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Immunological and Physiological Differences Between Layer- and Broiler Chickens after Concurrent Intratracheal Administration of Lipopolysaccharide and Human Serum Albumin

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Abstract: Layers and broilers were concurrently intratracheally challenged with 0.5 mg Lipopolysaccharide (LPS) and 0.1 mg Human Serum Albumin (HuSA) at 3 weeks of age. Specific total and isotype-specific (IgM, IgG, IgA) Antibody (Ab) responses to HuSA during 3 weeks following immunization, cellular *in vitro* mitogen responses to Concanavalin A (Con A) and specific cellular responses *in vitro* to different dosages of HuSA, blood serotonin (5-HT) levels, plasma Corticosterone (CORT) levels at 6 weeks of age and *ex vivo* nitric oxide (NO) production in the presence of LPS, respectively, were measured in all birds. Higher *in vitro* cellular responses to HuSA, but not Con A, were found in the broilers than in the layers. Also higher total, IgM and IgG antibody responses to HuSA were found in the broilers. Higher *ex vivo* NO production was found in the layers. A heavier spleen weight was found in the broilers, but relative spleen weight was higher in the layers. The broilers grew much heavier and also maintained a higher growth during the first 24 and 48 h after i.t. challenge with LPS and HuSA. No breed effect was found for body temperature responses after i.t. challenge. Blood 5-HT levels and plasma CORT levels were significantly higher in the layers. Number and type of significant correlations between 5-HT levels, cachectin response to LPS, antibody levels and cellular immunity differed between breeds. Our data suggest comparable immune responses to i.t. HuSA challenge in broilers and layers of similar age and confirm the earlier reported higher humoral immune response in broilers. On the other hand, the cachectin response to LPS differed between broilers and layers. Our results do not confirm the earlier reported higher cellular immune response of layers. Different significant relationships between physiological parameters in broilers and layers were found. Our results suggest that selection for enhanced growth does not necessarily affect specific immune competence of poultry.

Key words: LPS, HuSA, layer, broiler, intratracheal, immune response

INTRODUCTION

Genetic selection of poultry resulted in two major different chicken breeds; broilers and layers which substantially differ in Body Weight (BW) gain, longevity, nutritional needs and probably also immune responsiveness. A previous comparative study between one broiler (Ross) and one layer line (White Leghorn) suggested that broilers are more specialized in mounting strong short (innate) term humoral (IgM) immune responses, while layers are specialized in a long term humoral (IgG) immune response in combination with a strong cellular response to the model antigen Trinitrophenyl-conjugated Keyhole Limput Hemocyanin (TNP-KLH) after systemic immunization (Koenen *et al*., 2002). Also other non-comparative studies in broilers (Parmentier *et al*., 2008) and (selected) layer lines suggested that breeding towards a higher body weight (gain) of broilers negatively affected the humoral immune response while the genetic changes of layers towards egg production had less negative impact on the birds immune system. A higher body weight (gain) in layer lines was also related with lower immune competence (Parmentier *et al*., 1996; Siegel and Gross, 1980; Siegel *et al*., 1982). A dysfunction of the broiler's specific cellular and humoral immune system has been proposed to underlie the health problems and enhanced disease sensitivity after pressure on the immune system (Miller *et al*., 1992; Koenen *et al*., 2002).

Broilers and layers differ with respect to housing and nutrition demands. To compare immune reactivity, layers and broilers should be studied under one housing condition, which may, however, be disadvantageous for one of the breeds. Poultry houses contain high levels of airborne endotoxins originating from faeces, feed, skin, dust, plants and mould which could cause health problems in poultry (Appleby *et al*., 2004; Powers *et al*., 2005). A important airborne endotoxin is Lipolysaccharide (LPS), a component of the cell wall of gram negative bacteria (Chapman *et al*., 2005;

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Lorenzoni and Wideman, 2008). LPS is constantly challenging the birds lungs and air sacs via the air and by microbiota in the intestine (Wideman *et al*., 2009). LPS, either systemically or intratracheally administered has immunomodulating features in layers (Maldonado *et al.*, 2005; Parmentier *et al*., 2008; 2004; Ploegaert *et al*., 2007) and broilers (Lai *et al*., 2009). Although in both chicken types, refractile responses to airborne LPS were found (Lai *et al*., 2009; Parmentier *et al*., 2008), these responses were more pronounced in layers. Therefore, we compared in the present study the (immune) responsiveness to a concurrent airway challenge of one layer breed and one broiler breed of the same age with a specific (HuSA) and an innate (LPS) antigen. LPS may not only affect natural and specific humoral immune competence (Star *et al*., 2007), but may also induce a variety of behavioural, hormonal and physiological changes (Shini *et al*., 2008; Star *et al*., 2007), such as an elevation in plasma corticosterone concentration with immune suppressing consequences and consequently increase of the Heterophil to Leukocyte (H/L) ratio (Al-Ghamdi, 2008; Altan *et al*., 2003; McFlane and Curtis, 1989; Shini *et al*., 2008).

In the present study, specific (humoral and cellular) and innate (*ex vivo* NO release) immune responses after concurrent intratracheal immunization with LPS and Human Serum Albumin (HuSA) were studied in broiler (Ross) and layer (Lohmann Brown) type chickens of similar age, which were housed and fed as practised in broiler husbandry. In addition, we measured BW gain, blood serotonin (5-HT) and plasma CORT levels.

MATERIALS AND METHODS

Chickens: Eighteen 1-day old female Lohmann Brown layer chicks and seventeen one 1-day old female Ross broiler chicks were used. Per line, chicks were grouped in a pen of 2 x 1.5 m^2 with a saw dusted floor. For both broilers and layers, pens were kept at temperatures normal for broiler housing meaning a starting temperature of 32°C at arrival, that decreased with 2°C per week until a constant temperature of 21°C was reached. Birds were housed with a light schedule of 16/8 h light/ dark, respectively. All birds were fed *ad libitum* with standard broiler diet (204 g/kg crude protein, 2,859 kcal/kg metabolizable energy). Water was provided *ad libitum* via drinking nipples. All birds were vaccinated with (all live) vaccines for Newcastle disease, Infectious Bursal disease (Gumboro) and Infectious Bronchitis at hatch according to broiler management.

The experiment was approved by the Animal Welfare Committee of Wageningen University according to Dutch law.

Reagents: *Escherichia coli* derived lipopolysaccharide (L2880, serotype 055:B5) and human serum albumin (Lot 8763) were from Sigma-Aldrich Inc. (St. Louis, MO 63103).

Experimental design: At sixteen days of age, five birds of each breed received a microchip temperature transponder (Bio Medic Data Systems, Seaford, DE) for recording Body Temperature (BT) using a DAS-6007 Bio Medic Data System reader. At 20 days of age, all birds received a concurrent intratracheal (i.t.) injection with 0.1 mg HuSA and 0.5 mg LPS in 0.5 ml PBS using a blunted needle. All birds were regularly weighed and blood samples were regularly collected from the wing vein. BT was measured one h prior to and subsequently 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 24, 48 and 96 h after i.t. challenge with HuSA and LPS at 3 weeks of age. At 6 weeks of age, all birds were euthanized and the spleen was dissected and weighed.

Antibody assays: Total antibody and isotype specific IgM, IgG and IgA antibody titers to HuSA in plasma from all birds were determined by ELISA at days 0, 2, 7, 14 and 21 after i.t. immunization with HuSA and LPS at 3 weeks of age. Briefly, 96 well plates were coated with 4 µg/ml HuSA. After subsequent washing with tap water containing 0.05% Tween, the plates were incubated for 1 h at room temperature with serial four-step dilutions of plasma in PBS containing 0.5% horse serum and 0.05% Tween. Binding of total antibodies to HuSA was detected after 1 h of incubation at room temperature with 1:20,000 in PBS (containing 0.5% horse serum and 0.05% Tween) diluted rabbit anti-chicken IgG_{H+L} coupled to peroxidase (RACh/IgG_{H+L}/PO, Nordic, Tilburg, The Netherlands). IgM, IgG and IgA antibodies binding to HuSA, were determined at all days as well. After incubation with serial dilutions of plasma and subsequent washing, bound isotype-specific antibodies to HuSA were detected using 1:20,000 diluted goat anti-chicken IgM coupled to PO (GACh/IgM/PO) directed to the µ heavy chain of IgM (Bethyl, Montgomery, TX), or 1:20,000 diluted goat anti-chicken IgG_{Fc} coupled to PO (Bethyl), or 1:20,000 diluted GACh/IgA/PO (Bethyl), respectively. After incubation with the conjugate and subsequent washing, 100 µL substrate-buffer (containing aqua dest, 10% tetramethylbenzidin-buffer and 1.33% tetramethylbenzidin) per well were added and incubated for 10 min at room temperature. The reaction was stopped with 1.25 M H_2SO_4. Extinctions were measured with a Multiscan spectrophotometer (Labsystems, Helsinki, Finland) at a wavelength of 450 nm. Titers were expressed as the log_2 values of the dilutions that gave an extinction closest to 50% of Emax, where Emax represents the highest mean extinction of a standard positive (pooled) serum present on every microtiter plate.

***In vitro* cellular immunity:** A whole blood stimulation test was used to determine specific cellular reactivity *in vitro*. Briefly, heparinized blood from all birds obtained at 6 weeks of age was diluted 1:60 in RPMI-1640 culture

medium (N6846, Biowittaker, Cambrex) and 100 µl was added per well of a 96-well flat bottom culture plate. Triplicate cultures were incubated with either 100 µl Con A (20 µg/ml), or 10-, 50- and 100 µg/ml HuSA, respectively, or RPMI culture medium (control) for 48 h in a humidified incubator at 41°C with 5% CO_2. Then 0.4 µCi ^{3}[H]-thymidine were added per well and incubated overnight. Plates were stored at -20°C before harvesting. After thawing of the plates, they were harvested onto fiberglass filters and filters were counted by liquid scintillation spectroscopy. Data are presented as stimulation index (SI = stimulated counts (cpm/control counts (cpm)).

***Ex vivo* nitric oxide production:** An *ex vivo* oxide production test was used to determine activity of Peripheral Blood Leucocytes (PBL) in response to LPS. PBL were isolated from 1 ml heparinized blood, collected on day 0, 7, 14 and 21 post i.t. challenge from 8 layers and 10 broilers using a discontinuous Histopaque gradient (Sigma) with density 1.191 in 2 ml Eppendorf tubes. After centrifugation for 2 min at 12.000 g, PBL were collected from the interphase and washed twice in 1 ml RPMI-1640 containing penicillin (13.2 µg/ml) and streptomycin (20 µg/ml) and finally dissolved in 1 ml of the same tissue culture medium. Sixty nine well flat-bottomed culture plates were filled with 100 µg/ml RPMI medium containing 20 µg LPS and 100 µl of isolated cells in triplicate. Cells of each sample were incubated for 48 h at 41°C, 5% CO_2 and 100% humidity. After incubation, 50 µl culture supernatant was transferred to the wells of flat-bottomed microtiter plates and combined with 50 µl Griess reagent (10) in a 1:1 ratio. After 10 min incubation at RT the absorbance of each well was measured at 510 nm. Sodium nitrate in a dilution series was used as a standard to determine nitrite concentration in supernatants.

Blood serotonin levels: Blood 5-HT levels were determined as described earlier (Bolhuis *et al*., 2009) in all plasma samples obtained at 6 weeks of age. Most of avian blood 5-HT is localized in platelets (Sorimachi *et al*., 1970) and blood 5-HT concentration correlates with that in platelets (Bolhuis *et al*., 2009). Whole blood samples (1 ml), collected in EDTA-containing tubes, were placed on ice and stored at -70°C until analysis. The 5-HT concentration in blood was determined by a fluorescence assay, based on a protocol for assessing 5-HT in human blood (Yuwiler *et al*., 1970). One ml of blood was pipetted in 50 ml centrifuge tubes and 2 ml of 0.9% NaCl solution, 1 ml of an ascorbic acid solution (3% in distilled water, saturated with KCl and EDTA) and 5 ml of a phosphate buffer (2 M K_2HPO4, saturated with KCl and adjusted to pH 10 with KOH) was added, followed by 20 ml of n-butanol. The tubes were shaken thoroughly for 5 min and centrifuged at 895 g for 15 min. Fifteen ml of butanol layer was transferred to a second tube containing 2 ml of 0.1 M HCl and 25 ml of cyclohexane and tubes were shaken for 20 s and centrifuged for 4 min at 895 rpm. The butanol-cyclohexane layer was removed and 1 ml of the acidic phase was pipetted in a tube containing 0.3 ml of 12 M HCl and vortexed for 3 s. Fluorescence was determined in a Perkin-Elmer 2000 Fluorescence spectro-photometer at 283 and 540 nm. A standard curve was prepared by taking 0.1, 0.2, 0.3, 0.4 and 0.5 ml of serotonin hydrochloride (Sigma-Aldrich) dissolved in Krebs-Ringer-phosphate buffer (0.2755 µmol/ml), to a volume of 1 ml with 0.9% NaCl solution and subsequently the procedure as described above was followed.

Plasma corticosterone levels: Levels of plasma corticosterone were determined in all plasma samples obtained at 6 weeks of age using a radioimmunoassay kit (IDS, Inc. Bolton, UK) according to the manufacturers procedures and described before (Buyse *et al*., 1987).

Statistics: (Total and isotype-specific IgM, IgG and IgA) antibody titers to HuSA, growth from 2-6 weeks of age and *ex vivo* NO production were analyzed by a two-way ANOVA for the effect of breed (layer or broiler), time and their interaction using the repeated measurement procedure with a 'bird nested within breed' option. Body weight gain (growth) per moment, *in vitro* whole blood lymphocyte stimulation, (relative) spleen weight, NO production, body temperature, blood 5-HT content and plasma corticosterone levels, respectively, at 6 weeks of age were analyzed by a one-way ANOVA for the effect of breed. Pearson's correlations between levels of 5-HT, corticosterone and antibodies in blood, cellular immunity *in vitro* and body weight (gain) were separately calculated for broiler and layer birds. All analyses were according to SAS Institute procedures (SAS Institute, 1990).

RESULTS

Total antibody titers to HuSA: Fig. 1A shows the kinetics of the total specific antibody titers to HuSA of Lohmann Brown layers and Ross broilers after concurrent i.t. challenge with HuSA and LPS at 3 weeks of age. Both breeds responded with increased levels of antibodies binding HuSA. Highest titers for both breeds were found at day 14 post challenge.

Average total antibody titers to HuSA during the whole 3-week observation period after concurrent i.t. immunization with HuSA and LPS were significantly affected by a breed effect and a breed * time interaction (Table 1, $p<0.05$). Significantly higher levels of total antibodies to HuSA were found in the Ross broilers. Total antibody titers to HuSA were significantly higher in broilers at days 14 and 21 post challenge (Fig. 1A).

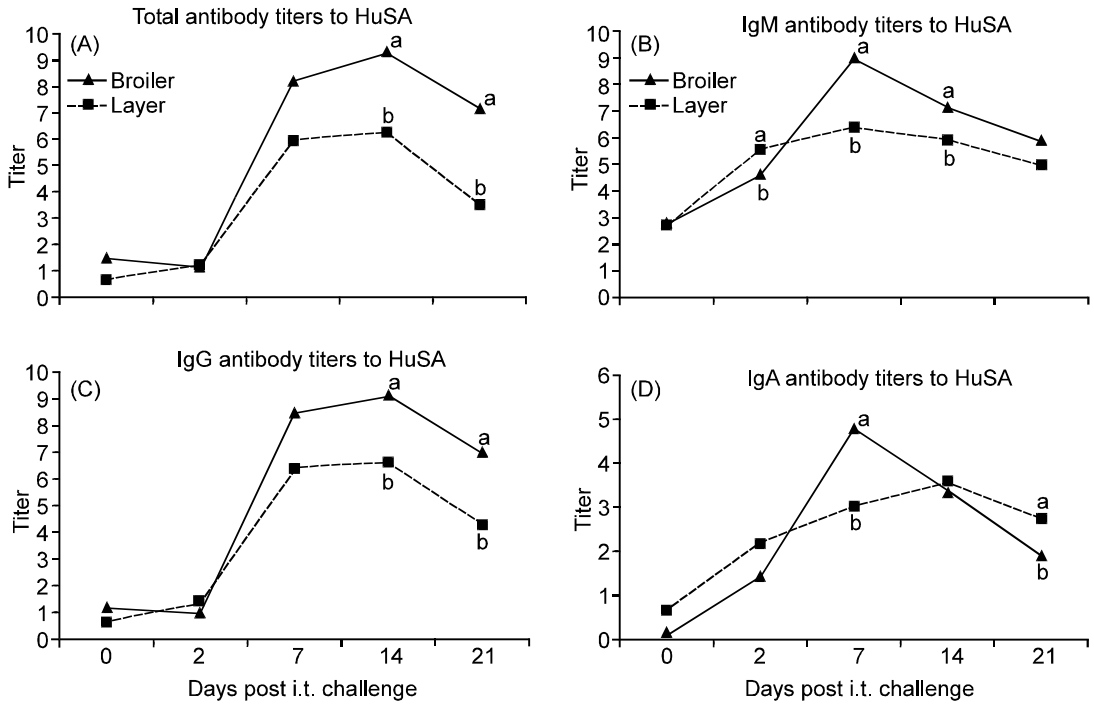

Fig. 1: The time course of the mean systemic total (A), IgM (B), IgG (C) and IgA (D) antibody titers to HuSA of Lohmann Brown (n = 18, dotted lines) or Ross broiler chicks (n = 17, solid lines) during 3 weeks after intratracheal challenge with HuSA and LPS at 3 weeks of age. a,b reveals significant difference (p<0.05) per moment

Table 1: Total and isotype-specific IgM, IgG and IgA plasma antibody titers[1] directed to HuSA during 3 weeks after primary concurrent intratracheal immunization with HuSA and LPS at 3 weeks of age

Breed[2]	IgTotal	IgM	IgG	IgA
L	3.52	5.11	3.86	2.44
B	5.44	5.85	5.34	2.31
SEM	0.39	0.20	0.38	0.19
Main effects[3]				
Breed	**	*	**	NS
	B>L	B>L	B>L	
Time	***	***	***	***
Time*Breed	**	***	**	**

[1]Least squares means ± SEM of the complete observation period as calculated by repeated measurement procedures. Titers are log_2 of the reciprocal of the antibody dilution. [2]Breed: L: Lohmann Brown layer, B: Ross broiler. [3]Breed = Breed effect, Time = time effect; Time * Breed = Time by Breed interaction. *p<0.05, **p<0.01, ***p<0.001, NS non significant

IgM antibody titers to HuSA: Figure 1B shows the kinetics of the IgM antibody titers to HuSA of Lohmann Brown layers and Ross broilers after concurrent i.t. challenge with HuSA and LPS at 3 weeks of age. Both breeds responded with increased levels of IgM antibodies binding HuSA. Highest titers for both breeds were found at day 7 post challenge (Fig. 1B).

Average IgM antibody titers to HuSA during the whole 3-week observation period after concurrent i.t. immunization with HuSA and LPS were significantly affected by a breed effect and a breed * time interaction (Table 1, p<0.05). Significantly higher levels of IgM antibodies to HuSA were found in the broilers. At day 2 post challenge higher levels of IgM binding HuSA were found in the layers, whereas at days 7 and 14 significantly higher IgM titers to HuSA were found in the broilers (Fig. 1B).

IgG antibody titers to HuSA: Figure 1C shows the kinetics of the IgG antibody titers to HuSA of Lohmann Brown layers and Ross broilers after concurrent i.t. challenge with HuSA and LPS at 3 weeks of age. Both breeds responded with increased levels of IgG antibodies binding HuSA. Highest titers for both breeds were found at d 14 post challenge.

Average IgG antibody titers to HuSA during the whole 3-week observation period after concurrent i.t. immunization with HuSA and LPS were significantly affected by a breed effect and a breed * time interaction (Table 1, p<0.05). Significantly higher levels of IgG antibodies to HuSA were found in the broilers. At days 7 and 14 significantly higher IgG titers were found in the broilers (Fig. 1C).

IgA antibody titers to HuSA: Figure 1D shows the kinetics of the IgA antibody titers to HuSA of Lohmann brown layers and Ross broilers after concurrent i.t. challenge with HuSA and LPS at 3 weeks of age. Both breeds responded with increased levels of antibodies

binding HuSA. Highest titers for broilers were found at 7 days post challenge and for layers at 14 days post challenge.

Average IgA antibody titers to HuSA during the whole 3-week observation period after concurrent i.t. immunization with HuSA and LPS were significantly affected by a breed * time interaction (Table 1, $p<0.05$). IgA titers binding HuSA were significantly higher in broilers at day 7 post challenge and in layers at day 21 post challenge (Fig. 1D).

Cellular immune responses *in vitro:* *In vitro* responses to Con A and three concentrations of HuSA of whole blood cultures at 6 weeks of age are shown in Table 2. No significant breed effects were found for the Stimulation Index (SI) in the presence of Con A. At all concentrations of HuSA significantly higher SI were found in whole blood cultures of the Ross broilers as compared to the Lohmann brown layers.

***Ex vivo* nitric oxide production:** Figure 2 shows the nitric oxide levels after stimulation of PBL with LPS *in vitro*. Overall no difference in average NO production during the complete observation period between the breeds was found. However, a significant time * breed interaction ($p<0.05$), which was due to the enhanced NO production levels of Lohmann brown layers at day 14 post challenge as compared to the Ross broilers.

Body weight (gain): As expected, much higher BW and higher BWG was found in the Ross broilers as compared to the Lohmann Brown layers. BWG of the broilers from 2-6 weeks of age (1673 gram) was significantly higher than BWG from 2-6 weeks of age of the Layers (362 gram) (SEM 25 gram, $p<0.001$). A breed effect was found after concurrent challenge with HuSA and LPS at 3 weeks of age. Whereas a decrease in BWG was found at 24 h after challenge in layers (-1,4 gram), a positive BWG (37 gram) was found in the broilers after challenge (SEM 2,2 gram, $p<0.001$).

(Relative) spleen weight: Spleen weights were significantly higher in the Ross broilers (2.11 gram) as compared to the Lohmann Brown layers (1.32 gram), SEM 0.07, $p<0.001$. Relative spleen weight (weight of spleen/BW at six weeks of age) was significantly higher in the layers (0.0027) as compared to the broilers (0.0011), SEM 0.0001, $p<0.001$.

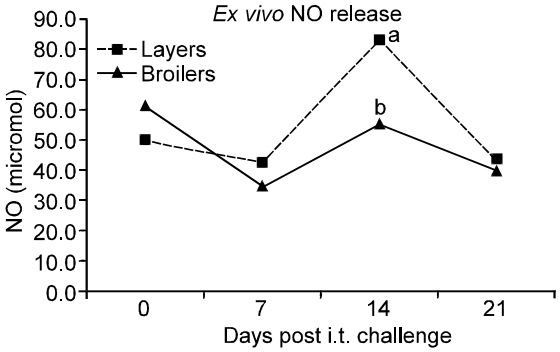

Fig. 2: Average nitric oxide production in µmol of Lohmann Brown (n = 8, dotted lines) or Ross broiler chicks (n = 10, solid lines) during 3 weeks after intratracheal challenge with HuSA and LPS at 3 weeks of age, a,b reveals significant difference ($p<0.05$) per moment

Blood serotonin levels: Blood 5-HT levels at 6 weeks of age were significantly higher ($p<0.001$) in the Lohmann Brown layers (87.5 nmol/ml) as compared to the Ross broilers (45.3 nmol/ml), SEM 3.0.

Plasma corticosterone levels: Plasma corticosterone levels at 6 weeks of age were significantly higher in Lohmann Brown layers (8.50 ng/ml) than in Ross broilers (3.81 ng/ml), SEM 0.81, $p<0.01$.

Body temperature: Body Temperature (BT) and delta BT from Lohmann brown layers and Ross broilers during the first days post challenge are shown in Fig. 3A and 3B, respectively. Basal temperatures before challenge did not differ between broilers and layers. Average body temperature during the first 4 days post i.t. challenge was higher in the layers, but not significantly. Average BT was affected by a significant time * breed interaction, after challenge BT rose higher and faster in layers than

Table 2: *In vitro* proliferation[1] of whole blood cells at 6 weeks of age after concurrent intratracheal immunization with HuSA and LPS at 3 weeks of age in the presence of 20 µg/ml concanavalin (Con) A, or HuSA (10, 50 or 100 µg/ml), respectively

	BG	Con A[3]		$HuSA_{10}$		$HuSA_{50}$		$HuSA_{100}$	
Breed[2]	cpm	SI[1]	(cpm)	SI	(cpm)	SI	(cpm)	SI	cpm
L	718	12.07	8635	1.20	629	1.15	604	1.18	620
B	673	10.39	6790	1.54	834	1.54	827	1.51	813
SEM		1.68		0.09		0.09		0.11	
Breed		NS		**		**		*	
				B>L		B>L		B>L	

[1]Least squares means ± SEM of SI. [2]Breed: L: Lohmann Brown layer, B: Ross broiler.
[3]Con A = concanavalin A, $HuSA_{10}$ = 10 µg/ml HuSA, $HuSA_{50}$ = 50 µg/ml HuSA, $HuSA_{100}$ = 100 µg/ml, BG = background cpm, cpm = counts per minute. *$p<0.05$, **$p<0.01$, NS non significant

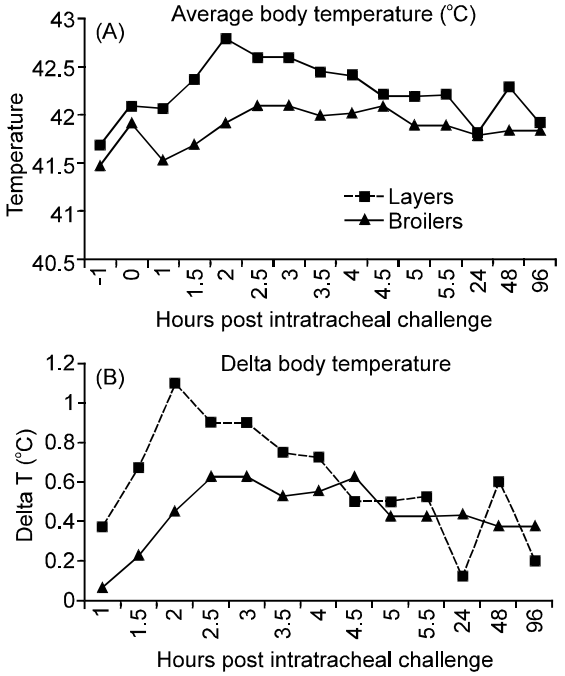

Fig. 3: Average body temperature (A) and average delta body temperature (B) of Lohmann Brown (dotted lines) or Ross broiler chicks (solid lines) during 4 d after intratracheal challenge with HuSA and LPS at 3 weeks of age.

in broilers ($p<0.05$). as well as delta body temperature during the first 4 h post challenge was higher for layers than for broilers ($p<0.05$).

Correlations between immune parameters, serotonin, body weight (gain) and corticosterone: Correlations between immune parameters and physiological parameters (growth, 5-HT and CORT content) and cachectin response (BW loss at 24 h after i.t. challenge with HuSA and LPS) are shown in Table 3. In Ross broilers, the 24 h cachectin response (Table 3, delta) was negatively related with the IgM and IgG antibody responses to HuSA at 14 d after challenge, but positively correlated with the cellular immune response to HuSA *in vitro* (HuSA50, Table 3, bold). 5-HT content of the blood was positively correlated with the IgM and IgG antibody titers to HuSA, but negatively correlated with the cellular immune response *in vitro* to HuSA. No correlations between corticosterone levels in the blood and any other parameter was found in broilers. In Lohmann brown layers, the cachectin response was negatively correlated with the cellular immune response to HuSA *in vitro* (Table 3, lower half, bold). 5-HT content was also positively correlated with spleen weight and the total, IgM and the IgG, respectively, antibody responses to HuSA. Also in layers, no significant correlations between blood corticosterone content and any other parameter measured was found. In both breeds, no significant correlations between body weight gain and other parameters were found.

DISCUSSION

In the current study levels of specific (humoral and cellular), innate immunity (*ex vivo* NO production) and temperature responses to a concurrent airborne challenge with LPS and HuSA, body weight (gain) until the age of 6 weeks and levels of blood 5-HT and plasma Corticosterone (CORT) levels were measured in one layer breed (Lohmann Brown) and one broiler breed (Ross) of similar age and kept under similar conditions. Blood 5-HT levels were measured since they have been related with misbehaviour (feather pecking) in layers and BW gain in broilers, respectively (Carew *et al*., 1983). Relations between behavioural responses and 5-HT were also reported for broilers (Kostal and Savory, 1996; Shea-Moore *et al*., 1996). Plasma CORT levels may be

Table 3: Correlations between immune and physiological parameters in broilers and layers.

Broiler (n=17)	Delta	BWG	SW	rSW	5-HT	HuSA IgT_{14}	HuSA IgM_{14}	HuSA IgG_{14}	HuSA IgA_{14}	ConA	$HuSA_{50}$	CORT
Layer (n = 18)												
Delta	x	0.27	-0.13	-0.28	-0.55*	-0.23	-0.58*	-0.50*	-0.18	-0.29	0.48*	0.24
BWG	-0.05	x	0.19	-0.17	-0.01	0.15	-0.08	0.32	-0.36	-0.04	0.12	-0.27
SW	-0.41	0.32	x	0.92***	-0.01	0.25	0.11	0.18	-0.08	-0.14	0.31	-0.12
rSW	-0.37	-0.27	0.81***	x	0.04	0.21	0.18	0.08	0.01	-0.07	0.18	-0.08
5-HT	-0.32	0.13	0.48*	0.36	x	0.35	0.98***	0.56*	0.00	0.27	-0.54*	-0.11
HuSA IgT_{14}	-0.16	0.37	0.56*	0.31	0.55*	x	0.38	0.49*	-0.16	-0.31	-0.21	0.29
HuSA IgM_{14}	-0.43	0.05	0.43	0.36	0.40*	0.51*	x	0.55*	0.00	0.23	-0.45	-0.08
HuSA IgG_{14}	0.20	0.17	-0.28	-0.37	0.97***	0.33	-0.04	x	-0.18	-0.20	-0.44	0.00
HuSA IgA_{14}	-0.21	0.08	0.44	0.42	0.17	0.08	0.13	-0.21	x	0.21	0.25	0.24
ConA	0.08	0.01	-0.49*	-0.50*	-0.35	-0.24	-0.37	0.23	-0.25	x	-0.25	-0.38
HuSA50	-0.54*	0.08	0.33	0.27	-0.12	-0.07	-0.09	-0.44	0.26	0.08	x	0.24
CORT	0.00	-0.45	-0.10	0.14	-0.08	0.44	-0.03	-0.40	-0.03	0.20	20	x

Delta = growth during 24 h after concurrent intratracheal challenge with HuSA and LPS at 3 weeks of age, BWG = growth from 2 weeks of age to 6 weeks of age, SW = spleen weight at 6 weeks of age, rSW = relative spleen weight at 6 weeks of age, 5-HT = plasma serotonin content at 6 weeks of age, HuSA IgT_{14}, HuSA IgM_{14}, HuSA IgG_{14} and HuSA IgA_{14} are levels of plasma total and isotype antibodies binding HuSA at 14 d after intratracheal challenge, Con A and $HuSA_{50}$ represent proliferation of whole blood cells to con A and 50 µg/ml HuSA *in vitro*, CORT is blood corticosterone level at 6 weeks of age. *$p<0.05$, **$p<0.01$, ***$p<0.001$

indicative for the stress level of the bird related to breed or immune (LPS, HuSA) challenge. It has to be kept in mind, however, that levels of 5-HT and CORT in the blood were measured at one moment: 6 weeks of age, indicating a status, whereas the immune parameters and BWG exemplified responsiveness of the birds. In this study, birds were housed in pens with temperatures and fed with diets as practised in broiler husbandry. Layers and broilers differ in nutritional and temperature demands especially during early life, but for comparison husbandry procedures for broilers were chosen. A concurrent i.t. challenge with LPS and HuSA was chosen for two reasons. First, i.t. challenge with HuSA was shown to result in production of HuSA specific antibody responses in layers (Parmentier *et al*., 2008) and broilers (Lai *et al*., 2009). Second, when administered via the i.t. route LPS not only modulated the immune response of both layers (Miller *et al*., 1992) and broilers (Lai *et al*., 2009; Wideman *et al*., 2004), but LPS may also elevate plasma CORT concentrations and consequently increase of the Heterophil to Leukocyte (H/L) ratio (Al-Ghamdi, 2008; Altan *et al*., 2003; Shini *et al*., 2008). In addition, refractile responses to i.t. administered LPS with respect to BWG appeared less pronounced in broilers (Lai *et al*., 2009) than in layers (Parmentier *et al*., 2008). The LPS challenge dose was 100 times higher than the amount that is normally inhaled by a healthy chicken per day (approximately 1 µg) kept under routine husbandry (battery or floor) conditions. Earlier we found that this dose affected growth and immune responses in layers and broilers (Lai *et al*., 2009; Parmentier *et al*., 2008). Concentrations of airborne endotoxins (LPS) and beta-glucans ranging from 240-13,400 EU/m^3 (Endotoxin Units, 1 EU/m^3 = 0.1 ng/m^3) were found in chicken farms (Douwes *et al*., 2004; Powers *et al*., 2005), but levels up to 63 µg/m^3 were also reported (Pomorska *et al*., 2007).

Broilers and layers substantially differ in Body Weight (BW) gain, duration of life, nutritional needs and probably also immune mechanisms. Pronounced differences in immune responses were, however, also found within and between layer breeds (White Leghorns and Lohmann Brown) after similar treatments as in the current study (Parmentier *et al*., 2006). A previous comparative study between one broiler (Ross) and one layer line (White Leghorn) suggested that broilers are more specialized in mounting strong short term innate humoral (IgM) immune responses, while layers are specialized in a long term (specific) humoral (IgG) immune response in combination with a strong cellular response to the systemically administered antigen TNP-KLH (Koenen *et al*., 2002). In addition, these authors suggested a negative relation between BW and immune competence. It was concluded that genetic and possibly nutritional changes in broiler chickens have put faster growing broilers in a disadvantageous situation in terms of humoral immune function. Studies directed to broilers (Miller *et al*., 1992) or layers (Parmentier *et al*., 1996; Siegel *et al*., 1982) also suggested that higher body weight (gain) of broilers and layers negatively affected their humoral immune response, whereas enhanced egg production had less negative impact to the bird's humoral immune system. The high BWG of broilers might negatively affect their immune system and as a consequence enhance health problems and disease sensitivity (Koenen *et al*., 2002). However, selection for egg production might have affected behaviour and stress responses in layers as exemplified by Feather Pecking (FP) which is more common in certain (high egg producing) layer breeds than in others (Kjaer and Sørensen, 1997; Kjaer, 1995). Furthermore, (low) levels of blood 5-HT (4) and low 5-HT neurotransmission (Van Hierden *et al*., 2004) have been related with enhanced fear-related behaviour and FP in layers.

In the present study we found higher levels of specific total, IgM and IgG, respectively, antibodies directed to HuSA and higher levels of *in vitro* proliferation of whole blood leucocytes as a parameter of cellular immunity in the presence of HuSA in Ross broilers than in Lohmann Brown layers of similar age, whereas *ex vivo* NO production in the presence of LPS was higher in the layers. Thus, we conclude that there is no fundamental difference between the current broiler (Ross) and layer (Lohmann Brown) breeds during the first 6 weeks of age, kept under similar conditions, with respect to their specific and innate (primary) immune competence after a concurrent challenge with HuSA and LPS at the respiratory mucosal level. Whether this is true for secondary immune responses remains unknown. In the current study the broiler (Ross) breed, as expected, became much heavier and grew substantially faster than the Lohmann Brown layers, but the higher BW(G) was not related with a lower humoral nor cellular primary immune competence. Only at one moment we found a significantly higher immune (*ex vivo* NO release) response in layers, whereas antibody responses and *in vitro* cellular immunity to HuSA were significantly higher in the broiler birds. In the broilers a higher spleen weight was found albeit the relative spleen weight was lower in broilers than in layers. The currently studied broilers appeared less sensitive to the LPS challenge, with respect to their 24 and 48 h cachectin responses as compared to the Lohmann brown layer birds which revealed a negative growth after the LPS challenge. This is in accordance with the observation that body temperature increase post challenge in layers was higher than in broilers.

Lower blood 5-HT content (Bolhuis *et al*., 2009) and low 5-HT neurotransmission (Van Hierden *et al*., 2004) have been related with enhanced feather pecking in layers. Tryptophan supplemented diet decreased aggression in broiler breeder males (Shea-Moore *et al*., 1996). In

broilers, a high 5-HT content in the brain negatively affected water intake and food intake (Denbow *et al*., 1982), whereas 5-HT has been proposed as a negative modulator of the synthesis of plasma triiodothyronine, plasma thyroxine and growth hormone (Carew *et al*., 1983), whereas serotonergic agonists and antagonists affected degree of sedation of broilers (Kostal and Savory, 1996). In the current study we found much lower blood 5-HT contents in the Ross broilers (about half of the content found in Lohmann brown layers) and a negative relation between 5-HT and the 24-h cachectin response to LPS, which are not contra dictionary to the proposed role of 5-HT in BWG. In the layers, however, we also found a negative (though not significant) relation between the cachectin response and 5-HT. Misbehaviour such as feather pecking or toe pecking or feather damage were not found in the broiler nor the layer cages, but birds were still young. Also plasma Corticosterone (CORT) levels were significantly higher in the current layers than in broilers of similar age. Plasma CORT levels were measured at 3 weeks after concurrent i.t. challenge with HuSA and LPS. High CORT levels and lower levels of specific immune responses in layers were found earlier (Hangalapura *et al*., 2004). In the current study, the Ross broilers had lower CORT levels and higher specific humoral and cellular immune responses than Lohmann Brown layers, but no correlations between CORT and immune responses were found in both breeds. These data, together with the lower cachectin responses at 3 weeks of age, suggest that under the given housing conditions, Ross broilers appeared less sensitive towards environmental stimuli, such as LPS, than Lohmann Brown layer birds, which may rest on their genetic drive to maintain body weight gain. Whether similar results would have been found with other broiler or layer breeds, or when both bird types were housed under layer housing conditions remains to be established. It has to be kept in mind that we did not measure CORT and 5-HT levels in non-immunized layers and broilers at six weeks of age. CORT and 5-HT levels were determined in birds 3 weeks after experimental antigenic exposure. It is unlikely that under the current housing conditions control birds would not be challenged by antigen. It is therefore reasonable to expect that levels of CORT or 5-HT would have been very different in such control birds.

Correlation analyses suggested positive relations between 5-HT content of the blood and humoral immune response parameters in both breeds, but antigen specific cellular immunity *in vitro* was negatively correlated with 5-HT. The cachectin response to LPS was positively correlated with specific cellular immunity in the broilers, but negatively in the layers. In both breeds growth did not significantly affect the cellular and humoral immune parameters. The current data suggested that selection for growth or egg lay may have differentially affected cellular immune pathways after a LPS-induced cachectin response and suggest that the cellular immune pathway in the broilers may be less prone to LPS-induced effects than these in the currently studied layers, but the broilers appeared not immunodeficient as compared to the layers.

Taken together, we found no evidence of a lower primary immune competence in broiler birds as compared to layer birds during the first 6 weeks of life, albeit this study was limited to a comparison between one common layer and one common broiler breed kept under broiler conditions. On the contrary, the current broiler breed showed higher specific immune responses. Due to their genetic make up, broilers might be less sensitive to environmental stimuli such as LPS (lower CORT, lower 5-HT levels, lower *ex vivo* NO production and lower cachectin response (BW loss and temperature)), but more responsive to specific immunological stimuli (total antibody and T-cell responses to HuSA). Whether this underlies their proposed enhanced disease susceptibility remains to be elucidated.

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Abbreviations:
CORT : corticosterone
HuSA : human serum albumin
i.t. : intratracheal
LPS : lipopolysaccharide
5-HT : serotonine.