The Journal of Experimental Biology 214, 3378-3385

doi:10.1242/jeb.056499

RESEARCH ARTICLE

The development of endotoxin tolerance, and the role of hypothalamo-pituitary-adrenal function and glucocorticoids in Pekin ducks

Manette Marais[1,*], Shane K. Maloney[2] and David A. Gray[1]

[1]School of Physiology, Faculty of Health Science, University of the Witwatersrand, Johannesburg 2193, South Africa and [2]Physiology, School of Biomedical, Biomolecular, and Chemical Sciences, University of Western Australia, Perth, Western Australia 6009, Australia

*Author for correspondence (manette.marais@wits.ac.za)

Accepted 27 June 2011

SUMMARY

Endotoxin tolerance represents a state of abated immunological responsiveness to pyrogens, which, in mammals, leads to the decline or abolition of the fever response. The development of endotoxin tolerance in birds is not well understood; consequently, the impact of repeated pathogenic exposure on the avian febrile response, and thus on the ability of birds to fight recurrent infection, is not known. We determined the effect of repeated injections of lipopolysaccharide (LPS) on the febrile response of Pekin ducks. We gave ducks five injections of LPS, spaced 1, 4 or 10 days apart, and recorded their core body temperature with abdominally implanted temperature data loggers. Once we established that Pekin ducks developed endotoxin tolerance, we investigated the effect of repeated injections of LPS on the central and peripheral segments of the hypothalamo-pituitary-adrenal (HPA) axis in an attempt to elucidate the role of glucocorticoids in the modulation of the febrile response during the tolerant period. When our ducks became tolerant to LPS, they had significantly higher basal levels of plasma corticosterone (CORT, the principal glucocorticoid in birds), and their HPA response to treatment with LPS was blunted. We propose that the augmented levels of basal plasma CORT resulted from sensitized HPA function, and this, in turn, contributed to the development of endotoxin tolerance. Regulation of the circulating level of CORT might be a possible target for the re-establishment of appropriate immune responses in birds.

Key words: endotoxin tolerance, fever, glucocorticoid, lipopolysaccharide, Pekin duck.

INTRODUCTION

Birds and mammals develop fever when injected with bacterial endotoxin, lipopolysaccharide (LPS) from the cell walls of gram-negative bacteria (Blatteis et al., 2000; Cartmell and Mitchell, 2005; D'Alecy and Kluger, 1975; Gray et al., 2005; Macari et al., 1993; Maloney and Gray, 1998; Nomoto, 2003; Romanovsky et al., 2005). In mammals, repeated exposure to LPS within 1 to 10 days results in a reduction and, in some instances, the total abolition of the febrile response to the same stimulus (Kawasaki et al., 1987; Roth et al., 1997; Roth et al., 1994). This modulation of the febrile response is part of a temporary relegated immune response to gram-negative pathogens, a phenomenon known as endotoxin tolerance (Roth et al., 1997; Roth et al., 1994; West and Heagy, 2002). As yet, little is known about the development of endotoxin tolerance in birds. Consequently, the impact of repeated pathogenic exposure on the avian febrile response, and the ability of birds to fight recurrent infection, is not known.

In mammals, endotoxin tolerance results from: (1) an increase in adrenal glucocorticoid (GC) secretion due to modulation of the hypothalamo-pituitary-adrenal (HPA) axis (Beishuizen and Thijs, 2003), and (2) an attenuated release of the pro-inflammatory cytokines that are known to mediate the febrile response, specifically, tumor necrosis factor (TNF)-α, interleukin (IL)-1 and IL-6 (Blatteis, 2004; Cartmell and Mitchell, 2005; Harden et al., 2006; Harden et al., 2008; Nomura et al., 2000; Romanovsky et al., 2005; Rothwell et al., 1996). Although GCs are known to suppress the synthesis and release of pro-inflammatory cytokines from circulating immune cells (Coelho et al., 1995), GC-independent biochemical pathways, which inhibit the production and release of these cytokines during a state of tolerance, have been identified (Cavaillon and Adib-Conquy, 2006; Fan and Cook, 2004). In birds, GCs are the only known modulator of the febrile response (Gray et al., 2005), but whether GCs contribute to the development of endotoxin tolerance in birds is not known.

The present study was designed to ascertain firstly whether birds develop endotoxin tolerance to doses of LPS known to mimic mild to moderate bacterial infections (Maloney and Gray, 1998). Once we established that the birds had a reduced febrile response when they were treated repeatedly with LPS, we investigated the role of GCs in the development of endotoxin tolerance and the effect of repeated LPS injections on HPA function in birds.

For our study we chose Pekin ducks as experimental birds to represent the avian class. Pekin ducks are galloanserines, a group thought to be the basal lineage from which all modern-day birds evolved (vanTuinen et al., 2000). Previous studies have confirmed that the underlying mechanisms responsible for the fever response in Pekin ducks are similar to those present in other bird species (Gray et al., 2005; Maloney and Gray, 1998). For instance, pro-inflammatory cytokines as well as prostaglandins have been identified as likely mediators of fever in diverse avian species (Gray et al., 2005; Johnson et al., 1993b; Macari et al., 1993; Nakamura et al., 1998). Moreover, similarities between LPS fevers in Pekin ducks and LPS fevers in mammals have been identified (D'Alecy

and Kluger, 1975; Johnson et al., 1993b; Maloney and Gray, 1998). There is convincing evidence that prostaglandin E_2 fulfils the role of final mediator of the mammalian febrile response, and studies with birds have also identified prostaglandins as having an important role in the avian febrile mechanism (Johnson et al., 1993b; Macari et al., 1993; Romanovsky et al., 2005; Roth et al., 2004).

Here we describe the development of endotoxin tolerance in Pekin ducks by reporting the results from three experimental series of repeated LPS injections at varying time intervals. We also show that repeated LPS treatments modulated the HPA axis and that corticosterone (CORT, the principal GC in birds) likely contributed to the development of endotoxin tolerance in our birds.

MATERIALS AND METHODS

Experimental design

Experimentation was conducted over a period of 12 months and comprised three studies: (1) an investigation into the effect of repeated LPS injections on the febrile response; (2) an investigation into the role of CORT in the development of endotoxin tolerance in Pekin ducks and; (3) an investigation into the effect of repeated LPS injections on HPA function. We completed the first study and analyzed the data before we began the other studies.

Animals

Thirty-two adult Pekin ducks (*Anas platyrhynchos* Linnaeus 1758), 11 male and 21 female, with body mass between 2.5 and 3.4 kg, were used for the experiments. Sixteen ducks were randomly assigned to study 1 and eight ducks each to studies 2 and 3. The ducks were housed as a flock in an indoor pen and exposed to a 12 h:12 h day:night cycle (lights on at 6:00 h), an average night-time temperature of 21°C and a daytime temperature of 23°C. They had *ad libitum* access to dry chicken food enriched with minerals and vitamins, as well as drinking and bathing water.

Body temperature measurements

Core body temperatures were recorded at 20 min intervals using miniature temperature data loggers with a resolution of 0.02°C (Tidbit, Onset Computer, Bourne, MA, USA). The data loggers were calibrated (from 38 to 44°C at 1°C intervals) against a certified precision thermometer (Quat 100, Heraeus, Germany), coated with wax (Sasol Wax, Sasolburg, South Africa) and then implanted into the abdominal cavity of each duck. For surgery and data logger implantation, the ducks were anaesthetised with 2% Isoflurane (Safe Line Pharmaceuticals, Wadeville, Johannesburg, South Africa) in oxygen. The ducks were allowed 2 weeks after surgery before the start of experimentation.

Injections containing endotoxin

LPS from *Escherichia coli* (Sigma-Aldrich, St Louis, MO, USA) was dissolved in sterile isotonic saline and diluted to 350 μg ml^{-1}. Immediately before injection ducks were weighed and a volume containing 100 μg kg^{-1} of LPS was drawn up and adjusted with saline to a volume of 1 ml. The dose of LPS was based on the results of previous fever studies in birds and has been shown to give a strong, but sub-maximal fever response (Gray et al., 2005; Johnson et al., 1993a; Maloney and Gray, 1998; Nomoto, 1996). All injections were given intramuscularly (i.m.) between 10:00 and 11:00 h on days of experimentation.

Blood collection

For studies 2 and 3 we needed to collect blood from our ducks for plasma CORT analysis. Disturbing ducks was previously shown to significantly increase plasma CORT within 3 min (Harvey et al., 1980). For this reason, all blood collection for the purpose of obtaining normal levels of plasma CORT was done within 3 min of entering the housing pen. Once a bird was caught, we drew venous blood from the leg vein. All blood was collected in heparinised containers (Becton Dickinson, Johannesburg, South Africa), immediately placed on ice, and centrifuged at 3000 rpm for 10 min; thereafter the plasma was removed and stored at –20°C until analysis.

Plasma CORT analysis

The CORT levels of plasma samples were determined by a commercially available radioimmunoassay (RIA) (MP Biomedicals, Orangeburg, NY). The instructions of the manufacturers were followed for analysis.

Experimental procedures

Study 1

The aim of the first study was to describe the effect of repeated LPS treatments on the febrile response in Pekin ducks. For this, we randomly chose 10 Pekin ducks from the flock and treated them with three series of LPS injections. Each series consisted of a total of five LPS injections (each containing 100 μg kg^{-1} LPS made up to 1 ml with sterile isotonic saline). The time interval between each LPS injection differed from series to series and was 1, 4 or 10 days. The order of experimental series was randomized and ducks were randomly assigned to a particular experimental series. By the end of experimentation all of the birds had received each experimental series once. Thus, in total each bird received 15 LPS injections over a period of 9 months.

To control for any effect of prior exposure to LPS on the body temperature response to injection, we scheduled a control saline injection before the first, and after the last, LPS injection. The time interval between the saline injections and the LPS injections was the same as the time interval between LPS injections. For instance, if ducks were given LPS every 4 days, then one saline injection was given 4 days before the first LPS injection and one saline injection was given 4 days after the last LPS injection. Thus, in total each bird also received six saline injections.

To control for the effect of repeated injections on the body temperature of Pekin ducks, we randomly chose six naïve ducks (birds that have never before been injection with LPS) from the flock and gave each of them an i.m. injection of 1 ml isotonic saline for seven consecutive days. Injections were given at 10:00 h every day. After each injection the birds were released in their pen and left undisturbed for 24 h. We allowed a washout period of 21 days between experimental series.

At the end of the first study it was clear that our ducks had a significantly reduced febrile response when they were treated repeatedly with LPS (see results, Fig. 1), and we commenced with the other studies.

Study 2

To determine whether CORT contributed to the development of endotoxin tolerance observed in ducks in the first study, we gave the group of ducks (N=8) assigned to study 2 five injections of 100 μg kg^{-1} LPS (or saline) for five consecutive days and collected venous blood (2 ml each time) *via* venipuncture for CORT analysis at different times: immediately before the first and the fifth LPS (or saline) injection (time 0, for determination of basal levels of plasma CORT), and at 3 and 8 h after the first and fifth LPS (or saline) injection. The results of study 1 showed that during a state of

tolerance the peak of the fever was modulated and the duration of fever was shorter. So, we wanted to ascertain whether the level of plasma CORT at the time of the febrile peak was elevated and influenced the febrile peak. Likewise, we wanted to ascertain whether an elevated level of plasma CORT influenced the time to the resolution of fever and at the same time see whether plasma CORT was upregulated at the end of fever. Time points of 3 and 8 h post-injection coincide with the peak and the resolution of the fever response in ducks. Because handling the ducks for blood collection potentially affects the level of plasma CORT, we designed our experiment so that blood collection was performed at only one time point post LPS (or control saline) treatment. Thus, each duck was made tolerant with a series of LPS injections twice, once for blood collection at 3 h and once for blood collection at 8 h after the first and fifth LPS treatment. To appropriately control for the effect of treatment, *per se*, on the level of plasma CORT, we also gave ducks two series of saline injections and then followed the same protocol for blood collection as described for LPS. Ducks were randomly chosen to receive these series of injections. At the end of experimentation all ducks had received all the injections (10 containing LPS and 10 containing saline).

We continuously monitored core body temperatures of this group of ducks with abdominally implanted data loggers. However, handling the ducks for blood collection confounds temperature data because the ducks develop a stress hyperthermia when they are caught (Gray et al., 2008). Therefore, the body temperature data obtained from these ducks were used to confirm that the ducks had body temperature responses similar to those of the ducks in the first study.

Study 3

In birds, the amount of CORT that can be elicited by various HPA stimulants provides a measure of HPA sensitivity (Kuenzel and Jurkevich, 2010; Mikhailova et al., 2007; Spelman et al., 1995). We used the protocol of Spelman et al. (Spelman et al., 1995) to test for adrenal sensitivity with an intravenous (i.v.) injection of synthetic adrenocorticotrophic hormone (ACTH, 0.25 mg) (Novartis, Johannesburg, South Africa) (Spelman et al., 1995). A dose of 0.25 mg of synthetic ACTH was shown to increase plasma CORT levels maximally in ducks (approximately the same weight and age as our birds) 1 to 2 h post injection and we therefore opted to collect blood from our ducks 1 h post ACTH injection (Spelman et al., 1995).

In ducks, peripheral co-administration of arginine vasotocin (AVT) and corticotrophin releasing hormone (CRH) was shown to synergistically stimulate ACTH and subsequent CORT release in ducks (Kuenzel and Jurkevich, 2010; Mikhailova et al., 2007). We followed the protocol of Mikhailova et al. (Mikhailova et al., 2007) to test the pituitary sensitivity of our ducks. Briefly, AVT (Sigma-Aldrich, Johannesburg, South Africa) and ovine CRH (oCRH; Sigma-Aldrich) were each made up to a dose of 3 μg kg^{-1} in 0.5 ml sterile isotonic saline. The dosages of AVT and CRH were then combined and injected into the leg vein and we collected blood 30 min post injection. Mikhailova found AVT + CRH administration to cause maximal HPA stimulation and CORT release 30 min post treatment (Castro et al., 1986; Mikhailova et al., 2007).

To determine whether endotoxin tolerance affected the peripheral and/or central segment of the HPA axis, we tested the adrenal sensitivity and, on another occasion, the pituitary sensitivity of ducks (N=8) before and 24 h after they were made tolerant using four consecutive daily injections of 100 μg kg^{-1} LPS. From the results of study 1, we knew that ducks made tolerant with daily injections of LPS developed a reduced fever following the fifth LPS treatment, and we wanted to ascertain whether the state of HPA axis sensitivity at the time of the fifth LPS injection contributed to the reduction of this febrile response. Thus, we designed our experiment to measure the HPA axis sensitivity at the time point coinciding with the fifth LPS injection. For this reason, the ducks assigned to this study received four consecutive daily injections of LPS and then the HPA sensitivity was measured 24 h after the fourth injection – the same time that the ducks in study 1 received the fifth LPS treatment. Because we measured the adrenal and pituitary sensitivity of all the ducks on separate occasions, ducks were made tolerant with a series of LPS injections twice: once to measure adrenal and once to measure pituitary sensitivity. The order of the sensitivity tests was randomized.

To control for the effect of repeated injections, *per se*, on HPA sensitivity, ducks also received two series of saline injections: one to measure adrenal and one to measure pituitary sensitivity. Ducks were randomly given each of the series of injections and we allowed a washout period of 21 days between each series of injections.

We obtained baseline levels of circulating CORT by collecting blood at the time of injecting HPA stimulants. Similar to the protocol of blood collection described in study 2, we drew venous blood from the leg vein of ducks within 3 min of entering the pen. Immediately thereafter ducks were injected with ACTH or with AVT combined with CRH (AVT + CRH). Each duck was then restrained in a custom-made canvas sling that kept the wings secured around the body, but left the head uncovered and the feet to hang freely until we collected blood from the leg vein on the other leg (the leg not used for blood collection when we entered the pen).

At the end of experimentation, the ducks were killed with an overdose of sodium pentobarbitone (Eutha-naze, Centaur Laboratories, RSA, Chapel Hill, NC, USA) injected i.v. according to the Animal Ethics Committee guidelines. The implanted data loggers were retrieved and the data were downloaded for analysis. The procedures of this study were approved by the Animal Ethics Committee of the University of the Witwatersrand (application no. 2008/31/04).

Data analysis

All data are reported as means ± s.d. or means ± s.e.m. (as indicated).

Study 1

We calculated thermal response indices (TRIs) evoked by all treatments by subtracting, for each duck, the mean body temperature for the 2 h prior to injection from the body temperatures following treatment. We omitted the stress hyperthermia and calculated 10 h TRIs from 2 to 12 h post injection.

To ascertain whether repeated LPS treatments affected the febrile response in Pekin ducks, we compared the mean of the TRIs following each LPS treatment within and between each series with a repeated-measures two-way ANOVA.

To ascertain whether prior repeated exposure to LPS affected body temperature responses to an injection, *per se*, we compared the mean of the TRIs evoked by saline treatment given prior to and post LPS treatment, within and between the different series, using a repeated-measures two-way ANOVA.

To ascertain whether repeated injections, *per se*, affect normal body temperature, we compared the mean of the TRIs following the first to the seventh injection of saline using a repeated-measures ANOVA.

Study 2

Because ducks were randomly assigned to the series of treatments (LPS or saline) we determined the effect of experimental order on

the level of plasma CORT in our ducks by comparing the level of plasma CORT evoked by the first and fifth LPS treatment in ducks first exposed to saline with those first exposed to LPS, using a repeated-measures two-way ANOVA. Thereafter we compared the mean of plasma CORT levels for our group of ducks immediately before the first and the fifth LPS (or saline) injection and the plasma CORT levels in ducks at 3 and 8 h after the first and fifth LPS (or saline) injection with a repeated-measures two-way ANOVA and Bonferroni *post hoc* tests when the ANOVA indicated significant differences between means.

Study 3

The experiment was randomized and we determined the effect of experimental order on the level of plasma CORT evoked in our ducks with HPA stimulants using a repeated-measures two-way ANOVA. Thereafter we compared the mean of plasma CORT levels elicited by injections of ACTH and AVT + CRH given before and after a series of LPS or control saline injections using a repeated-measures two-way ANOVA and Bonferroni *post hoc* tests when the ANOVA indicated significant differences between means.

All comparisons were deemed significantly different at $P<0.05$.

RESULTS

On days when they were not handled, the normal mean (±s.d.) daytime body temperature of the ducks was 41.1±0.2°C. The ducks developed a transient, stress hyperthermia when handled, prior to treatment; however, this resolved within 60 to 90 min.

Effect of repeated injection of LPS on body temperature

There were significant differences in the body temperatures evoked by LPS within and between experimental series. Summarising the responses as TRI showed that the treatments differed significantly (main effect of treatment, $F_{4,135}=32.15$, $P<0.0001$; main effect of time, $F_{2,135}=23.10$, $P<0.0001$; interaction, $F_{8,135}=4.52$, $P<0.0001$; Fig. 1A–C). Bonferroni *post hoc* analyses showed that when LPS injections were given every day, or every 4 days, the third, fourth and fifth injections evoked significantly smaller TRIs than the TRI following the first injection in each series (Fig. 1A,B). There was no change in the TRIs evoked by LPS when the ducks were treated every 10 days (Fig. 1C).

Effect of prior exposure to LPS on body temperature responses to injection

The TRIs evoked by saline injections given before each experimental series did not differ from each other, or from the TRIs evoked by the saline injection after the LPS treatment series, within or between each series (repeated measures two-way ANOVA, main effect of treatment, $F_{2,54}=0.7$, $P=0.50$; main effect of saline before *versus* saline after, $F_{1,54}=0.03$, $P=0.86$; interaction, $F_{2,54}=0.95$, $P=0.39$; Fig. 1D).

Effect of repeated injection of saline on body temperature

The TRIs evoked by the first through the seventh injection of saline did not differ significantly from each other (repeated-measures one-way ANOVA, $F_{6,5}=1.8$, $P=0.13$, $N=6$; Fig. 1E).

Role of CORT in endotoxin tolerance

The order in which ducks were treated due to randomization did not affect plasma CORT concentrations post LPS (main effect of order, $F_{1,24}=0.27$, $P=0.61$; main effect of treatment, $F_{3,24}=3.33$, $P=0.03$; interaction, $F_{3,24}=0.69$, $P=0.57$). The data were therefore pooled for further analysis.

The treatment that the ducks received, either a series of LPS or saline, and the time point post treatment significantly affected the level of plasma CORT in our ducks (repeated-measures two-way ANOVA, main effect of treatment, $F_{3,84}=20.0$, $P<0.0001$; main effect of time, $F_{2,84}=5.91$, $P=0.004$; interaction, $F_{6,84}=1.57$, $P=0.16$; Fig. 2). *Post hoc* analysis showed that, prior to the first injections of the series of LPS or saline (time 0), the circulating level of CORT did not differ between the experimental groups. The mean ± s.d. basal levels of CORT in the two experimental groups were 79.06±34.43 and 68.79±29.20 ng ml^{-1}, respectively ($t_7=0.64$, $P=0.54$).

CORT levels measured 3 or 8 h after the first injection of saline did not differ significantly from the basal CORT ($t_7=0.06$, $P=0.95$ and $t_7=0.19$, $P=0.85$, respectively). There was also no change in the basal levels of CORT after four consecutive saline injections ($t_7=0.50$, $P=0.63$) or a change in CORT either 3 or 8 h following the fifth saline treatment ($t_7=0.92$, $P=0.39$ and $t_7=0.46$, $P=0.66$, respectively).

On the first day of LPS treatment there was a significant increase in CORT at 3 h ($t_7=3.88$, $P=0.006$), and the circulating levels had returned to basal by 8 h ($t_7=0.94$, $P=0.38$).

After four consecutive LPS injections, immediately prior to the fifth LPS treatment, the mean (±s.d.) concentration of plasma CORT in the ducks was 143.23±41.32 ng ml^{-1}. This was significantly higher than the basal level of CORT measured before the first LPS treatment ($t_7=3.5$, $P=0.01$). At 3 or 8 h after the fifth LPS injection there was no significant change in CORT from the already elevated levels observed at time 0 ($t_7=1.29$, $P=0.24$ and $t_7=0.37$, $P=0.72$) (see Fig. 2 for plasma CORT amounts measured at different times).

HPA axis sensitivity

The order in which ducks were exposed to treatment, either following a series of saline or LPS injections first, did not affect the amount of CORT evoked by the HPA stimulants (repeated-measures two-way ANOVA, main effect of experimental order, $F_{1,24}=0.02$, $P=0.90$; main effect of treatment, $F_{3,24}=6.55$, $P=0.002$; interaction $F_{3,24}=0.3$, $P=0.82$). The data were therefore pooled for further analysis.

The series of treatments that the ducks received prior to testing HPA axis sensitivity, either saline or LPS, significantly affected the level of plasma CORT evoked by injections of ACTH and AVT + CRH [repeated-measures two-way ANOVA, main effect of treatment, $F_{5,84}=24.57$, $P<0.0001$; main effect of time (before *vs* after a series of injections) $F_{1,84}=18.54$, $P<0.0001$; interaction, $F_{5,84}=3.45$, $P=0.007$; Fig. 3].

Bonferroni *post hoc* analysis showed that: (1) prior to treatment with LPS or saline, both AVT + CRH and ACTH evoked significant increases in plasma CORT concentrations ($t_7=2.48$, $P=0.04$ and $t_7=2.62$, $P=0.03$ for AVT + CRH prior to saline and LPS series, respectively, and $t_7=4.32$, $P<0.001$ and $t_7=4.96$, $P<0.001$ for ACTH prior to saline and LPS series, respectively); (2) repeated injections of saline did not affect the basal level of plasma CORT in our ducks ($t_7=0.61$, $P=0.56$); and (3) saline injections did not affect the amount of plasma CORT evoked by either AVT + CRH or by ACTH ($t_7=0.02$, $P=0.98$ for AVT + CRH prior to saline treatment *vs* AVT + CRH post saline treatment, and $t_7=0.002$, $P=1.0$ for ACTH prior to saline treatment *vs* ACTH post saline treatment).

In contrast, repeated injections of LPS resulted in: (1) significantly higher basal levels of plasma CORT ($t_7=2.45$, $P=0.04$); (2) significant increases in the amount of CORT evoked by AVT + CRH and by ACTH ($t_7=4.14$, $P=0.009$ for AVT + CRH prior to LPS treatment *vs* AVT + CRH post LPS treatment, and $t_7=3.83$,

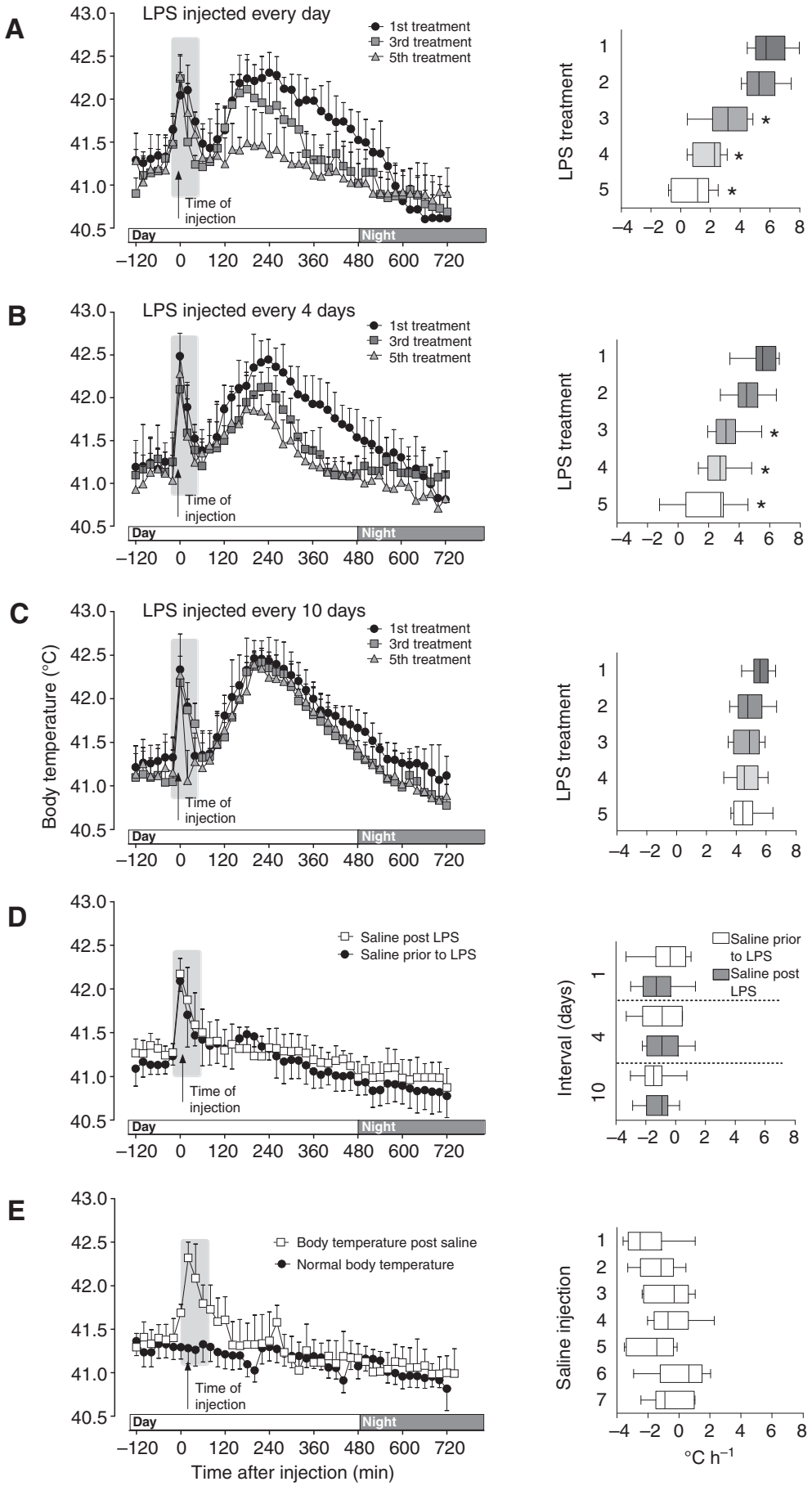

Fig. 1. Left: (A–C) mean (±s.d.) core body temperatures recorded every 20 min in Pekin ducks (*N*=10) after injections of lipopolysaccharide (LPS); (D) core body temperatures evoked by saline injections, given before and after each series of LPS treatments; and (E) core body temperatures of ducks given daily injections of saline. The thermal response indices (TRIs) following saline injections did not differ significantly from each other, and the profiles of body temperature (D, E) are the means (±s.d.) of the body temperatures evoked by the saline injections in each series. The black circles in E show the normal core body temperature of ducks on a day when they were left undisturbed. The arrow indicates the time of injection and the grey shading shows the stress hyperthermic response that ducks develop when they are handled for treatment. Right: mean (±s.d.) TRIs for the different LPS or saline treatments in the corresponding experimental series. Asterisks indicate significantly smaller than the TRI evoked by the first LPS treatment ($P<0.05$, repeated-measures two-way ANOVA and Bonferroni *post hoc* tests).

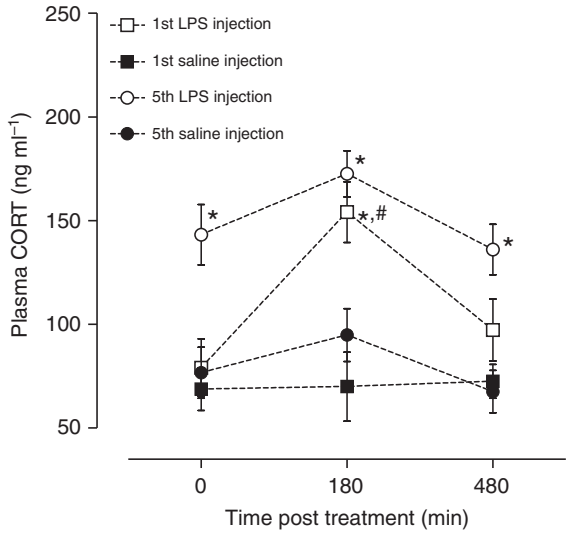

Fig. 2. Mean (±s.e.m.) concentration of plasma corticosterone (CORT, ng ml^{-1}) in Pekin ducks at different times after treatment with LPS (open symbols) or saline (filled symbols). Squares represent the first treatment in the series and circles represent the fifth treatment in the series. *, Plasma CORT concentrations significantly different from those evoked by the control treatment at the same time interval; #, CORT levels significantly different from time 0 following that specific treatment.

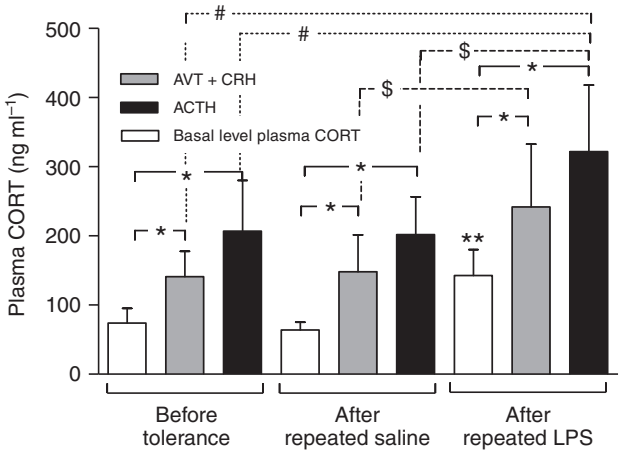

Fig. 3. Plasma CORT levels in Pekin ducks. White bars show the basal level (mean ± s.d.) of CORT (ng ml^{-1}) in ducks before the hypothalamo-pituitary-adrenal (HPA) axis was stimulated on a day before the ducks had received any treatment and after a series of LPS or saline injections. Grey and black bars show the level (mean ± s.d.) of plasma CORT that was evoked by the HPA stimulants arginine vasotocin given with corticotropic releasing hormone (AVT + CRH; grey bars) and adrenocorticotropic releasing hormone (ACTH; black bars). *, Significant difference between the level of CORT following HPA stimulation and the basal level within that treatment; **, significant difference from basal level measured before treatment and the basal level after a series of saline injections; #, significant difference between the corresponding treatments before tolerance and after a series of LPS injections; $, significant difference between the CORT levels evoked by repeated saline and by repeated LPS injections.

P=0.002 for ACTH prior to LPS treatment *vs* ACTH post LPS treatment); and (3) no difference in the amount of plasma CORT that was evoked by AVT + CRH and by ACTH (t_7=2.03, P=0.08).

DISCUSSION

Endotoxin tolerance causes a suppression of the innate immune response. This can be protective or detrimental, as a refractory immune response safeguards the host against endotoxic shock, but, at the same time, renders the host vulnerable to secondary infections (Cavaillon and Adib-Conquy, 2006). Manipulating the development of endotoxin tolerance is therefore considered a target for re-establishing appropriate immune responses (Cavaillon and Adib-Conquy, 2006; Fan and Cook, 2004). Although researchers have thus far focused their attention on the development of endotoxin tolerance in mammals, knowledge on the manifestation of endotoxin tolerance in birds could be valuable for ecologists and for the poultry industry. Also, information on the mechanism responsible for endotoxin tolerance in birds may well contribute to our understanding of the evolutionary development of this phenomenon.

From our results it is clear that the febrile response in Pekin ducks became modulated when they were repeatedly given amounts of LPS that mimic mild to moderate bacterial infections (Maloney and Gray, 1998). Attenuated, or abolished, febrile responses are a well-described sign for the development of endotoxin tolerance (Roth et al., 1997; Roth et al., 1994). Fever is a component of the innate immune system's acute phase response and, therefore, reflects the robustness of innate immune reactions. The time frame in which endotoxin tolerance developed in our ducks depended on the period between endotoxin exposures, with LPS injections given on consecutive days resulting in the earlier, and more acute, immunological hypo-responsiveness.

It is likely that other bird species would develop tolerance in the same way that our ducks became hypo-responsive to endotoxin because: (1) the febrile mechanism is thought to be evolutionary conserved (Ewald, 1980); (2) the response of ducks, chickens, pigeons and sparrows to LPS is comparable (Adelman et al., 2010; Johnson et al., 1993a; Maloney and Gray, 1998; Nomoto, 1996); and (3) there is evidence that the physiological mechanism, underlying fever in Pekin ducks, is common to other avian orders (Gray et al., 2005; Johnson et al., 1993b; Nomoto, 2003). There are also similarities in the function of the innate immune response in birds and in mammals; for instance, there is evidence that the activation of the innate immune response *via* a family of toll-like receptors and the ensuing release of pro-inflammatory cytokines are equivalent in both phyla (Gray et al., 2005; Romanovsky et al., 2005). Despite these similarities, we show here that endotoxin tolerance in Pekin ducks is somewhat different to endotoxin tolerance in mammals. In mammals, the injection of 20 to 100 μg kg^{-1} of LPS at intervals 24 or 48 h apart typically abolishes the febrile response after the second or third treatment (Dias et al., 2005; Roth et al., 1997; Roth et al., 1994; Tedeschi et al., 2007; Tripp et al., 1998; Wakabayashi et al., 1994). In contrast, ducks continued to develop fever, albeit markedly reduced, even after five injections of 100 μg kg^{-1} of LPS, each given 24 h apart. A plausible explanation for this major difference is offered later in the discussion.

In birds, and in mammals, GCs are known to modulate the fever response after acute exposure to endotoxin (Gray et al., 2005). Similar to the results of previous studies with birds, we also detected a significant increase in the amount of circulating CORT in naïve ducks 3 h post LPS treatment, a time point that corresponds with the peak of fever (Gray et al., 2005; Shini et al., 2008). Gray et al. reported that the concentration of plasma CORT in naïve ducks treated with LPS returns to pre-injection levels within 6 h (Gray et al., 2005). In the present study we also found that 8 h after an acute challenge with LPS, plasma CORT concentrations were not significantly different from basal levels.

GCs are known to contribute to a state of endotoxin tolerance in mammals (Beishuizen and Thijs, 2003). As yet, the role of GCs in the development and maintenance of endotoxin tolerance in birds is not well understood. We found that tolerant ducks had significantly elevated levels of plasma CORT 24 h after they had been given LPS injections each day for the previous 4 days, and we think it is likely that this elevation in plasma CORT contributed to the attenuated fever response that followed the fifth LPS treatment. In mammals, GCs are known to downregulate the peripheral release of febrile mediating pro-inflammatory cytokines (Cavaillon and Adib-Conquy, 2006; Flohéa et al., 1998). It is likely that in birds CORT also inhibits the synthesis and/or release of pro-inflammatory cytokines, and we propose that the higher levels of plasma CORT in our ducks curbed the synthesis and/or release of pro-inflammatory cytokines that were liberated from circulating immune cells when the innate immune system was activated by the fifth LPS treatment. This, we propose, limited the amount of pro-inflammatory cytokines that crossed the blood–brain barrier to produce central mediators of the febrile response. Because the immunological mediators of fever were reduced prior to the central activation of the febrile mechanism, the febrile response in tolerant ducks, treated with a dose of $100\,\mu g\,kg^{-1}$ of LPS, would, in effect, be modulated in such a way that it represents a fever that would be evoked by a lower dose of LPS in naïve birds.

Rearte et al. proposed that, in mammals, GCs maintain, rather than initiate, endotoxin tolerance (Rearte et al., 2010). It is possible that the significantly elevated levels of basal CORT in our ducks also maintained tolerance. However, in contrast to our findings, tolerant rats had normal basal levels, but significantly elevated levels of plasma GCs after an immune challenge. The elevated basal CORT concentrations in our tolerant ducks might not have been as immunosuppresive as the enhanced liberation of GCs upon immune challenge reported in tolerant rats. These differences in HPA axis actions could account for the differences observed in the severity of febrile suppression in tolerant ducks and mammals.

From our investigations into the effect of repeated LPS exposure on the HPA axis it is clear that in tolerant ducks both the peripheral and the central components of the HPA axis were sensitized. We propose that the elevated basal levels of CORT in our ducks resulted from a general adaptation of the HPA axis. In mammals, endotoxin exposure is known to modulate the HPA axis, although the main site of action of endotoxin on GC secretion is contentious (Beishuizen and Thijs, 2003).

In tolerant mammals, GCs are not the only inhibitors of pro-inflammatory cytokines. Two cytosolic molecules have been identified (SHIP and IRAK-M) that have the ability to independently inhibit pro-inflammatory cytokine production during a state of tolerance (Harada et al., 2006; Sly et al., 2004). It is not yet known whether these or similar molecules exist or downregulate pro-inflammatory cytokines in tolerant birds.

Our ducks became increasingly tolerant to endotoxin as treatment progressed in each series. We suggest that in birds, both the amount of endotoxin and the frequency of exposure to endotoxin contribute to the development of tolerance. Although bolus injections of LPS do not mimic the accrual of endotoxin during bacterial infections, it is likely that higher exposure to pathogens and larger bacterial loads would result in a more rapid onset of tolerance in birds. This has important implications for wild bird populations and for the poultry industry because, in circumstances where birds are frequently exposed to pathogens, they likely become more susceptible to infection and disease. In addition, an elevated level of circulating CORT is known to mediate growth depression in poultry as it negatively affects feed efficiency and lowers the rate of growth (Klasing et al., 1987; Shini et al., 2008). Also, from our results it is clear that experiments investigating avian febrile responses should consider the dose of LPS, the number of consecutive injections and the time period between injections, to avoid confounded results.

Conclusions

With this study we confirm that endotoxin tolerance is an evolutionarily conserved physiological response to repeated pathogenic exposure in birds as well as mammals. We also show that the role of GCs in the development of endotoxin tolerance has been phylogenetically retained. In Pekin ducks, elevated levels of basal CORT result in tolerance, rather than an elevated release of CORT post LPS treatment.

ACKNOWLEDGEMENTS

The care provided for the animals by the Central Animal Service of the University of the Witwatersrand is gratefully appreciated. The financial assistance of the National Research Foundation (NRF) of South Africa towards this research is hereby acknowledged. Opinions expressed and conclusions arrived at are those of the author and are not necessarily to be attributed to the NRF.

REFERENCES

Adelman, J., Cordoba-Cordoba, S., Spoelstra, K., Wikelski, M. and Hau, M. (2010). Radiotelemetry reveals variation in fever and sickness behaviours with latitude in a free-living passerine. *Funct. Ecol.* **24**, 813-823.

Beishuizen, A. and Thijs, L. (2003). Review: endotoxin and the hypothalamo-pituitary-adrenal (HPA) axis. *J. Endotoxin Res.* **9**, 3-24.

Blatteis, C., Sehic, E. and Li, S. (2000). Pyrogen sensing and signalling: old views and new concepts. *Clin. Infect. Dis.* **31**, S168-S177.

Blatteis, C. M. (2004). The cytokine-prostaglandin cascade in fever production: fact or fancy? *J. Therm. Biol.* **29**, 359-368.

Cartmell, T. and Mitchell, D. (2005). The molecular basis of fever. In *Handbook of Stress and the Brain*, Vol. 15 (ed. T. Steckler, N. H. Kahn and J. M. Reul), pp. 193-227. Amsterdam: Elsevier.

Castro, M., Estivariz, E. and Iturizza, F. (1986). The regulation of the corticomelanotropic cell activity in aves. Effect of various peptides on the release of ACTH from dispersed, perfused duck pituitary cells. *Comp. Biochem. Physiol.* **83A**, 71-75.

Cavaillon, J. and Adib-Conquy, M. (2006). Bench-to-bedside review: endotoxin tolerance as a model of leukocyte reprogramming in sepsis. *Crit. Care* **10**, 233.

Coelho, M. M., Luheshi, G., Hopkins, S. J., Pela, I. R. and Rothwell, N. J. (1995). Multiple mechanisms mediate antipyretic action of glucocorticoids. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **269**, R527-R535.

D'Alecy, L. G. and Kluger, M. (1975). Avian febrile response. *J. Physiol.* **253**, 223-232.

Dias, M., Almeida, M., Carnio, E. and Branco, L. (2005). Role of nitric oxide in tolerance to lipopolysaccharide in mice. *J. Appl. Physiol.* **98**, 1322-1327.

Ewald, P. (1980). Evolutionary biology and the treatment of signs and symptoms of infectious disease. *J. Theor. Biol.* **86**, 169-176.

Fan, H. and Cook, J. (2004). Molecular mechanisms of endotoxin tolerance. *J. Endotoxin Res.* **10**, 71-84.

Flohéa, S., Fernándeza, E. D., Ackermann, M., Hirsch, T., Börgermann, J. and Schadeb, F. (1998). Endotoxin tolerance in rats: expression of TNF-a, IL-6, IL-10, VCAM-1 and HSP 70 in lung and liver during endotoxin shock. *Cytokine* **11**, 796-804.

Gray, D. A., Maloney, S. K. and Kamerman, P. R. (2005). Lipopolysaccharide-induced fever in Pekin ducks is mediated by prostaglandins and nitric oxide and modulated by adrenocortical hormones. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **289**, R1258-R1264.

Gray, D. A., Maloney, S. K. and Kamerman, P. R. (2008). Restraint increases afebrile body temperature but attenuates fever in Pekin ducks (*Anas platyrhynchos*). *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **294**, R1666-R1671.

Harada, K., Isse, K., Sato, Y., Ozaki, S. and Nakanuma, Y. (2006). Endotoxin tolerance in human intrahepatic biliary epithelial cells is induced by upregulation of IRAK-M. *Liver Int.* **26**, 935-942.

Harden, L., Plessis, I. D., Poole, S. and Laburn, H. (2006). Interleukin-6 and leptin mediate lipopolysaccharide-induced fever and sickness behaviour. *Physiol. Behav.* **89**, 146-155.

Harden, L., Plessis, I. D., Poole, S. and Laburn, H. (2008). Interleukin (IL)-6 and IL-1beta act synergisically within the brain to induce sickness behaviour and fever in rats. *Brain Behav. Immun.* **22**, 838-849.

Harvey, S., Merry, B. and Phillips, J. (1980). Influence of stress on the secretion of corticosterone in the duck (*Anas platyrhynchos*). *J. Endocrinol.* **87**, 161-171.

Johnson, R., Curtis, S., Dantzer, R., Bahr, J. and Kelley, K. (1993a). Sickness behavior in birds caused by peripheral or central injection of endotoxin. *Physiol. Behav.* **53**, 343-348.

Johnson, R. W., Curtis, S. E., Dantzer, R. and Kelley, K. W. (1993b). Central and peripheral prostaglandins are involved in sickness behaviour in birds. *Physiol. Behav.* **53**, 127-131.
Kawasaki, H., Moriyama, M., Ohtani, Y., Naitoh, M., Nariuchi, H. and Tanaka, A. (1987). Restoration of normal febrile response to endotoxin in pyrogen-tolerant rabbits by injection with human beta interferon. *Infect. Immun.* **55**, 2574-2578.
Klasing, K., Laurin, D., Peng, R. and Fry, D. (1987). Immunologically mediated growth depression in chicks: influence of feed intake, corticosterone and interleukin-1. *J. Nutr.* **117**, 1629-1637.
Kuenzel, W. and Jurkevich, A. (2010). Molecular neuroendocrine events during stress in poultry. *Poult. Sci.* **89**, 832-840.
Macari, M., Furlan, R. L., Gregorut, F. P., Secato, E. R. and Guerreiro, J. R. (1993). Effects of endotoxin, interleukin 1-beta and prostaglandin injections on fever response in broilers. *Br. Poult. Sci.* **34**, 1035-1042.
Maloney, S. K. and Gray, D. A. (1998). Characteristics of the febrile response in Pekin ducks. *J. Comp. Physiol. B* **168**, 177-182.
Mikhailova, M. V., Mayeux, P. R., Jurkevich, A., Kuenzel, W. J., Madison, F., Periasamy, A., Chen, Y. and Cornett, L. E. (2007). Heterooligomerization between vasotocin and Corticotropin-Releasing Hormone (CRH) receptors augments CRH-stimulated 3′,5′-cyclic adenosine monophosphate production. *Mol. Endocrinol.* **21**, 2178-2188.
Nakamura, K., Mitaria, Y., Yoshioka, M., Koizumi, N., Shibahara, T. and Nakajima, Y. (1998). Serum levels of interleukin-6, a_1-acid glycoprotein, and corticosterone in two-week-old chickens inoculated with *Escherichia coli* lipopolysaccharide. *Poult. Sci.* **77**, 908-911.
Nomoto, S. (1996). Diurnal variations in fever induced by intravenous LPS injection in pigeons. *Pflugers Arch.* **431**, 987-989.
Nomoto, S. (2003). Role of prostaglandin E_2 and indomethacin in the febrile response of pigeons. *Jpn. J. Physiol.* **53**, 253-258.
Nomura, F., Akashi, S., Sakao, Y., Sato, S., Kawai, T., Matsumoto, M., Nakanishi, K., Kimoto, M., Miyake, K., Takeda, K. et al. (2000). Cutting edge: endotoxin tolerance in mouse peritoneal macrophages correlates with down-regulation of surface toll-like receptor 4 expression. *J. Immunol.* **164**, 3476-3479.
Rearte, B., Landoni, V., Laborde, E., Fernandez, G. and Isturiz, M. (2010). Differential effects of glucocorticoids in the establishment and maintenance of endotoxin tolerance. *Clin. Exp. Immunol.* **159**, 208-216.
Romanovsky, A., Almeida, M., Aronoff, D., Ivanov, A., Konsman, J., Steiner, A. and Turek, V. (2005). Fever and hypothermia in systemic inflammation: recent discoveries and revisions. *Front. Biosci.* **10**, 2193-2216.
Roth, J., McClellan, J. L., Kluger, M. J. and Zeisberger, E. (1994). Attenuation of fever and release of cytokines after repeated injections of lipopolysaccharide in guinea-pigs. *J. Physiol.* **477**, 177-185.
Roth, J., Aslan, T., Storr, B. and Zeisberger, E. (1997). Lack of cross tolerance between LPS and muramyl dipeptide in induction of circulation TNF-a and IL-6 in guinea pigs. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **273**, 1529-1533.
Roth, J., Harre, E., Rummel, C., Gerstberger, R. and Hubschle, T. (2004). Signaling the brain in systemic inflammation: role of sensory circumventricular organs. *Front. Biosci.* **9**, 290-300.
Rothwell, N. J., Luheshi, G. N. and Toulmond, S. (1996). Cytokines and their receptors in the central nervous system: physiology, pharmacology and pathology. *Pharmacol. Ther.* **69**, 85-95.
Shini, S., Kaiser, P., Shini, A. and Bryden, W. (2008). Biological response of chickens (*Gallus gallus domesticus*) induced by corticosterone and a bacterial endotoxin. *Comp. Biochem. Physiol.* **149B**, 324-333.
Sly, L., Rauh, M., Kalesnikoff, J., Song, C. and Krystal, G. (2004). LPS-induced upregulation of SHIP is essential for endotoxin tolerance. *Immunity* **21**, 227-239.
Spelman, L., Fleming, W., Davis, G. and Stoskopf, M. (1995). Effect of exogenous adrenocorticotropic hormone administration on plasma corticosterone concentrations in American black ducks (*Anas rubripes*). *J. Wildl. Dis.* **31**, 135-141.
Tedeschi, V. P. N., Dias, M. B. and Branco, L. G. (2007). Reduced central c-fos expression and febrile response to repeated LPS injection into periodontal tissue in rats. *FASEB J.* **21**, 954.1.
Tripp, A., Roth, J., Störr, B., Voigt, K. and Zeisberger, E. (1998). Febrile tolerance develops in response to repeated administration of bacterial lipopolysaccharide but not of interleukin-1β in rats. *J. Therm. Biol.* **23**, 387-394.
vanTuinen, M., Sibley, C. and Hedges, S. (2000). The early history of modern birds inferred from DNA sequences of nuclear and mitochondrial ribosomal genes. *Mol. Biol. Evol.* **17**, 451-457.
Wakabayashi, G., Cannon, J., Gelfand, J., Clark, B., Aiura, K., Burke, J., Wolff, S. and Dinarello, C. (1994). Altered interleukin-1 and tumor necrosis factor production and secretion during pyrogenic tolerance to LPS in rabbits. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **267**, 329-336.
West, M. and Heagy, W. (2002). Endotoxin tolerance: a review. *Crit. Care Med.* **30**, S54-S73.