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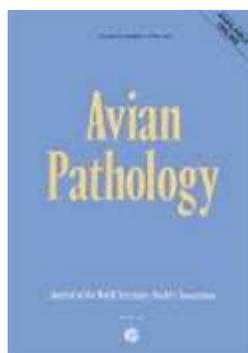
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**The influence of age and repeated LPS administration on body temperature and the relation with interleukin-6 and IgM antibodies in broiler chickens**

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The influence of age and repeated lipopolysaccharide administration on body temperature and the concentration of interleukin-6 and IgM antibodies against lipopolysaccharide in broiler chickens

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**Abstract**

Our objective was to create a standardized and reproducible inflammation model in chickens in order to study the pharmacodynamics of several anti-pyretic and anti-inflammatory drugs. We studied the influence of age and repeated lipopolysaccharide (LPS) administration on body temperature and the correlation of this with concentrations of interleukin 6 (IL-6) and IgM antibodies against LPS in plasma of chickens. Three and five week old broilers were injected intravenously with LPS from *Escherichia coli* O127: B8 at a dose of 1 mg/kg. LPS administration was repeated after 2 or 7 days. After the first dose of LPS, the body temperature was initially decreased below normal and then later increased above normal. The second dose of LPS reduced the level of hypothermia and the duration of the febrile phase. Three-week-old birds responded to LPS with a higher maximum body temperature and a greater area under the body temperature versus time curve than five-week-old chickens ( $P < 0.05$ ). Interleukin-6 reached its highest concentration 3 hours after LPS administration and returned to baseline levels after 9 hours. A second dose of LPS resulted in a significantly lower peak in IL-6. Significant higher levels of antibodies against LPS could be detected 7

days after LPS administration. However, there appeared to be no correlation between the reduced response to LPS and the presence of antibodies.

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## Introduction

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Our objective was to create a standardized and reproducible inflammation model in chickens in order to study the pharmacodynamics of several anti-pyretic and anti-inflammatory drugs. Lipopolysaccharide (LPS), a component of the cell wall of Gram-negative bacteria, is commonly used to induce an acute phase reaction in humans and animals because of its proinflammatory properties (Skarnes *et al.*, 1981; Morimoto *et al.*, 1987; Klosterhalfen *et al.*, 1992; Erroi *et al.*, 1993; Semrad, 1993; Franco *et al.*, 2000; Maxwell *et al.*, 2002; Haudek *et al.*, 2002). LPS, in combination with CD14, interacts with Toll-Like Receptor 4 (TLR4), leading to the activation of Nuclear Factor  $\kappa$ B and expression of inflammatory genes (Dil and Qureschi, 2002; Doyle and O'Neill, 2006). Several parameters can be determined to evaluate the progress of an acute phase reaction, including body temperature, and cytokine and acute phase protein concentrations. Body temperature responses after LPS administration can vary dramatically. Jones *et al.* (1981) and Baert *et al.* (2005) described a biphasic temperature reaction after LPS administration in 5 week old commercial broilers. Leshchinsky and Klasing (2001) found a significant difference in the body temperature responses after LPS administration between broilers and layers. Smith *et al.* (1978) injected 14-day-old chickens with LPS for one, three or six consecutive days. Body temperature was measured at 1, 2, 3, 4, 6, 24 and 48 hours after the last injection and the patterns seen included hypothermia, hypothermia followed by hyperthermia, and hyperthermia without hypothermia, respectively. These variations could be attributable to differences in ambient temperature, dosage, prior sensitization, age, route of administration or genetic line (Jones *et al.*, 1983; Cheng *et al.*, 2004). Significantly, higher concentrations of interleukin-6 (IL-6) in plasma, and IL-6, IL-8, IL-18, IL-1 $\beta$ , myelomonocytic growth factor (MGF) and interferon- $\gamma$  (IFN $\gamma$ ) in spleen cells, can be seen after LPS administration (Nakamura *et al.*, 1998; Xie *et al.*, 2000; Sijben *et al.*,

2003; Leshchinsky & Klasing, 2003). Alpha-1 acid glycoprotein, ceruloplasmin, transferrin, fibronectin and serum amyloid A are important acute phase proteins in serum that are known to increase in concentration in poultry during an inflammatory response (Curtis & Butler, 1980; Nakamura *et al.*, 1998; Takahashi *et al.*, 1998; Chamanza *et al.*, 1999). Repeated administration of LPS reduces the response, but the mechanisms responsible for this phenomenon have not yet been elucidated. Several mechanisms have been suggested, including the suppression of activation of NF $\kappa$ B and a high proportion of non-transactive p50 homodimers, upregulation of soluble TNF receptor 1, which binds and clears TNF, modulation of Toll-Like Receptor expression, and increased expression of intracellular mediators involved in negative regulation of TLR signalling as well as a decrease in TLR4 tyrosine phosphorylation (Qu *et al.*, 2003; Wang *et al.*, 2004; Medvedev *et al.*, 2006; Medvedev *et al.*, 2007). The reduced response to LPS comprises two phases: the early phase, which is a refractory state evident within hours after LPS administration and which lasts for up to one week, and the late phase, which occurs only after weeks to months and which is restricted to the LPS serotype used for initial injection. Experiments in guinea pigs and rats demonstrate that attenuation of the febrile response after repeated LPS administration is associated with reduced production of cytokines, such as IL-1 $\beta$ , IL-6 and TNF- $\alpha$  (Zeisberger & Roth, 1998; Medvedev *et al.*, 2006).

The aims of this study were to investigate the influence of age and repeated LPS administration on body temperature and the correlation of this with concentrations of IL-6 and anti-LPS IgM antibodies in the plasma of unvaccinated broiler chickens.

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## Materials and methods

**Animals.** Sixty one-day-old, unvaccinated, Ross chicks of both sexes were obtained from a commercial hatchery. For the first two weeks all chicks were housed together. Temperature and relative humidity accorded with the requirements of young chickens and temperature was adjusted to prevent huddling (ETS 123, 2004). Just before the start of the experiment, the chickens were placed into 5 cages, with each cage containing twelve randomly selected birds. The cages had a floor area of 1 m<sup>2</sup> and had a 1 m high wooden frame covered with wire. The concrete floor was covered with wood shavings, which were changed every 2 days. Before entering the room containing the cages, staff decontaminated their footwear and put on a clean laboratory coat. The lighting schedule consisted of 12 hours dark and 12 hours light. Commercial pullet feed and spring water were available *ad libitum*. All experiments and procedures were approved by the Ethics Committee of the Faculty of Veterinary Medicine (EC 2005/06).

**Experimental design** The experiment was designed as a split plot, with the cage being a block. Each block (cage) contained 12 animals, 6 three-week-old chickens and 6 five-week-old chickens. Within each of the two age groups, two animals were assigned to receive LPS at day 0 and day 2, two animals were assigned to receive LPS at day 0 and day 7, one control animal received saline at day 0 and day 2, and a second control animal received saline at day 0 and day 7.

LPS from *Escherichia coli* O127: B8 (Sigma-Aldrich, Bornem, Belgium) dissolved in 0.9% saline was administered by intravenous injection at a dose of 1 mg/kg. The control animals were injected with the same amount of saline. Freshly dissolved LPS from the same

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batch was used for each experiment. The endotoxin level in this batch was 1,500,000 units/mg. Body temperature was measured by inserting a thermocouple probe (Physitemp Instruments Inc., Clifton, NJ, USA) 5 cm into the cloaca, before and at 1, 2, 3.5, 5, 6.5, 8, 12 and 24 h after LPS administration. The area under the body temperature versus time curve ( $AUC_{0 \rightarrow 24h}$ ) was calculated in order to evaluate possible effects of age and/or treatment. Other responses calculated were the mean body temperature (meanT), the maximum temperature (maxT) and the time at which the maximum temperature was reached (Tmax).

Blood was withdrawn into heparinised tubes (Venoject<sup>®</sup>, Terumo Corp., Tokyo, Japan) from the leg vein before and at 3, 6, 9, 12 and 24 h after LPS administration to determine IL-6 concentrations. Just before the first and second LPS dose, blood was withdrawn for the determination of the concentration of IgM antibodies against LPS. Plasma was separated by centrifugation (1100g for 10 min. at 5°C) and samples were stored at -20°C until assayed.

**Measurement of IL-6:** Concentrations of IL-6 were measured using IL-6 dependent murine 7TD1 hybridoma cells (Van Snick *et al.*, 1986). Briefly, cells were cultured in 96 well microtitre plates for 72 h in medium containing different dilutions of the plasma samples. A colorimetric hexosaminidase assay was used to assess the number of living cells, which is related to the amount of IL-6 in the media (Landegren, 1984). The concentration of IL-6 in plasma samples was calculated from a standard curve obtained using dilutions of recombinant mouse IL-6. The results were corrected for differences in the responses of the 7TD1 cells to mouse and chicken IL-6 standards, with mouse IL-6 being twice as active as chicken IL-6 (data not shown). The IL-6 data were logarithmically transformed to normalise the distribution (Snedecor & Cochran, 1989). The plasma IL-6 concentrations were compared 3 h

after the administration of LPS or saline, as this was the time point at which the IL-6 concentration was highest.

**Measurement of anti-LPS IgM antibodies.** ELISA plates containing 150  $\mu$ l LPS solution per well (30  $\mu$ g/ml in bicarbonate buffer at pH 9.6, using the same LPS that was used to inoculate the birds) were incubated overnight at 4 °C. Plates were washed three times with PBST (phosphate buffered saline (PBS) containing 0.01 % (v/v) Tween 20) and blocked with 5 % bovine serum albumin (BSA) in PBS for 1 h at 37 °C. Plates were washed and plasma from experimental birds (at 1/100 and 1/400 dilutions in 0.5 % BSA-PBST) added to the wells, then incubated for 1 h at room temperature. Subsequently, the plates were washed three times with PBST and horseradish peroxidase (HRP)-labelled goat-anti-chicken IgM antibody added to each well (Bethyl, Montgomery Texas, USA; 1/5000 in 0.5 % BSA-PBST), and incubated for 1 h at room temperature. After washing, bound HRP was detected using 2,2'-azino-di(3-ethylbenzothiazolin-6-sulfinic) acid (ABTS) (KPL, Gaithersburg, Maryland, USA) and absorbances were measured at 405 nm using a Titertek microplate reader (MCC/340 Multiskan, Titertek, Alabama, USA). Negative controls (no chicken plasma, 0.5% BSA-PBST) were included on each plate. The mean of duplicate wells was determined and the value obtained for the negative control was subtracted from this.

**Statistical analysis.** All analyses of the different body temperature variables ( $AUC_{0 \rightarrow 24h}$ , meanT, maxT and Tmax), IL-6 (log transformed) and IgM were based on a mixed model with cage and animal within cage as the random effect and treatment, age and their interaction as categorical fixed effects (SAS Version 9.1). The treatment factors included saline at day 0, saline at day 2, saline at day 7, LPS at day 0, LPS at day 2 and LPS at day 7. A global test was performed at the 5% significance level for the treatment and age effects and their interaction.

If the interaction was significant, separate analyses were performed for the two age categories. The following contrasts were of interest: the overall difference between controls and LPS treated birds, the difference between controls and LPS treated birds at day 0, the difference between controls and LPS treated birds at day 2, between controls and LPS treated birds at day 7, between birds treated with LPS at day 0 and day 2, between birds treated with LPS at day 0 and day 7 and between birds treated with LPS at day 2 and day 7. Each of these comparisons was tested within the framework of the mixed model at a comparison wise Bonferroni-adjusted significance level of 0.0071 (0.05/7). The use of the mixed model implies that the contrasts are tested at the appropriate level. For example, the effect of age is tested by comparing chickens of different ages using cage as a block factor, whereas the difference between LPS administration at day 0 and day 2 is tested within a chicken, as these two treatments were applied to the same chicken. Additional analyses, also based on the mixed model, were done to investigate whether IgM concentrations at day 0 differed significantly between three- and five-week-old chickens.

## Results

The global statistical analysis revealed a significant influence of the age of the birds on the reaction to LPS ( $P < 0.05$ ). Therefore, more detailed analyses were performed for each of the two age categories.

**Body temperature.** After LPS administration, the body temperature was initially below normal and then was later above normal after both the first and second LPS doses in both three- and five-week-old birds. The second LPS administration reduced the hypothermic

phase of the temperature curve and the duration of the fever phase, as shown in Figure 1. The graph of the body temperature of the control birds was obtained by averaging the measurements for all three-week-old control birds. Although similar results were obtained for both age groups, three-week-old birds had a higher maxT after LPS administration. The AUC<sub>0→24h</sub> and maxT of three-week-old and five-week-old birds are shown in Tables 1 and 2.

**IL-6:** The concentrations of IL-6 in plasma after the first and second doses of LPS in three-week-old birds are shown in Figure 2. The graph of IL-6 concentrations in control birds was obtained by averaging the measurements for all three-week-old control birds.

The concentration of IL-6 was significantly higher after the first dose of LPS, peaking 3 h after administration and returning to baseline levels 9 h after administration in both three-week-old and five-week-old birds ( $P < 0.0001$ ). IL-6 concentrations were significantly lower after the second dose of LPS in both ages of birds, irrespective of whether the LPS was administered after a 2 or 7 day interval.

**IgM concentrations.** Significantly higher concentrations of IgM antibodies against LPS could be detected 7 days after the first dose of LPS in both age groups ( $P < 0.0001$ ). Before the first dose of LPS three-week-old birds did not have detectable concentrations of IgM against LPS, with the absorbances in the ELISA below the threshold level of 0.222. Two days after the first dose there was a detectable increase in IgM antibodies against LPS, although the concentrations were not significantly different from those seen in the control group. Before the second dose at day 7 the three-week-old control birds had detectable concentrations of IgM against LPS. The five-week-old birds had detectable concentrations of IgM against LPS before the first dose of LPS was administered.

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## Discussion

Intravascular administration of minimal doses of LPS elicits monophasic fevers, a moderate dose is known to produce biphasic fevers, and high doses of endotoxin elicit persistent hypothermia (Skarnes *et al.*, 1981; Lang *et al.*, 1985; Morimoto *et al.*, 1987; Massart-Leën *et al.*, 1992). Chickens are very resistant to the deleterious effects of LPS and since ambient temperature, dosage, prior sensitization, age, route of administration, serotype and genetic line may be involved in the febrile response to endotoxin, it is very difficult to compare different studies (Berczi *et al.*, 1966; Adler & Damassa, 1978; Jones *et al.*, 1983; Dogan *et al.*, 2000; Cheng *et al.*, 2004).

Smith *et al.* (1978) reported the attenuation of hypothermia after repeated intravenous injection of 14 day old chickens with endotoxin from *Salmonella gallinarum* at a dose of 15 mg/kg. The first injection of LPS produced hypothermia. When LPS was given for 3 consecutive days, hypothermia was followed by hyperthermia. After six consecutive doses of LPS there was no hypothermia, only hyperthermia. A fall in body temperature was also demonstrated by Pittman *et al.* (1976) after intravenous administration of endotoxin from *Salmonella abortus equi* to female Leghorn chickens, but the temperature was only recorded for 180 min after administration. Jones *et al.* (1981) injected five-week-old commercial broiler cockerels intravenously with LPS from the same serotype of *E. coli* that we used in our study and demonstrated a biphasic febrile response. Baert *et al.* (2005) saw similar response. Fraifeld *et al.* (1995) saw a peak in body temperature 3 hours after intraperitoneal administration of 1 mg of *E. coli* O127:B8 LPS (corresponding to 0.8 mg/kg) to three-week-old chickens. In our study the same serotype and dose of LPS as was used by Jones *et al.* and Baert *et al.* was injected into broiler chickens. In contrast to their findings, we saw a monophasic febrile response that was preceded by hypothermia. After the second dose of

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LPS, the hypothermia, and the length of the febrile phase, was attenuated. A possible explanation for these differences could be variation in the endotoxin concentration in the different batches of LPS. The concentration of endotoxin in LPS from *E. coli* O127:B8 (Sigma-Aldrich) can vary from 500,000 to 2,250,000 units per mg. For this reason we used LPS from the same batch for the whole experiment. The febrile response following endotoxin administration is also affected by age. In the study of Jones *et al.* (1983) broiler cockerels of 1-8 weeks of age received an intravenous injection of *E. coli* O127:B8. Seven and eight week old birds developed the highest and most persistent fever. Younger birds had lower increases in body temperature, after an initial hypothermic response. In contrast we found that older birds had a less pronounced reaction to LPS. Gregorut *et al.* (1992) injected 10- to 50-day-old birds with LPS from *E. coli* O122:B8 and *S. typhimurium*. They found no significant effect of age on the febrile response, but there was a slight tendency towards a reduced peak in fever and a more rapid response with increasing age.

In our study, ambient temperature was adjusted according to animal behaviour and ethical guidelines. Three-week-old chickens housed at temperatures below their thermoneutral zone did not develop fever (unpublished data).

Repeated administration of LPS results in a reduced response, characterized by a decrease in immune responses (e.g. the production of a variety of cytokines) and metabolic changes (e.g. fever, weight loss, shock and mortality). The reduced response to LPS comprises two phases. The early phase results in a down-regulation of monocyte and macrophage function and is evident within hours after LPS administration. Repeated administration of LPS leads to attenuation of almost all pathophysiological effects mediated by proinflammatory cytokines. In guinea pigs and rats, the reduced response to LPS consists of attenuation of the febrile response and is associated with a reduced production of cytokines (Zeisberger & Roth, 1998; Medvedev *et al.*, 2006). IL-6, IL-1 $\beta$  and TNF- $\alpha$  have been

implicated as key mediators of fever in several animal models. IL-1 $\beta$ , IL-6 and other cytokines have been characterized as endogenous pyrogens or fever-inducers (Leon *et al.*, 1999). Changes in IL-6 concentration are believed to follow the course of the body temperature changes. In guinea-pigs the peak of the IL-6 activity and the febrile phase coincide (Roth *et al.*, 1994). In our study the concentration of IL-6 peaked at 3 hours after LPS administration and preceded the febrile phase, returning to baseline concentrations 9 hours after LPS administration. The peak IL-6 concentration in plasma was significantly lower after the second dose of LPS, demonstrating a reduced response to LPS. Nakamura *et al.* (1998) injected 2 week old birds with LPS from *E. coli* and detected the highest concentrations of IL-6 at 3 h after LPS administration, but the concentrations remained high for 2 days after LPS administration. After administration of LPS from *S. typhimurium* at a dose of 5 mg/kg to broiler chickens, the IL-6 concentration in plasma was elevated at 12 and 24 hours (Xie *et al.*, 2000). The attenuation of the IL-6 concentrations in plasma in LPS-tolerant chickens is similar to that seen in LPS-tolerant mice, in which the induction of IL-6 and other cytokines is extremely low (Erroi *et al.*, 1993). In guinea-pigs repeated intramuscular injection of LPS reduced the peak in IL-6 concentrations in plasma at 3 hours after administration (Roth *et al.*, 1994).

The late phase is characterized by the production of anti-LPS antibodies and occurs weeks or months after LPS administration. The antibodies are directed against the O chain of LPS, so in the late phase there is no cross-tolerance between different LPS serotypes (Zeisberger & Roth, 1998; Medvedev *et al.*, 2006). In our study, the first dose of LPS induced the production of IgM antibodies in plasma. The five-week-old birds had IgM antibodies against LPS prior to administration of LPS. These antibodies were most probably induced by intestinal *E. coli*, which are present in every animal. The existence of such naturally occurring antibodies against LPS has been demonstrated in several animal species, including chickens

and mice (Reid *et al.*, 1997; Hangalapura *et al.*, 2003; Siwek *et al.*, 2006), with the concentration increasing with age (Parmentier *et al.*, 2004). Siwek *et al.* (2006) demonstrated an increase in antibodies against LPS between the ages of 5 and 18 weeks in layer birds. This is in accordance with our findings in three- and five-week-old broilers. These antibodies are often dismissed in immunological analyses as “background” and their role remains unclear. One of the possibilities is that they may be involved in the clearance of LPS (Ochsenbein *et al.*, 1999). However, in our experiment there seemed to be no correlation between the reduced response to LPS and the presence of antibodies, as a second dose of LPS reduced the reaction against LPS irrespective of whether it was administered after 2 or 7 days. As in mice, it could be suggested that factors other than antibodies play a critical role in this reduced response against endotoxin, including the production of anti-inflammatory cytokines and upregulation of inhibitory molecules that prevent intracellular Toll-Like receptor 4 signalling (Cavaillon & Adib-Conquy, 2006).

In conclusion, we developed a reproducible and standardized model of LPS-induced inflammation in broiler chickens. A second administration of LPS reduced the hypothermic phase of the body temperature-time curve and the duration of the febrile phase, at both three and five weeks of age. Moreover, after a second challenge with LPS the peak in concentration of IL-6 was significantly decreased. Antibodies did not seem to play a role in the reduced response to LPS.

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## References

- Adler, H.E. & Damassa, A.J. (1978). Toxicity of endotoxin to chicks. *Avian Diseases*, 23, 174-178.
- Baert, K., Duchateau, L., De Boever, S., Cherlet, M., De Backer, P. (2005). Antipyretic effect of oral sodium salicylate after an intravenous *E. coli* LPS injection in broiler chickens. *British Poultry Science*, 46, 137-143.
- Berczi, I., Bertók, L., Bereznai, T. (1966). Comparative studies on the toxicity of *Escherichia coli* lipopolysaccharide endotoxin in various animal species. *Canadian Journal of Microbiology*, 12, 1070-1071.
- Cavaillon, J.M. & Adib-Conquy, M. (2006). Bench-to-bedside review: Endotoxin tolerance as a model of leukocyte reprogramming in sepsis. *Critical Care*, 10, 233.
- Chamanza, R., Toussaint, M.J.M., van Ederen, A.M., van Veen, L., Hulskamp-Koch, C., Fabri, T.H.F. (1999). Serum amyloid A and transferrin in chicken. A preliminary investigation of using acute-phase variables to assess diseases in chickens. *Veterinary Quarterly*, 21, 158-162.
- Cheng, H.W., Freire, R., Pajor, E.A. (2004). Endotoxin stress responses in chickens from different genetic lines. 1. Sickness, behavioural, and physical responses. *Poultry Science*, 83, 707-715.
- Curtis, M.J. & Butler, E.J. (1980). Response of caeruloplasmin to *Escherichia coli* endotoxins and adrenal hormones in the domestic fowl. *Research in Veterinary Science*, 28, 217-222.
- Dil N., Qureshi M.A. (2002). Involvement of lipopolysaccharide related receptors and nuclear factor kappa B in differential expression of inducible nitric oxide synthase in chicken macrophages from different genetic backgrounds. *Veterinary Immunology and Immunopathology*, 88, 149-161
- Dogan, M.D., Ataoglu, H., Akarsu, E.S. (2000). Effects of different serotypes of *Escherichia coli* lipopolysaccharide on body temperature in rats. *Life Sciences*, 67, 2319-2329.

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- Doyle S.L., O'Neill L.A. (2006). Toll-like receptors: from the discovery of NFkappaB to new insights into transcriptional regulations in innate immunity. *Biochemical Pharmacology*, *72*, 1102-1113.
- Erroi, A., Fantuzzi, G., Mengozzi, M., Sironi, M., Orencole, S.F., Clark, B.D., Dinarello, C.A., Isetta, A., Gnocchi, P., Giovarelli, M., Ghezzi, P. (1993). Differential regulation of cytokine production in lipopolysaccharide tolerance in mice. *Infection and Immunity*, *61*, 4356-4359.
- ETS 123 (2004). Draft Appendix A of the European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS No. 123). Guidelines for accommodation and care of animals (Article 5 of the Convention).
- Fraifeld, V., Blaicher-Kulick, R., Allan Degen, A., Kaplanski, J. (1995). Is hypothalamic prostaglandin E<sub>2</sub> involved in avian fever? *Life Sciences*, *56*, 1343-1346.
- Franco, R.F., de Jonge, E., Dekkers, P.E.P., Timmerman, J.J., Spek, C.A., van Deventer, S.J.H., van Deursen, P., van Kerkhoff, L., van Gemen, B., ten Cate, H., van der Poll, T., Reitsma, P.H. (2000). The in vivo kinetics of tissue factor messenger RNA expression during human endotoxemia: relationship with activation of coagulation. *Blood*, *96*, 554-559.
- Gregorut, F.P., Baptista, L.C., Paulim, A.S., Guerreiro, J.R., Secato, E.R., Macari, M. (1992). Influence of age on the febrile response to *E. coli* and *S. typhimurium* endotoxins in growing pullets. *British Poultry Science*, *33*, 769-774.
- Hangalapura, B.N., Nieuwland, M.G.B., de Vries Reilingh, G., Heetkamp, M.J.W., van den Brand, H., Kemp, B., Parmentier, H.K. (2003). Effects of cold stress on immune responses and body weight of chicken lines divergently selected for antibody responses to sheep red blood cells. *Poultry Science*, *82*, 1692-1700.
- Haudek, S.B., Natmessing, B.E., Fürst, W., Bahrami, S., Schlag, G., Redl, H. (2003). Lipopolysaccharide dose response in baboons. *Shock*, *20*, 431-436.

- Jones, C.A., Edens, F.W., Denbow, D.M. (1983). Influence of age on the temperature response of chickens to *Escherichia coli* and *Salmonella typhimurium* endotoxins. *Poultry Science*, 62, 1553-1558.
- Jones, C.A., Edens, F.W., Denbow, D.M. (1981). Rectal temperature and blood chemical responses of young chickens given *E. coli* endotoxin. *Poultry Science*, 60, 2189-2194.
- Klosterhalfen, B., Hörstmann-Jungemann, K., Vogel, P., Flohé, S., Offner, F., Kirkpatrick, C.J., Heinrich, P.C. (1992). Time course of various inflammatory mediators during recurrent endotoxemia. *Biochemical Pharmacology*, 43, 2103-2109.
- Landegren, U. (1984). Measurement of cell numbers by means of the endogenous enzyme hexosaminidase. Applications of detection of lymphokines and cell surface antigens. *Journal of Immunological Methods*, 67, 379-388.
- Lang, C.H., Bagby, G.J., Spitzer, J.J. (1985). Glucose kinetics and body temperature after lethal and nonlethal doses of endotoxin. *American Journal of Physiology*, 248, R471-8.
- Leon, L.R., Kozak, W., Rudolph, K., Kluger, M.J. (1999). An antipyretic role for interleukin -10 in LPS fever in mice. *American Journal of Physiology*, 276, R81-89.
- Leshchinsky, T.V. & Klasing, K.C. (2001). Divergence of the inflammatory response in two types of chickens. *Developmental & Comparative Immunology*, 25, 629-638.
- Leshchinsky, T.V. & Klasing, K.C. (2003). Profile of chicken cytokines induced by lipopolysaccharide in modulated by dietary  $\alpha$ -tocopheryl acetate. *Poultry Science*, 82, 1266-1273.
- Massart-Leën, A.M., Burvenich, C., Vandeputte-Van Messom, G., Hilderson, H. (1992). Partial prostaglandin-mediated mechanism controlling the release of cortisol in plasma after intravenous administration of endotoxins. *Domestic Animal Endocrinology*, 9, 273-283.

- Maxwell, J.R., Ruby, C., Kerkvliet, N.I., Vella, A.T. (2002). Contrasting the role of costimulation and the natural adjuvant lipopolysaccharide during the induction of T cell immunity. *Journal of Immunology*, 168, 4372-4381.
- Medvedev, A.E., Sabroe, I., Hasday, J.D., Vogel, S.N. (2006). Tolerance to microbial TLR ligands: molecular mechanisms and relevance to disease. *Journal of Endotoxin Research*, 12, 133-150.
- Medvedev, A.E., Piao, W., Shoenfelt, J., Rhee, S.H., Chen, H., Basu, S., Wahl, L.M., Fenton, M.J., Vogel, S.N. (2007). Role of TLR4 tyrosine phosphorylation in signal transduction and endotoxin tolerance. *The Journal of Biological Chemistry*, 282, 16042-16053.
- Morimoto, A., Murakami, N., Nakamori, T., Watanabe, T. (1987). Evidence for separate mechanisms of induction of biphasic fever inside and outside the blood-brain barrier in rabbits. *Journal of Physiology*, 383, 629-637.
- Nakamura, K., Mitarai, Y., Koizumi, N., Shibahara, T., Nakajima, Y. (1998). Serum levels of interleukin-6,  $\alpha_1$ -acid glycoprotein and corticosterone in two-week-old chickens inoculated with *Escherichia coli* lipopolysaccharide. *Poultry Science*, 77, 908-911.
- Ochsenbein, A.F., Fehr, T., Lutz, C., Suter, M., Brombacher, F., Hengartner, H., Zinkernagel, R.M. (1999). Control of early viral and bacterial distribution and disease by natural antibodies. *Science*, 286, 2156-2159.
- Parmentier, H.K., Lammers, A., Hoeckman, J.J., De Vries Reilingh, G., Zaanen, I.T.A., Saverkoul, H.F.J. (2004). Different levels of natural antibodies in chickens divergently selected for specific antibody response. *Developmental & Comparative Immunology*, 28, 39-49.
- Pittman, Q.J., Veale, W.L., Cockeram, W., Cooper, K.E. (1976). Changes in body temperature produced by prostaglandins and pyrogens in the chicken. *American Journal of Physiology*, 230, 1284-1287.

- Qu J., Zhang J., Pan J., He L., Ou Z., Zhang X., Chen X. (2003). Endotoxin tolerance inhibits lipopolysaccharide-initiated acute pulmonary inflammation and lung injury in rats by the mechanism of Nuclear Factor –  $\kappa$ B. *Scandinavian Journal of Immunology*, 58, 613-619.
- Reid, R.R., Prodeus, A.P., Khan, W., Hsu, T., Rosen, F.S., Carroll, M.C. (1997). Endotoxin shock in antibody-deficient mice. *Journal of Immunology*, 159, 970-975.
- Roth, J., McClellan, J.L., Kluger, M.J., Zeisberger, E. (1994). Attenuation of fever and release of cytokines after repeated injections of lipopolysaccharide in guinea-pigs. *Journal of Physiology*, 477, 177-185.
- Senrad, S.D. (1993) Comparative efficacy of flunixin, ketoprofen, and ketorolac for treating endotoxemic neonatal calves. *American Journal of Veterinary Research*, 54, 1511-1516.
- Sjibben, J.W.C., Klasing, K.C., Schrama, J.W., Parmentier, H.K., van der Poel, J.J., Savelkoul, H.F., Kaiser, P. (2003). Early in vivo cytokine genes expression in chickens after challenge with *Salmonella typhimurium* lipopolysaccharide and modulation by dietary n-3 polyunsaturated fatty acids. *Developmental & Comparative Immunology*, 27, 611-619.
- Siwek, M., Buitenhuis, B., Cornelissen, S., Nieuwland, M., Knol, E.F., Crooijmans, R., Groenen, M., Parmentier, H., van der poel, J. (2006). Detection of QTL for innate: Non-specific antibody levels binding LPS and LTA in two independent populations of laying hens. *Developmental & Comparative Immunology*, 30, 1-8.
- Skarnes, R.C., Brown, S.K., Hull, S.S., McCracken, J.A. (1981). Role of prostaglandin E in the biphasic fever response to endotoxin. *The Journal of Experimental Medecine*, 154, 1212-1224.
- Smith, I.M., Licence, S.T., Hill, R. (1978). Haematological, serological and pathological effects in chicks of one or more intravenous injections of *Salmonella gallinarum* endotoxin. *Research in Veterinary Science*, 24, 154-160.
- Snedecor, G.W. & Cochran, W.G. (1989). *Statistical methods*, Eight edition. Blackwell publishing.

- Takahashi, K., Miyake, N., Ohta, T., Akiba, Y., Tamura, K. (1998). Changes in plasma  $\alpha_1$ -acid glycoprotein concentration and selected immune response in broiler chickens injected with *Escherichia coli* lipopolysaccharide. *British Poultry Science*, 39, 153-155.
- Van Snick, J., Cayphas, S., Vink, A., Uyttenhove, C., Coulie, P.G., Rubira, M.R., Simpson, R.J. (1986). Purification and NH<sub>2</sub>-terminal amino acid sequence of a T-cell-derived lymphokine with growth factor activity for B-cell hybridomas. *Proceedings of the National Academy of Science of the United States of America*, 83, 9679.
- Wang M., Meng X., Tsai B., Wang J., Turrentine M., Brown J.W., Meldrum D.R. (2004). Preconditioning up-regulates the soluble TNF receptor I response to endotoxin. *Journal of Surgical Research*, 121, 20-24.
- Xie, H., Rath, N.C., Huff, G.R., Huff, W.E., Balog, J.M. (2000). Effects of Salmonella typhimurium lipopolysaccharide on broiler chickens. *Poultry Science*, 79, 33-40.
- Zeisberger, E. & Roth, J. (1998). Tolerance to pyrogens. *Annals of the New York Academy of Science*, 856, 116-131.

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**Table 1.** *Differences in  $AUC_{0 \rightarrow 24h}$  and  $maxT$  after the first and second dose of LPS in chickens at the age of 3 weeks.*

	$AUC_{0 \rightarrow 24h} \pm SD$ ( $^{\circ}C \times h$ )		$maxT \pm SD$ ( $^{\circ}C$ )	
	LPS	Control	LPS	Control
Day 0 (n=20)	1002.9 $\pm$ 1.1 <sup>A</sup>	994.6 $\pm$ 1.5 <sup>C</sup>	42.39 $\pm$ 0.06 <sup>A</sup>	41.85 $\pm$ 0.08 <sup>C</sup>
Day 2 (n=10)	992.8 $\pm$ 1.6 <sup>B</sup>	993.6 $\pm$ 2.1 <sup>B</sup>	42.03 $\pm$ 0.09 <sup>B</sup>	41.64 $\pm$ 0.11 <sup>C</sup>
Day 7 (n=10)	996.7 $\pm$ 1.5 <sup>B</sup>	992.8 $\pm$ 2.1 <sup>B</sup>	42.34 $\pm$ 0.08 <sup>A</sup>	41.56 $\pm$ 0.11 <sup>C</sup>

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**Table 2:** *Differences in  $AUC_{0 \rightarrow 24h}$  and  $maxT$  after a first and second LPS challenge in chickens at the age of 5 weeks.*

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	$AUC_{0 \rightarrow 24h} \pm SD$ ( $^{\circ}C \times h$ )		$maxT \pm SD$ ( $^{\circ}C$ )	
	LPS	Control	LPS	Control
Day 0 (n=20)	$995.1 \pm 1.0^A$	$987.9 \pm 1.5^B$	$42.16 \pm 0.06^A$	$41.55 \pm 0.08^B$
Day 2 (n=10)	$995.5 \pm 1.5^A$	$993.4 \pm 2.1^A$	$42.08 \pm 0.08^A$	$41.75 \pm 0.11^A$
Day 7 (n=10)	$992.0 \pm 1.5^A$	$989.3 \pm 2.1^A$	$42.15 \pm 0.08^A$	$41.62 \pm 0.11^B$

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**Figure 1.** *Mean (+ SD) body temperature versus time plot after a first and second dose of LPS in chickens at the age of 3 weeks (1<sup>st</sup> challenge n=20, 2<sup>nd</sup> challenge n=10, control n=20). The second dose of LPS was administered 2 or 7 days after the first dose of LPS.*

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**Figure 2.** *Plasma IL-6 concentrations (mean + SD) after a first and second dose of LPS in chickens at the age of 3 weeks (1<sup>st</sup> challenge n=20, 2<sup>nd</sup> challenge n=10, control n=20). Means with an asterisk differ significantly from those of the control group ( $P<0.0001$ ). The second LPS dose was administered 2 or 7 days after the first dose.*

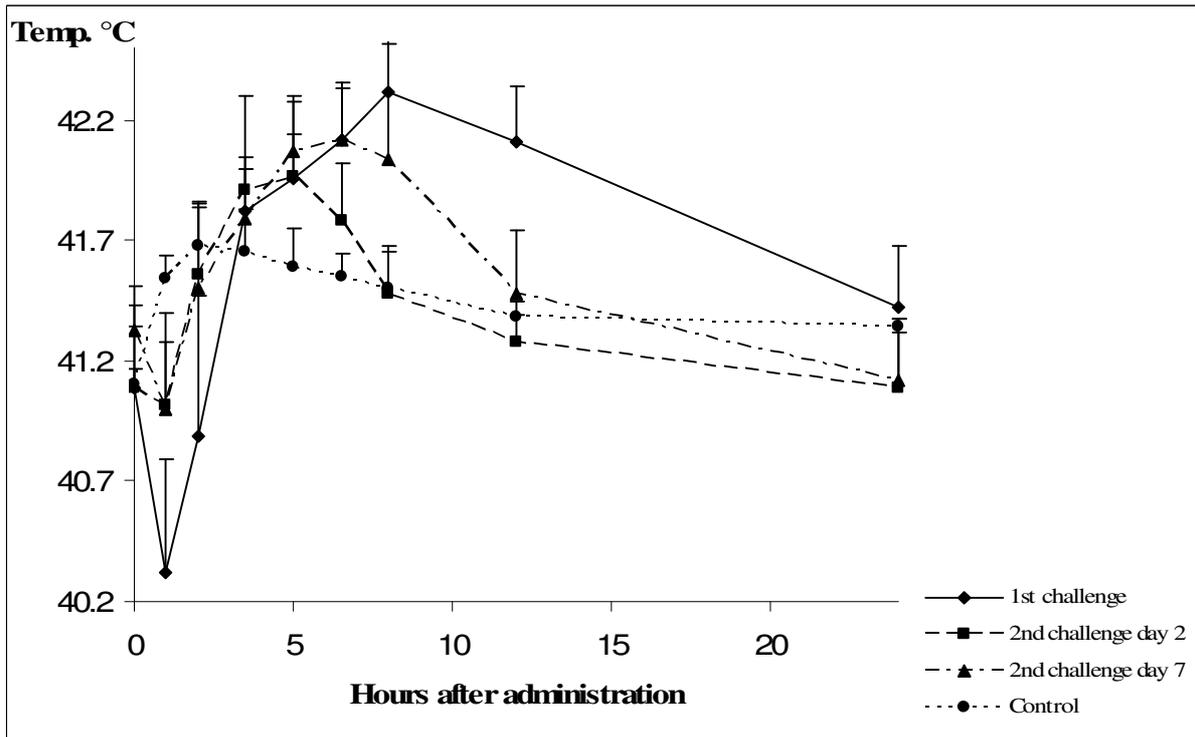


Figure 1. Mean (+ SD) body temperature versus time plot after a first and second dose of LPS in chickens at the age of 3 weeks (1<sup>st</sup> challenge n=20, 2<sup>nd</sup> challenge n=10, control n= 20). The second dose of LPS was administered 2 or 7 days after the first dose of LPS.

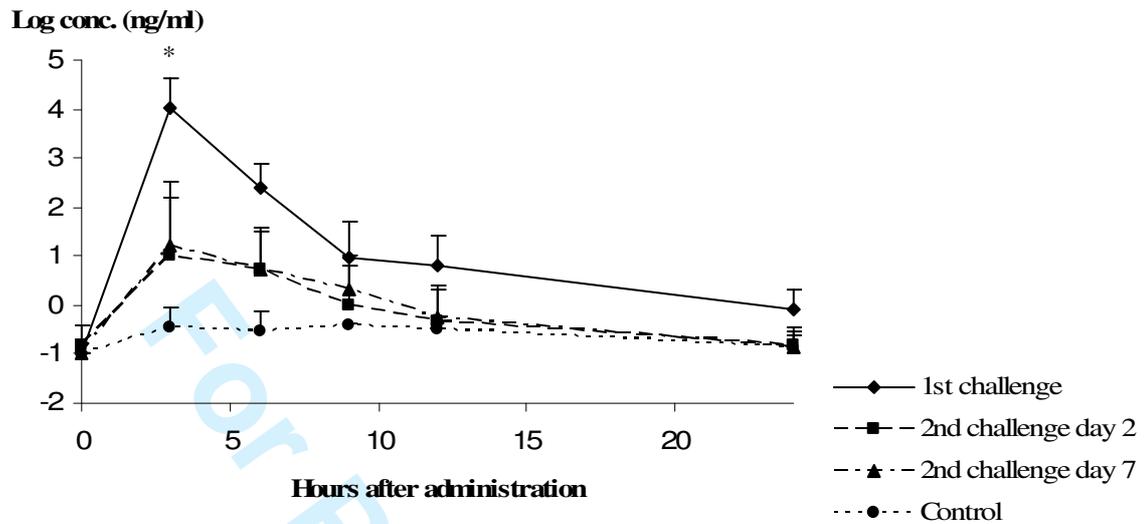


Figure 2. Plasma IL-6 concentrations (mean + SD) after a first and second dose of LPS in chickens at the age of 3 weeks (1<sup>st</sup> challenge n=20, 2<sup>nd</sup> challenge n=10, control n= 20). Means with an asterisk differ significantly from those of the control group ( $P < 0.0001$ ). The second LPS dose was administered 2 or 7 days after the first dose.