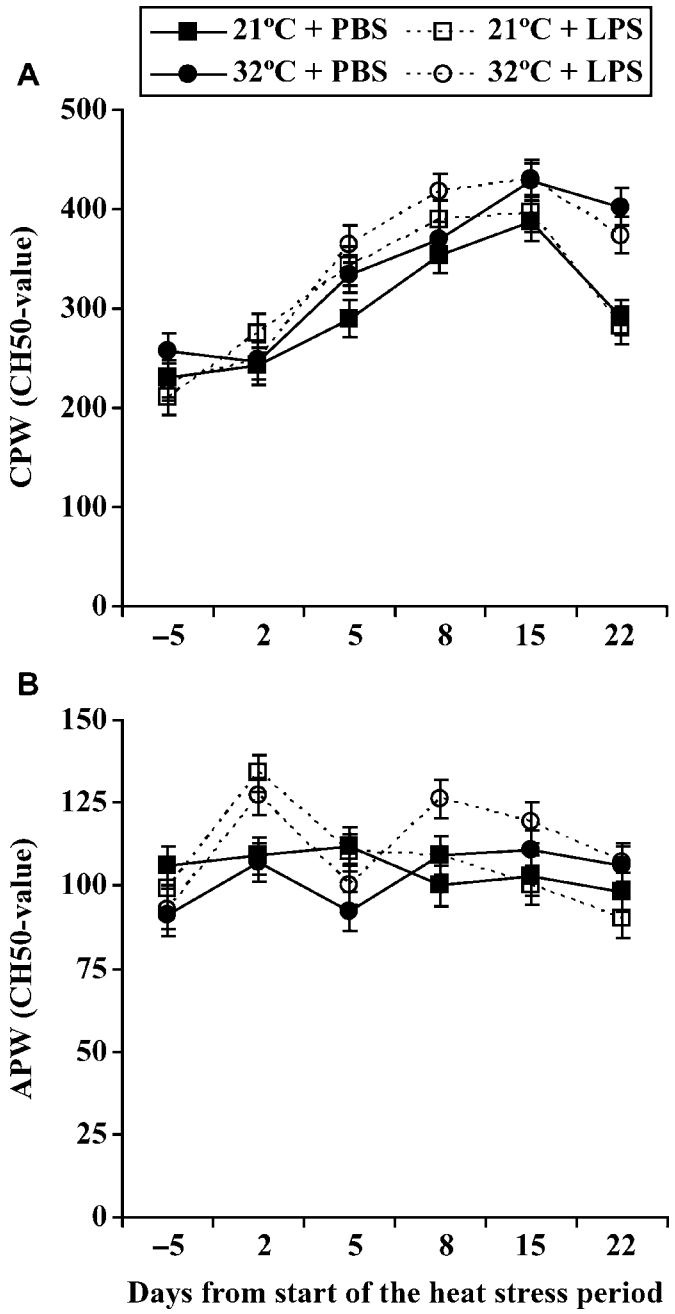

Figure 3. Effect of heat exposure, administration of *Escherichia coli* lipopolysaccharide (LPS), or combined exposure to both stressors on activity of the (A) classical complement pathway (CPW) and (B) alternative complement pathway (APW) of laying hens (least squares mean ± SE). Heat exposure was maintained for 23 d, with the start of the heat stress period at d 0. Lipopolysaccharide was i.v. injected at d 1. Within each treatment group (n = 80 hens per treatment group, except for treatment group 32°C + LPS, where n = 77), 4 genetically different purebred layer lines, characterized by natural humoral immune competence and survival rate, were equally represented.

2006), which may be related to the more innate character of APW, as opposed to the more specific character of CPW, although CPW activity was not influenced by HuSA immunization (data not shown). These data suggest that natural Ab and hemolytic complement activity were stimulated by LPS, which confirms the findings of Reid et al. (1997) and Fischer et al. (1997) that the innate immune system is sensitive to microbial components and that it plays an important role in the host defense against bacterial products.

In contrast to the enhancement of natural Ab and complement by LPS administration, specific Ab titers to HuSA were decreased by LPS administration. These results correspond with the reported decreasing effects of LPS on specific Ab titers to BSA, KLH, and HuSA (Parmentier et al., 1998, 2004b, 2006; Maldonado et al., 2005). The dose of LPS and the stimulation of dendritic cells by LPS might affect the balance of Th-1- and Th-2-stimulating cytokines (Langenkamp et al., 2000; Boonstra et al., 2003), and changes in the balance between Th-1 and Th-2 can cause a shift in stimulation of natural or specific immune responses.

In the present study, exposure to heat stimulated natural and specific immune responses. Previous studies on the effect of heat stress on specific immune responses were not consistent. Most studies have described a decreasing effect of heat stress on specific immune responses (Thaxton et al., 1968; McFarlane and Curtis, 1989; Zulkifli et al., 2000; Mashaly et al., 2004), but enhancing effects (Heller et al., 1979) and no effects at all (Donker et al., 1990) were also described. These differences may depend on the length and intensity of heat exposure (Kelley, 1985) or the breed of chicken (Regnier et al., 1980) because genetic variation in heat tolerance is known to exist within species (Mahmoud and Yaseen, 2005), as also indicated by the different response levels of the lines used in the present study. To our knowledge, the enhancing effect of heat stress on natural Ab and complement activity has not been published before. Hangalapura et al. (2003, 2004) observed that the innate immune system of chickens exposed to cold stress (10°C) responded with enhanced natural Ab responses. These data, as well as the present study, suggest a stimulating effect of high or low temperature on innate immune competence. Hangalapura et al. (2003, 2004), however, found that the innate immune system responded immediately to changes in temperature, whereas in our study the effect of heat stress on the innate immune response was extended. Stimulation of the immune system by heat was affected by time, indicating that the effect of heat exposure was not present during the whole experimental period. Our data show that innate and specific immune responses were not affected by heat exposure during the first week, but were mainly affected during the second and third weeks after the start of heat stress.

Our data suggest that LPS and heat stress affected the natural and specific humoral immune competence of laying hens. However, the data also indicate that, based on natural and specific immune competence, hens were able to cope with single or combined heat stress and LPS administration. Furthermore, LPS and heat stress initiated sequential responses over time, with an earlier effect of short-term LPS exposure (within the first and second week) and a later effect of long-term heat exposure (within the second and third week). It has yet to be investigated whether this indicates a priority setting

of the chicken between acute or nonacute life-threatening situations or, alternatively, the ability to differentiate between an immunogenic and a physical stressor.

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