Effects of corticosterone on innate and humoral immune functions and oxidative stress in barn owl nestlings

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SUMMARY

The costs of coping with stressful situations are traded-off against other functions such as immune responses. This trade-off may explain why corticosterone secretion reduces immune reactions. Corticosterone differentially affects various immunity components. However, which component is suppressed varies between studies. It remains unclear whether the trade-off in energy, nutrition, autoimmunity or oxidative stress accounts for differential immunosuppression. In this study, we investigated whether corticosterone differentially affects the constitutive innate and humoral acquired immunity. We used barn owl nestlings, implanting 50% with a corticosterone-releasing pellet and the other 50% with a placebo pellet. To measure the effect on humoral immunity we vaccinated 50% of the corticosterone-nestlings and 50% of the placebo-nestlings with the antigens 'Tetravac' and the other 50% were injected with PBS. To assess the costs of elevated corticosterone, we measured body mass and resistance to oxidative stress. Administration of corticosterone increased corticosterone levels whereas vaccination induced the production of antibodies. Corticosterone reduced the production of antibodies, but it did not significantly affect the constitutive innate immunity. Corticosterone reduced body growth and resistance to oxidative stress. Under stressful conditions barn owl nestlings seem to keep the constitutive innate immunity, whereas elevated corticosterone levels negatively affected inducible immune responses. We found evidence that mounting a humoral immune reaction is not costly in terms of growth, but reduces the resistance to oxidative stress independently of corticosterone administration. We suggest that humoral immunity is suppressed because the risk of immunopathologies may be disproportionately high when mounting an antibody response under stressful situations.

Key words: constitutive innate immune system, corticosterone, humoral immunity, immunocompetence, oxidative stress, stress.

INTRODUCTION

As a response to stressful situations, glucocorticoids, such as corticosterone in birds, are secreted to induce behavioural and physiological changes allowing an organism to cope with the demanding situation (Wingfield and Hunt, 2002). This physiological reaction to a stressful situation is generally called 'stress'. Previous studies showed that a variety of stressors affect parasite and pathogen defence mechanisms of animals, usually leading to a downregulation of immune responses (El-Lethey et al., 2002; Laudenslager et al., 1988; Wick et al., 1993) [but there are contrasting results (see Dhabhar and McEwen, 1996; Irwin et al., 1989; Jessop et al., 1987)].

A frequently cited explanation for the stress-induced reduction in immunity is that the induction and maintenance of immune activity is costly (e.g. in terms of energy, nutrients, autoimmunity or oxidative stress) and hence there are trade-offs with other demanding functions (Gustafsson et al., 1994; Sheldon and Verhulst, 1996; Svensson et al., 1998; Hanssen et al., 2004; Moret and Schmid-Hempel, 2000). Thus, the suppression of the immune system during exposure to stress may allow organisms to reallocate resources to other physiological processes, thereby enhancing immediate survival. In periods of stress, the risk of immunopathology and autoimmune reactions leading to tissue damage may be particularly pronounced and costly (Adams, 1996; Råberg et al., 1998; Levin and Antia, 2001), and thus downregulation of the immune system

can be momentarily beneficial. An additional gain of a reduced immune activity evoked by stress is the avoidance of oxidative stress (von Schantz et al., 1999; Svensson et al., 1998), because a higher metabolic rate induced by a stressful situation entails the production of free oxygen radicals, which have a damaging effect on cellular processes. Since the immune system is divided into different compartments with a complex network of regulation, stress is likely to differentially affect different components, as recently shown in birds (Bourgeon and Raclot, 2006; Ilmonen et al., 2003; Lochmiller et al., 1993). In these studies corticosterone implantation decreased humoral acquired but not cellular acquired immunity whereas external stressors had the contrary effect of a reduced T-cell response but no effect on antibody production.

Here we investigate the effect of an elevated plasma concentration of the stress hormone corticosterone on one component of each of the constitutive innate and humoral acquired immune systems in barn owl (*Tyto alba* Scopoli 1769) nestlings. This is one of only a few studies testing the effect of corticosterone on immune function in wild animals. We chose these two components because the innate constitutive system is the first line of defence and humoral immunity is induced against specific pathogens. We simulated a physiological stress situation by implantation of a corticosterone-releasing pellet, which elevates plasma corticosterone level within a physiological range (Müller et al., 2009), while other individuals serving as controls were implanted with a placebo pellet. In this way, the effect

of an experimental elevation of circulating corticosterone over several days could be studied without any direct deleterious effect of a natural stressor like food deprivation or disturbance that induce an elevation of corticosterone (Wingfield and Kitaysky, 2002). To assess the effect of corticosterone on humoral acquired immunity, we vaccinated 50% of the corticosterone-nestlings and 50% of the placebo-nestlings with a cocktail of four non-pathogenic antigens (Tetravac) and the other nestlings with a control physiological solution PBS. Our prediction was that corticosterone negatively affects the production of antibodies specifically directed towards the vaccine Tetravac. We also tested whether corticosterone suppresses innate constitutive immunity. Natural antibodies in the blood recognise invading particles like foreign blood cells and bind them, thereby initiating the complement enzyme cascade, which results in cell lysis. Thus, agglutination arises from natural antibodies only, whereas lysis reflects the interaction of natural antibodies and complement proteins. Both scores can be interpreted as an index of the strength of the constitutive innate immune system, with higher scores indicating a more effective immune response (Matson et al., 2005). Therefore, our prediction is that corticosterone lowers the scores of the haemolysis and agglutination assays in individuals implanted with corticosterone compared with a placebo pellet independently of whether nestlings were vaccinated with Tetravac or not. In addition, we checked the assumption that corticosterone depresses nestling body mass increase and resistance to oxidative stress.

MATERIALS AND METHODS
Experimental procedure

The study was carried out in western Switzerland in an area covering 190 km^2 where 150 nest-boxes placed on barns were available for breeding barn owls. We checked boxes regularly to monitor clutches and hatching dates. In 2006, when the present study was carried out, breeding conditions were the poorest for the last 20 years resulting in only 27 clutches producing only 73 chicks at fledging. For comparison, in 2002 86 clutches gave 285 fledglings. We marked nestlings by clipping off the tip of claws before they were big enough to be ringed with an aluminium ring. Age of nestlings was determined by their wing length shortly after hatching (Roulin, 2004).

Our experimental design necessitated four barn owl nestlings per nest, and for this reason we selected the four oldest individuals of each nest. Nestlings hatch every 2.5 days resulting in a pronounced within-brood age hierarchy. For this reason, we used only the four oldest nestlings in 21 nests (mean brood size at the first visit was 4.3±0.9, range: 3–6). When the oldest chick of each brood was 26.6±2.3 days old (hereafter referred to as day 0), we selected two individuals per nest to implant a corticosterone-releasing pellet under the skin of the flank. The implant consisted of a biodegradable carrier-binder containing 15 mg corticosterone that is known to release this hormone over 7 days in rats (Innovative Research of America, Sarasota, Florida). Implants of various doses were tested before in captive barn owls and kestrels to find the dose, which increases corticosterone to about 15–20 ng ml^{-1} above baseline levels. This increase is within the physiological range of this species (for details, see Müller et al., 2009). Body mass of corticosterone-implanted nestlings at implantation was 271.4±44.0 g (mean ± s.d.; range: 186–351 g). The two other siblings served as controls by the implantation of a placebo pellet containing only the biodegradable carrier-binder. Hereafter individuals implanted with corticosterone are denoted 'cort-nestlings' while those implanted with a placebo pellet 'placebo-nestlings'.

Two days after implantation of the pellets, 50% of the cort-nestlings and 50% of the placebo-nestlings were vaccinated subcutaneously in the neck with 100 μl of the vaccine Tetravac (TETRAVAC$^©$ vaccine, Aventis Pasteur MSD, Switzerland). This vaccine includes a cocktail of antigens (diphtheria 60 i.u. ml^{-1}, tetanus 80 i.u. ml^{-1}, pertussis 50 μg ml^{-1}, filamentous haemagglutinin 50 μg ml^{-1}, type 1 poliovirus D antigen 80 i.u. ml^{-1}, type 2 poliovirus D antigen 16 i.u. ml^{-1} and type 3 poliovirus D antigen 64 i.u. ml^{-1}) mixed in a solution containing 0.30 mg of aluminium hydroxide which boosts humoral immune responses (Schijns, 2000). The other 50% of the nestlings were injected in the neck with a phosphate-buffered saline solution (PBS). Hereafter, vaccinated individuals were referred to as 'Tetravac-nestlings' and control nestlings 'PBS-nestlings'. With our experimental design we therefore created four groups of nestlings differing in blood corticosterone level and humoral immune stimulation (22 cort- and Tetravac-nestlings, 19 cort- and PBS-nestlings, 18 placebo- and Tetravac-nestlings and 18 placebo- and PBS-nestlings). Age at implantation did not differ between the four treatments (one-way ANOVA, $F_{3,73}$=0.98, P=0.41) neither did rank in the within-brood age hierarchy (one-way ANOVA, $F_{3,73}$=0.58, P=0.63). Nestlings were weighed at each nest visit on days 0, 2, 4, 12, 17 and 26 post-implantation. Sex of nestlings was identified using molecular markers as described by Py et al. (Py et al., 2006). Mortality until fledging was not increased by implantation (six out of 41 cort-nestlings *versus* four out of 36 placebo-nestlings; χ^2=0.251, P>0.6) or by vaccination (four out of 40 vaccinated-nestlings *versus* six out of 37 PBS-nestlings; χ^2=0.717, P>0.4).

Assessment of plasma corticosterone concentration

We took a 70 μl blood sample by puncturing the brachial vein on day 0 just before implantation, on day 2 just before vaccination, and on day 12. Samples were collected with heparinised capillary tubes, immediately centrifuged using a portable centrifuge in the car and the plasma stored in liquid nitrogen. After transport to the laboratory, the samples were stored at –20°C until analyses the following autumn. An increase in circulating corticosterone after a stressful situation is detected after 3 min (Romero and Reed, 2005) (B.A., unpublished data), and for this reason we measured baseline corticosterone level in 160 of the 216 samples (74%) collected within 3 min of first opening the nest-box (day 0: 40 cort-, 33 placebo-nestlings; day 2: 25 cort-, 21 placebo-nestlings; day 12: 21 cort-, 19 placebo-nestlings). Plasma corticosterone concentrations were determined using an enzyme immunoassay (Munro and Stabenfeldt, 1984; Munro and Lasley, 1988). Corticosterone was extracted from plasma with 4 ml dichloromethane (5 μl plasma diluted with 195 μl water). All samples were run in triplicates. The dilution of the corticosterone antibody [Chemicon Int., Temecula, CA, USA; cross-reactivity: 11-dehydrocorticosterone 0.35%, progesterone 0.004%, 18-OH-DOC 0.01%, cortisol 0.12%, 18-OH-B 0.02% and aldosterone 0.06%] was 1:8000. HRP (1:400,000) linked to corticosterone served as enzyme label and ABTS [2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)] as substrate. The concentration of corticosterone in plasma samples was calculated by using a standard curve run in duplicate on each plate. Plasma pools from chicken with a low and a high corticosterone concentration were included as internal controls on each plate. Intra-assay variation ranges from 9 to 11% and inter-assay variation from 12 to 21%, depending on the concentration of the internal controls. For details on the assay see Müller et al. (Müller et al., 2006).

Assessment of humoral acquired immunity

To measure the quantity of antibodies specifically directed against the vaccine Tetravac, we took blood samples on day 2 (just before individuals were injected with Tetravac or PBS) as well as on days 12 and 17. The analyses were run with an enzyme-linked immunoabsorbent assay [ELISA 'sandwich' (Crowther, 2001; Gasparini et al., 2009). As solid phase we used microtitre plates with 8×12 wells. Each well was coated with 100 µl of Tetravac diluted 1:50 in PBS and incubated for 2 h at room temperature. After washing the plates five times with PBS–Tween 0.05%, we added in each well 200 µl of PBS-Tween containing 5% milk. Plates were incubated for 2 h at room temperature and then washed five times. Plasma samples were diluted 1:40 in PBS–Tween containing 5% milk and 100 µl were distributed into each well. Plates were incubated overnight at 6°C and washed five times the following day. Each plate was saturated with 100 µl anti-chicken IgG diluted 3000-fold, marked with peroxydase for 2 h at room temperature and washed five times. 100 µl of peroxydase substrate (*o*-phenylenediamine dihydro-chloride, $0.4\,\mathrm{mg\,ml^{-1}}$) were added to each

sample and after 15 min at room temperature in the dark the reaction was stopped with 50 µl of $1\,\mathrm{mol\,l^{-1}}$ HCl. Optical density was read at 490 nm with a spectrophotometer. Results were expressed as the amount of antibodies directed against the Tetravac vaccine, on an arbitrary scale. As a standard, a mixture of numerous positive samples was measured at three dilutions to correct the OD values for a possible plate effect. After calibration with the standard, the repeatability between OD values of the same samples was high within (ANOVA, $R^2_{adj}=0.83$, $F_{14,30}=11.04$, $P<0.001$, $N=30$) and good between plates (ANOVA, $R^2_{adj}=0.62$, $F_{16,34}=4.303$, $P=0.002$, $N=34$).

Assessment of constitutive innate immunity

We collected a blood sample on day 0 from 65 individuals, on day 6 (70 individuals) and on day 17 (60 individuals). Agglutination and lysis titres were determined using the haemolysis–haemagglutination assay as described by Matson et al. (Matson et al., 2005) with a small modification, i.e. by using 20 µl of plasma, 20 µl of PBS and 10 µl of a 1% rabbit red blood cell suspension. For each sample,

Table 1. Mixed model analyses for repeated measures for testing the effect of vaccination and corticosterone implantation on immunity, body mass change and resistance to oxidative stress

Variable	Estimate (s.e.)	d.f.	F	P
Antibody production (OD)				
Implantation (placebo)	0.0074 (0.0027)	1,53	7.79	0.0073
Vaccination (tetravac)	0.0021 (0.0034)	1,53	0.390	0.5348
Day		2,130	0.847	0.4313
Day 12	0.0060 (0.0046)			
Day 17	0.0006 (0.0056)			
Vaccination×day		2,130	19.9	<0.0001
Vaccination×day 12	0.0304 (0.0064)			
Vaccination×day 17	0.0395 (0.0077)			
Agglutination scores				
Day		2,110	46.6	<0.0001
Day 4	1.256 (0.1311)			
Day 17	0.3808 (0.1734)			
Body mass change (g day^{-1})				
Implantation (placebo)	16.07 (3.685)	1,53	19.0	0.0001
Day		4,270	18.5	<0.0001
Day 2–4	20.30 (3.199)			
Day 4–12	16.19 (2.637)			
Day 12–17	12.76 (2.694)			
Day 17–26	10.42 (2.587)			
Age on day 0	−0.4367 (0.084)	1,53	27.0	<0.0001
Implantation×day		4,270	5.32	0.0004
Implantation×day 2–4	−16.89 (4.700)			
Implantation×day 4–12	−17.36 (3.858)			
Implantation×day 12–17	−17.44 (3.941)			
Implantation×day 17–26	−16.99 (3.784)			
Resistance to oxidative stress ($T_{1/2}$; min)				
Implantation (placebo)	−1.409 (0.8364)	1,55	2.84	0.0976
Day		3,208	4.47	0.0046
Day 2	0.6845 (0.6576)			
Day 4	0.4452 (0.7850)			
Day 12	−1.585 (0.6530)			
Implantation×day		3,208	7.78	0.0001
Implantation×day 2	−0.2751 (0.9699)			
Implantation×day 4	1.232 (1.154)			
Implantation×day 12	3.909 (0.9562)			

Production of antibodies specifically directed against the vaccaine Tetravac (humoral immunity): 210 measurements of 76 nestlings from 21 nests.

Agglutination scores (constitutive innate immunity): 186 measurements of 74 nestlings from 21 nests.

Body mass change: 354 measurements of 76 nestlings from 21 nests.

Resistance to oxidative stress: 291 measurements of 77 nestlings from 21 nests.

The full fixed model included the variables implantation, vaccination, day, age, body condition, sex and rank. We present final models after backwards elimination of non-significant terms. Estimates of days refer to day 0.

d.f., degrees of freedom.

we scored lysis and agglutination from the digitised images, blind with respect to treatments. The scores of lysis were often 0, indicating that the concentration of complement proteins in the barn owl plasma was too low to lyse the rabbit red blood cells under the given concentration of bird plasma. Because of this imbalance, scores of lysis were recorded as either lysis (scores>0) or no lysis (scores=0).

Assessment of the resistance to oxidative stress

We investigated whether the corticosterone and Tetravac treatments reduced resistance to a standardized free radical attack. For this purpose, we estimated resistance to oxidative stress by using the KRL® diagnostic test derived from human medicine, adapted to bird physiology [temperature and osmolarity (Alonso-Alvarez et al., 2004)]. We submitted a whole blood solution to a thermo-controlled oxidative attack where all antioxidants present in blood interact to slow down red blood cell haemolysis. More specifically, $16\,\mu l$ of the fresh blood were added to $584\,\mu l$ KRL buffer ($150\,mmol\,l^{-1}$ Na$^+$, $120\,mmol\,l^{-1}$ Cl$^-$, $6\,mmol\,l^{-1}$ K$^+$, $24\,mmol\,l^{-1}$ HCO$_3^-$, $2\,mmol\,l^{-1}$ Ca^{2+}, $340\,mosmol\,l^{-1}$, pH 7.4) for the oxidative stress analysis. The resistance to oxidative stress was assessed as the time needed to haemolyse 50% of the red blood cells ($T_{1/2}$) exposed to a controlled free radical attack. Thus, higher values of $T_{1/2}$ stand for higher resistance to oxidative stress. The analyses were done within 2 days of taking each blood sample.

Statistics

Data on corticosterone concentration (ln-transformed), resistance to oxidative stress, amount of antibodies specifically directed towards Tetravac and agglutination scores were analysed in R 2.5.1 using (repeated-measures) linear mixed-effect models where nests and individual identities nested within nests were entered as two random factors. As fixed factors we implemented the number of days after the start of the experiment (i.e. when birds were implanted), implantation (corticosterone or placebo) and vaccination (injection of Tetravac or PBS) plus their interactions. We also added the following nestling characteristics: sex, rank in the within-brood age hierarchy, age of each nestling at the start of the experiment on day 0, and residual body mass extracted from a linear regression of body mass on the actual age of each nestling at day 0. We also included the interactions of these variables with implantation or vaccination, but because they were not significant, we removed them from the final analyses presented in Table 1. To analyse the effect of implantation on body mass change we did not introduce residual body mass as an independent variable. Non-significant terms ($P>0.01$) were eliminated stepwise backwards.

Lysis scores of foreign blood cells were analysed with a binary repeated mixed-effect model. We included age as covariate and day, implant and vaccination as factors. Nestling identity nested in site was included as random factor. We used Bayesian Information Criterion (BIC) for model selection of the main effects and interactions of implant and vaccination. To estimate P-values of the final model we used a likelihood ratio test. Values are means ± s.e.m., unless indicated otherwise.

RESULTS
Baseline corticosterone level

There was a significant effect of corticosterone implantation on baseline corticosterone levels in interaction with day (mixed model, $F_{2,79}$=46.3, $P<0.0001$). Before implantation on day 0 baseline corticosterone levels did not differ between the four treatments (post-hoc two-way ANOVA, $F_{1,69}$=0.291, P=0.59; overall mean:
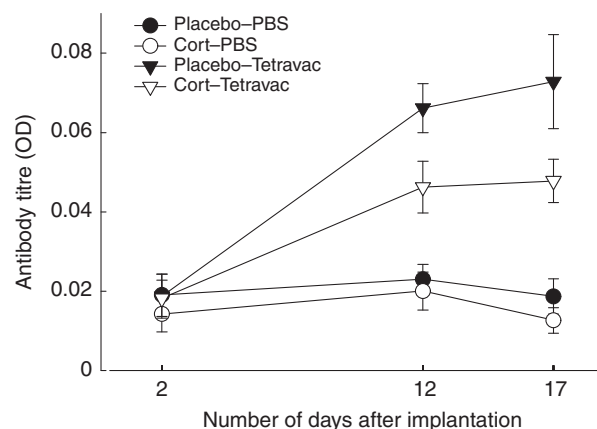

Fig. 1. Effects of corticosterone administration (Cort versus Placebo) and vaccination (Tetravac versus PBS) on the specific antibody production towards Tetravac. Error bars show ±1 s.e.m.

$8.2\pm1.3\,ng\,ml^{-1}$). At 2 days after implantation (day 2) baseline corticosterone concentrations were about fivefold higher in cort-nestlings than in placebo-nestlings (post-hoc two-way ANOVA, $F_{1,42}$=49.8, $P<0.001$; mean: $45.9\pm5.1\,ng\,ml^{-1}$ versus $5.2\pm0.8\,ng\,ml^{-1}$). At 12 days after implantation baseline corticosterone level did not differ between the four groups (post-hoc two-way ANOVA, $F_{1,36}$=0.06, P=0.81) and was back at about the same level as before the implantation in all four groups (post-hoc two-way ANOVA, $F_{1,112}$=0.06, P=0.81; overall mean: $7.8\pm0.7\,ng\,ml^{-1}$).

Humoral acquired and constitutive innate immunity

Before vaccination on day 2 there was no difference in the amount of antibodies specifically directed against Tetravac between the subsequently injected Tetravac-nestlings and PBS-nestlings (post-hoc t-test, $F_{1,69}$=0.27, P=0.79), but on days 12 and 17 Tetravac-nestlings produced more antibodies directed against the vaccine than PBS-nestlings (Fig. 1, Table 1). Antibody production after vaccination was negatively affected by the implantation of a corticosterone pellet compared with a placebo pellet (Table 1). In Tetravac-nestlings the negative effect of corticosterone administration was pronounced on day 12 and 17, whereas PBS administration did not result in any difference in antibody titre between cort- and placebo-nestlings (Fig. 1).

The agglutination scores differed between days 0, 4 and 17, but independently of the corticosterone treatment and vaccination (Table 1, Fig. 2; post-hoc t-test, for day 0 and 17: both $F_{1,63}$<–0.09, both P>0.19; day 4: $F_{1,67}$=1.9, P=0.066). The proportion of chicks showing lysis increased from day 0 to day 17 (Fig. 3; likelihood ratio=35.23, $P<0.001$). Administration of corticosterone and vaccination and their interactions with day had no detectable effect on the lysis scores (binary repeated mixed-effect model: all P-values >0.05). Post-hoc tests showed that the proportion of chicks showing lysis was significantly higher in cort-nestlings at day 4 (binomial logistic regression, z=–2.76, P=0.006) but not at day 0 (z=–1.11, P=0.27) and day 17 (z=0.28, P=0.78).

Body mass increase and resistance to oxidative stress

During the first 2 days after implantation nestlings implanted with a corticosterone-releasing pellet lost body mass while placebo-nestlings gained body mass (post-hoc t-test, $F_{1,73}$=–4.6, $P<0.0001$; Table 1, Fig. 4). From day 2 onwards, body mass change was not
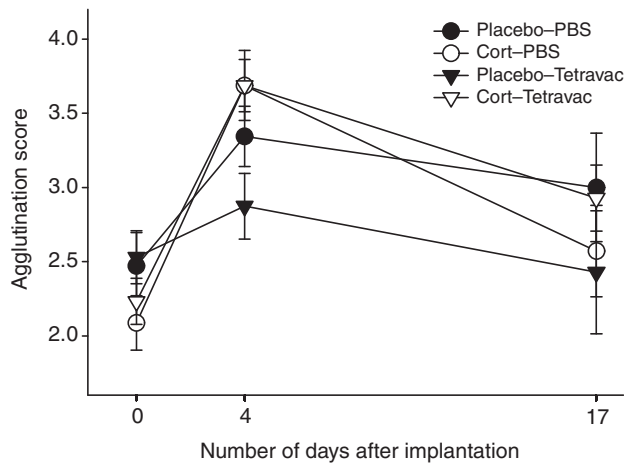
Fig. 2. Effects of corticosterone administration (Cort *versus* Placebo) and vaccination (vaccine *versus* PBS) on agglutination scores, reflecting the constitutive innate immunity. Error bars show ±1 s.e.m.

affected by corticosterone treatment (*post-hoc t*-test, day 2–4: $F_{1,70}=0.10$, $P=0.92$; day 4–12: $F_{1,68}=0.61$, $P=0.55$; day 12–17: $F_{1,68}=0.55$, $P=0.58$; day 17–26: $F_{1,65}=0.42$, $P=0.67$). Vaccination had no significant impact on body mass change either alone or in interaction with the corticosterone treatment (Table 1). *Post-hoc* analysis showed that on day 26, before fledging, body mass did not differ significantly between the four treatment groups (mixed model with implant, vaccination, sex, age on day 0 and the interaction of implantation with vaccination, all $F_{1,42}<0.80$, all $P>0.35$).

Corticosterone implants significantly affected the resistance to oxidative stress, but the overall effect of vaccination was not significant in the repeated measures mixed model analysis (Table 1). *Post-hoc* analysis of the effect of implantation and vaccination for each day (general linear models including the interaction implantation × vaccination) revealed that on day 12, both implantation of corticosterone (effect size –2.33±1.15, $P=0.004$) and Tetravac vaccination (effect size –2.04±1.22, $P=0.03$) significantly reduced the resistance to oxidative stress (Fig. 5) (interaction term not significant, $P=0.83$). On days 2 and 4, there were no significant effects of implantation or vaccination (all $P>0.06$). On day 0, the interaction

term implantation × vaccination was significant ($P=0.003$), indicating that the placebo-Tetravac group had by chance an unusually low resistance to oxidative stress (Fig. 5), although the birds had not yet been implanted or vaccinated on that day.

DISCUSSION
Corticosterone-mediated differential immunosuppression
The present study demonstrates that administering corticosterone, which mimics part of the physiological response to a stressor, caused a suppression of the humoral acquired immune system, but not of the constitutive innate immune system in barn owl nestlings. The elevation of the corticosterone level to 45.9±25.4 ng ml^{-1} (mean ± s.d.; range: 10.5–118.0 ng ml^{-1}) represented an increase within the physiological range, as the response to handling induced a rise in corticosterone to 58.2 ng ml^{-1}±25.3 ng ml^{-1} (mean ± s.d.; range: 11.25–118.03 ng ml^{-1}) (Müller et al., 2009).

The administration of corticosterone reduced the amount of specific antibodies produced against Tetravac in the plasma. Although circulating corticosterone was elevated for 2–3 days (for details, see Müller et al., 2009), the reduction in the immune response

Fig. 4. Effects of corticosterone administration (Cort *versus* Placebo) and vaccination (Tetravac *versus* PBS) on body mass increase between days 0 and 26 after corticosterone treatment was applied. Error bars show ±1 s.e.m.
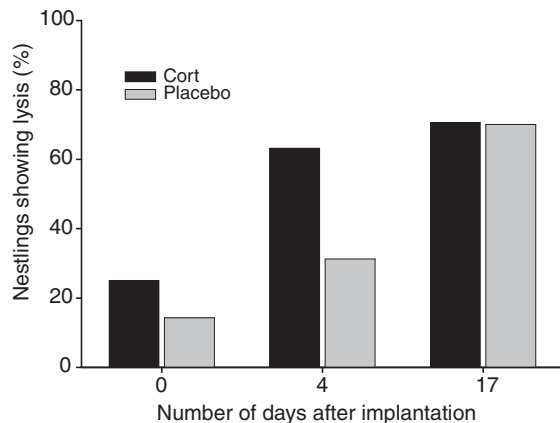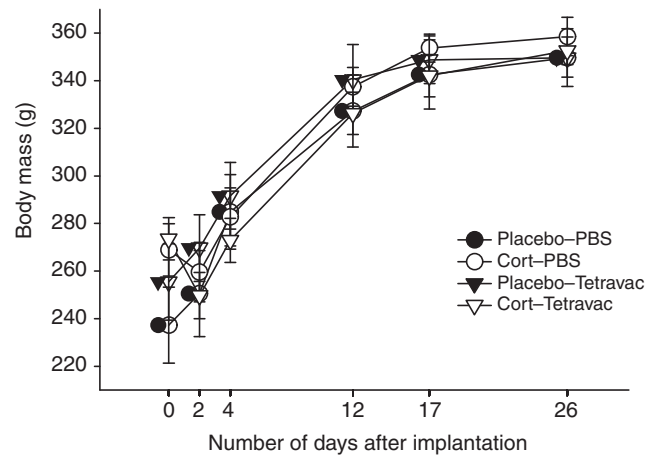

Fig. 3. Effects of corticosterone administration (Cort *versus* Placebo) on lysis of foreign blood cells. Bars indicate the percentage of nestlings showing lysis. Higher values indicate a more effective innate immune response.
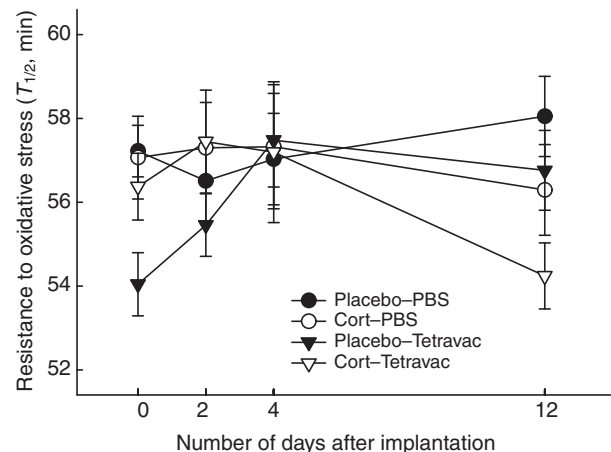

Fig. 5. Effects of corticosterone administration (Cort *versus* Placebo) and vaccination (Tetravac *versus* PBS) on the resistance to oxidative stress, measured as the time needed to haemolyse 50% of the red blood cells ($T_{1/2}$) exposed to a controlled free radical attack. Error bars show ±1 s.e.m.

was observed until fledging. This indicates that stressors, which elevate corticosterone levels, cause a long-lasting effect on IgG antibody levels, as also found in rats (Laudenslager et al., 1988), mice (Moynihan et al., 1990) and female eiders *Somateria mollissima* (Bourgeon and Raclot, 2006). The reduced antibody production against Tetravac through the experimental elevation of circulating corticosterone may be the result of a suppressed antibody production, possibly caused by lymphopenia, i.e. a deficit in B- and T-cells. Indeed, in humans and rats lymphopenia occurs after a session of 2 h of moderate stress as well as after the injection of corticosterone, an effect that is rapidly reversible (within 24 h) after cessation of the stressor (McEwen et al., 1997). However, in the present study corticosterone levels were elevated for 2–3 days, and thus such a long period may have profoundly decreased the number of B-cells and in turn the production of antibodies.

Assuming that our two indices of constitutive innate immunity, namely agglutination and lysis scores, were sensitive enough, we did not find evidence for an elevation in corticosterone level negatively affecting this immune component. Although the overall analysis revealed no significant effect, 4 days after corticosterone administration cort-nestlings showed higher lysis scores and a tendency for elevated agglutination scores. This suggests, contrary to expectations, a stronger innate immune response. A differential effect of corticosterone on various aspects of the immune system has already been reported in other bird studies. In female eiders implantation of a corticosterone-releasing pellet significantly decreased the total amount of circulating antibodies but did not affect the T-cell-mediated immune reaction (wing-web swelling after PHA injection) (Bourgeon and Raclot, 2006). Contrasting results were found in northern bobwhite *Colinus virginianus* chicks in which stress from protein malnutrition reduced the T-cell-mediated immune reaction (measured by wing-web swelling) to PHA injection, but not the humoral response, as determined by the antibody titres specifically directed to sheep red blood cell (SRBC) suspension (Lochmiller et al., 1993). In pied flycatchers *Ficedula hypoleuca,* nestlings of enlarged broods showed reduced T-cell-mediated immune responsiveness against PHA, whereas in their male parents corticosterone levels were elevated but had no effect on the production of specific antibodies against the vaccine SRBC (Ilmonen et al., 2003). Thus, birds under stress are able to selectively suppress only certain parts of the immune system and the other ones remain unaffected or even enhanced [as demonstrated in mammals (Jessop et al., 1987; Irwin et al., 1989; Dhabhar and McEwen, 1996)], possibly as part of a compensatory response (Apanius, 1998; Fowles et al., 1993).

Costs and benefits of a differential immune suppression by stress

An immune suppression under stressful conditions entails costs and benefits. Therefore, we would expect that corticosterone affects immune components differentially, and which component is negatively affected should depend on the costs and benefits of suppressing each component.

An obvious cost of a suppressed immune system is the inability to mount an adequate immune function, especially over such a long period of time as observed in the present study. The risk of infections is increased as well as the progression of diseases already present, leading to serious fitness costs to the point of reduced survival (Apanius, 1998; Dalton et al., 1993; Flynn et al., 1993). Barn owl nestlings with elevated corticosterone levels keep the first line of defence, the constitutive innate immune system, which at the same time has hardly any impact on production of memory antibodies

and thus on the secondary humoral immune response. They suppress the humoral acquired immune system, which has a more specific protective function for the body. Tissue injury, a possible risk of an activated immune system, causes the secretion of 'self' antigens and cytokines, which results in further tissue damage and may facilitate autoimmune reactions (Adams, 1996; Råberg et al., 1998; Svensson et al., 1998). This potential overshoot of the immune system can be a more important problem to the host than a parasite and, thus, should be prevented or confined by the suppression of immune responses (McEwen et al., 1997). Råberg et al. (Råberg et al., 1998) proposed that the risk of autoimmune reactions is higher in stressful situations than it is under relaxed conditions.

Immunity is widely assumed to be costly and therefore to trade-off with other resource-demanding processes, as caused by a response to stressful conditions. Several types of costs have been proposed to explain the trade-off between immune responses and other costly physiological processes, in particular energetic, nutrient and autoimmunity costs and oxidative stress (e.g. Lochmiller and Deerenberg, 2000; Råberg et al., 1998; Hanssen et al., 2004). In this study, we examined the costs of elevated corticosterone levels and the costs of mounting a humoral immune response in terms of body mass change and oxidative stress. Because we elevated corticosterone directly and not *via* a stressor, we did not confound the direct effect of a stressful situation (e.g. food restriction in nestlings) with the effect of corticosterone. Corticosterone-implanted nestlings lost, rather than gained, body mass during the days of elevated corticosterone and showed a reduced resistance to oxidative stress compared with chicks of the control group. Elevated corticosterone delayed growth temporarily, as in starlings (Love et al., 2005). Because nestling barn owls of the control group reached maximum body mass at about day 21 after implantation (44 days old), the corticosterone-implanted siblings had the opportunity to catch up in body mass and on day 26 after implantation, shortly before fledging, body mass did not differ significantly between the four groups (Fig. 4). Our study confirmed that elevated glucocorticoids impair the antioxidant defences (reviewed by von Schantz et al., 1999). Thus, increasing circulating corticosterone entailed clear costs in terms of a temporarily reduced growth rate and a reduced resistance to oxidative stress.

In contrast to the effects of corticosterone, we found that a challenge of the humoral immune system (Tetravac injection) did not result in measurable costs in terms of a temporarily reduced growth rate, but only in a somewhat reduced resistance to oxidative stress, independently of corticosterone administration. This result agrees with findings that the energetic costs of an immune response are not high enough to be traded-off against other demanding functions (Amat et al., 2006; Eraud et al., 2005; Verhulst et al., 2005; Gasparini et al., 2009; Svensson et al., 1998), but that the main proximate cost of an activation of the immune system may be a reduced resistance to oxidative stress (Hanssen et al., 2004).

Conclusions

Under elevated corticosterone, barn owls, as other animals, seem to differentially suppress the immune system, i.e. the humoral system but not the innate constitutive immune system. Thereby, birds balance the benefits of immune suppression with an acceptable risk of disease susceptibility and progression (Lochmiller and Deerenberg, 2000). The benefits of an immune suppression could be to reduce the costs, and therefore to trade-off with other resource-demanding processes. However, as found in our study, there were no measurable costs in terms of growth of mounting an immune response under relaxed conditions (i.e. in placebo-nestlings), but a

somewhat reduced resistance to oxidative stress. The suppression of the humoral response to an immune challenge under stressful-conditions (i.e. in cort-nestlings) is therefore to a large extent a direct cost of the elevated corticosterone concentration. It seems more probable that avoiding harmful secondary effects of mounting an immune response, such as oxidative stress and immunopathology, may be a major function of the differential suppression of the immune system in stressful situations.

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