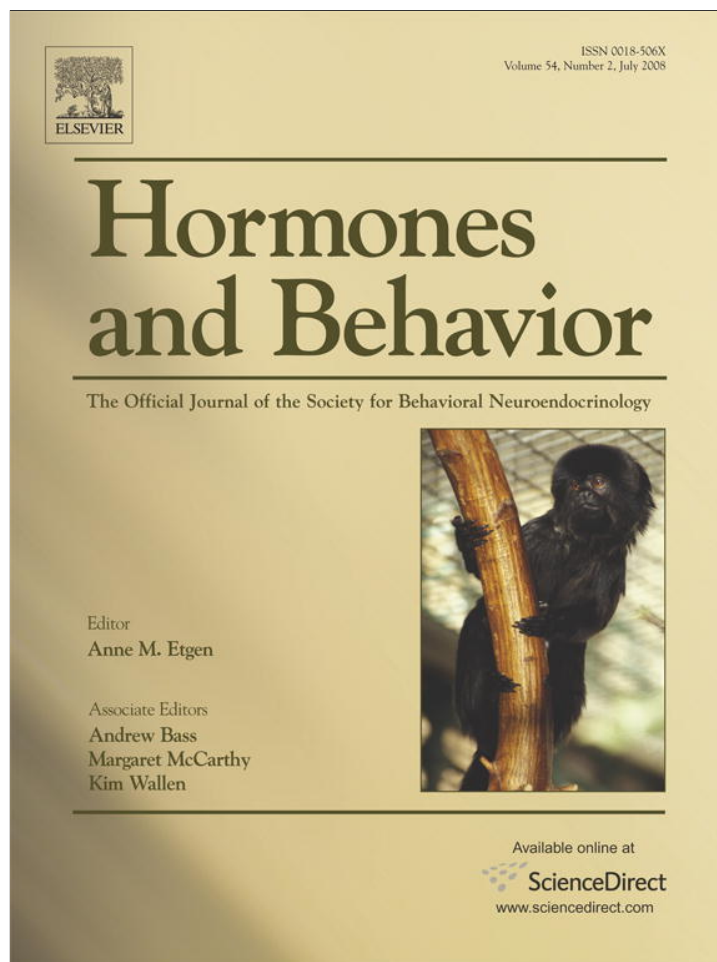


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Increased sibling competition does not increase testosterone or corticosterone levels in nestlings of the spotless starling (*Sturnus unicolor*)

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Abstract

Nestling begging in passerine birds is a complex behaviour that is shaped by a multitude of ecological factors and could be physiologically mediated by varying levels of steroid hormones. Previous research has shown links between sibling competition and testosterone and corticosterone in several bird species. The spotless starling (*Sturnus unicolor*) is a medium sized passerine in which nestlings compete intensively for resources, often resulting in marked size hierarchies that can have profound effects on their fitness. We tested the hypothesis that an increase in sibling competition levels would result in increases in testosterone and corticosterone in this species. To this end we conducted a brood size manipulation, creating small, medium and large broods. This manipulation had the expected effect on morphology: nestling size and mass decreased with increasing brood size. Androgen levels varied in response to brood size manipulation but, contrary to expectations, the largest concentrations were found in reduced brood sizes. Corticosterone levels increased with increasing brood size, but this effect disappeared when we corrected for the time taken to process nestlings. Cell-mediated immune response was found to decrease with increasing brood size and testosterone levels. The results suggest that the proposed link between testosterone and corticosterone and sibling competition does not hold in this species, and underlines the diversity of species-specific responsiveness to steroids.

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Keywords: Testosterone; Corticosterone; Spotless starling; *Sturnus unicolor*; Sibling competition; Begging

Introduction

Plastic secretion of steroid hormones is a powerful tool that animals can use to adapt themselves to varying environmental conditions (Dufty et al., 2002). For instance, an increase in corticosteroids promotes a whole suite of physiological and behavioural responses that enhances the ability of animals to cope with stressful situations (Sapolsky, 1992; Wingfield and Kitaysky, 2002). Similarly, androgen increases during breeding allow males to face challenges of territory ownership or access to mates (Wingfield et al., 1990). These modifications of the steroid milieu are typically short-term and animals return to their normal behaviour when the specific context that has elicited the behaviour disappears. Maintaining elevated levels of androgens or

corticosteroids in the long-term can be extremely costly in terms of immune suppression (Folstad and Karter, 1992; Owen-Ashley et al., 2004).

However, for developing organisms, even short-term changes in the normal hormonal course may alter their developmental path, thus permanently modifying their phenotype. These effects are stronger when occurring early in development (Lindström, 1999). Thus, perinatal steroid modifications may permanently alter the morphology, behaviour and physiology of organisms (Clark and Galef, 1995; Hews et al., 1994). Since these plastic steroid responses have short and long-term effects, it is often difficult to disentangle their adaptive function from their pleiotropic effects (Ketterson and Nolan, 1992; Sinervo and Svensson, 1998).

In birds, nestlings of many species present important variations in corticosteroids and androgens that can be associated with begging and sibling competition. In some passerine species,

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increased testosterone levels (T) are associated with increases in begging intensity and sibling competition (Goodship and Buchanan, 2006; Naguib et al., 2004; Quillfeldt et al., 2006). Studies in gulls however, have found that experimental increases in T lead to a substantial decrease in begging and an increase in sibling aggression (Groothuis and Ros, 2005; Ros et al., 2002), as expected from the challenge hypothesis (Wingfield et al., 1990). In other species, begging intensity and sibling hierarchies are mediated by corticosterone (CORT), although here again, the evidence is often species-specific (Kitaysky et al., 2001b; Love et al., 2003; Nuñez de la Mora et al., 1996; Saino et al., 2003).

Altricial nestlings depend on parental feeding for survival, so we would expect that, if T and CORT increase begging effort and/or sibling competition, nestlings should adjust the levels of these hormones according to the level of sibling competition that they are exposed to. Such a mechanism would ensure that an adequate amount of resources is brought to the nest. To test this hypothesis, we conducted a brood size manipulation in the spotless starling (*Sturnus unicolor*). In this species, nestlings show intense competition for resources, often leading to strong differences in size that can have profound effects on their fitness (Cordero et al., 2001). Shortly after hatching, we cross-fostered nestlings to small, medium and large broods, and examined their circulating levels of T and CORT. Since these steroids can compromise immune defence (Folstad and Karter, 1992; Owen-Ashley et al., 2004), we also measured the strength of cell-mediated immune response in nestlings, in order to determine whether increases in these hormones are costly in terms of immune response.

Materials and methods

Field study procedures

The study was conducted in a mixed oak and ash woodland located near Soto del Real (Madrid, Spain) that is managed for cattle grazing. We have been studying a spotless starling population breeding in nest-boxes in this area since 2001. Nests were visited every second day until laying started, and daily when the expected hatching date neared. Cross-fostering was carried out when nestlings were 1–3 days of age (average = 1.93; SD = 0.40), depending on the number of nests available for brood manipulation. A total of 189 nestlings originating from 50 different nests were cross-fostered in this experiment. We created three treatments: large broods (6 nestlings), medium broods (4 nestlings) and small broods (2 nestlings). Sample size was evenly distributed among treatments and consisted of 17 large broods, 15 medium broods and 16 small broods. We made sure that there was no correlation between natural and manipulated brood size ($r_s = 0.17$, $N = 47$, $P = 0.24$). There were no differences between treatments in nestling age on the day of manipulation (mixed model with nest as random factor: $F_{2,46.2} = 0.58$, $P = 0.56$). Brood size manipulations were carried out by splitting broods and moving nestlings to other nests. On average, experimental broods consisted in 49.5% of their own original nestlings and 50.5% of cross-fostered nestlings.

Cell-mediated immune response was measured by the phytohemagglutinin (PHA) skin test (Smits et al., 1999): at day 14, nestlings were injected subcutaneously with 0.05 ml of a 5 mg/ml solution of PHA (Sigma Aldrich) in the wing web, after recording the width of this area with a thickness gauge (Mitutoyo Co., Japan). We measured the swelling induced by the PHA injection 24 h later. This test provides a useful measurement of immune activity showed by several types of immune cell, and correlates with measures of fitness in a variety of bird species (Martin et al., 2006). At day 15, we also recorded the nestlings' body mass, wing and tarsus length and took a blood sample of

approximately 0.8 ml from the jugular vein. Nestlings were removed from the nest-box one at a time, and the order of handling was carefully recorded as this was expected to influence CORT measurements. Samples were taken to the lab on cooled bags (+4 °C) and centrifuged at 10,000×. Plasma and red cell fractions were separated and frozen at -20 °C for hormone assays and molecular sexing, respectively.

Animal procedures were approved by local authorities (Consejería de Medio Ambiente Comunidad de Madrid). We adhered to research standards as outlined in the Guidelines for the Treatment of Animals in Behavioural Research (ASAB, 2006).

Hormone assays

Steroids were extracted from 0.3 ml plasma samples by a standard diethyl ether procedure. We resuspended steroids in assay buffer and assayed T and CORT concentrations using commercial EIA kits (IDS Octeia for CORT and Cayman 582701 for T). We analysed the linearity of our plasma samples by carrying out serial pool dilutions. All samples were measured in duplicate. Internal standards were also run in each plate to adjust for inter-assay differences. Inter- and intra-assay coefficients of variations were, respectively, 5.4% and 8.0% for CORT, and 17.2% and 8.9% for T. Reported cross-reactivities of other relevant steroids with the kits' antibodies were all negligible (<1%), except for a 27.4% cross-reactivity with 5 α -dihydrotestosterone (5 α -DHT) with the T antibody. However, as 5 α -DHT and T have similar receptor affinities, we consider that our measure of T is not biased by the eventual presence of 5 α -DHT in the samples.

Molecular sexing

DNA was extracted from blood cells using the PUREGENE protocol (Gentra Systems). Around 20 ng of each chick DNA was used in a polymerase chain reaction to amplify introns of the genes CHD-W and CHD-Z (Griffiths et al., 1998).

Statistics

We examined differences in body mass and size measurements using mixed models in which we included nest of origin and cross-fostered nest as random factors, and treatment, sex and the interaction of these two terms as fixed factors. Although in most models interactions between sex and treatment were not significant, we forced them into all our models because of a priori expectations of a significant interaction (Fargallo et al., 2002) and because the Akaike Information Criterion (AIC) value of the model including interactions was always the lowest (Littell et al., 1996). However, results do not change qualitatively if interactions are removed. Residuals from models were normally distributed.

Results

Biometry

On the day of manipulation there were no significant differences between experimental groups in nestling age, tarsus size or body mass (Table 1). However, at 15 days of age, the brood size manipulation had achieved the expected effect: nestlings becoming smaller and lighter with increasing brood size. Sexual differences in size and mass could also be observed at this age (Table 1): males were heavier (Fig. 1A) and had longer tarsi than females. However, no significant interactions between treatment and sex were found.

A limited number of recruits ($N = 36$) from this manipulation were captured in the study area during three years following the experiment (2004–2006). No differences were found in the latter

Table 1
Effects of brood size manipulations on measurements of males and females at manipulation day, 15 days of age and adulthood

Time period	Measurement	Males			Females			Statistics				
		N	Small broods	Medium broods	Large broods	N	Small broods	Medium broods	Large broods	Treatment	Sex	Interaction
Manipulation	Body mass	86	16.9 (1.0)	14.7 (0.8)	15.6 (0.7)	89	16.3 (0.19)	15.3 (0.9)	15.9 (0.7)	1.64	0.03	0.34
	Tarsus length	86	14.9 (0.5)	13.9 (0.4)	14.4 (0.3)	89	14.8 (0.4)	14.5 (0.4)	14.8 (0.3)	1.10	1.31	0.59
Day 15	Body mass	82	86.6 (2.1)	80.5 (1.8)	78.8 (1.5)	87	83.1 (2.0)	77.6 (1.9)	73.2 (1.5)	8.39***	18.57***	1.38
	Tarsus length	82	30.1 (0.2)	29.9 (0.2)	29.7 (0.1)	87	29.8 (0.2)	29.6 (0.2)	29.3 (0.1)	3.30*	6.60*	0.05
	Wing length	82	86.1 (1.3)	82.9 (1.1)	83.7 (0.9)	87	86.2 (1.3)	83.3 (1.1)	82.3 (0.9)	3.71*	0.14	0.89
Adulthood	Body mass	16	91.1 (3.0)	90.4 (2.4)	87.0 (2.6)	20	85.0 (5.9)	85.5 (2.4)	83.8 (2.4)	0.59	2.91	0.10
	Tarsus length	16	30.8 (0.3)	29.8 (0.3)	29.9 (0.7)	20	29.9 (0.2)	29.5 (0.2)	30.8 (0.3)	3.86*	5.0*	0.48
	Wing length	16	136.9 (1.2)	137.2 (1.2)	137.6 (1.2)	20	136.0 (2.7)	130.2 (0.9)	132.3 (0.9)	1.55	12.61**	1.65

Data are averages (SE). Statistics correspond to *F* values from mixed models which included the identity of nest of origin and manipulated tests as random factors. Sample sizes for small, medium and large groups are, respectively, at manipulation: 15, 28 and 43 (males), and 18, 25 and 46 (females); at day 15: 13, 27 and 42 (males), and 15, 25 and 45 (females); at adulthood: 5, 6 and 5 (males), and 2, 8 and 10 (females). **P*<0.05; ***P*<0.01; ****P*<0.001.

in terms of wing length and body mass, although differences in tarsus length were still statistically different between groups (Table 1).

Steroid levels and cell-mediated immune response

We expected T and CORT to increase with increasing brood size. Since the levels of the two steroids were not correlated

among them ($r_s=0.10$, $N=76$, $P=0.38$), we built individual models for each hormone. The final model for T concentration showed significant differences between groups, and no sexual differences or interaction with brood size (brood size: $F_{2,80.3}=3.99$, $P=0.02$; sex: $F_{1,91.5}=0.03$, $P=0.85$; brood size*sex: $F_{2,92}=0.33$, $P=0.72$). Male levels were almost identical to female levels (mean (SD): males: 20.36 pg/mg (12.3), females: 20.30 pg/mg (11.30)). Handling time had no effect on T levels

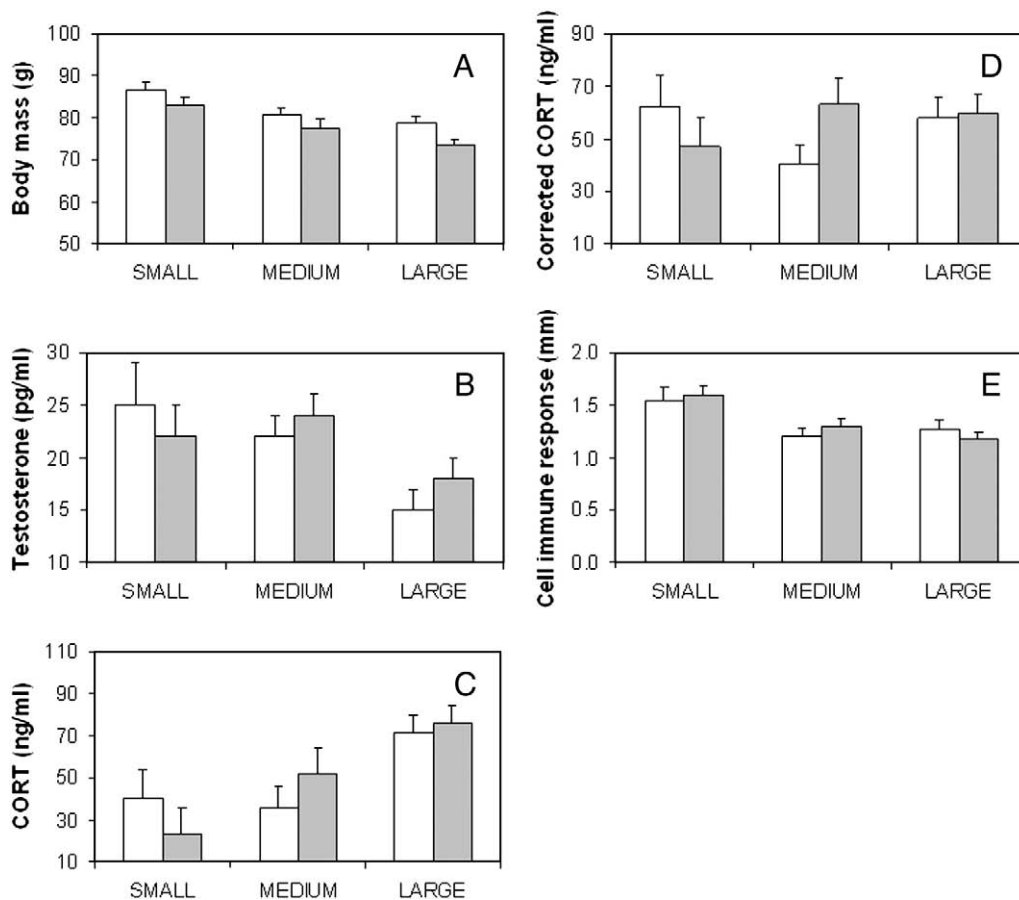


Fig. 1. Effects of the brood size manipulation on males (white bars) and females (grey bars) nestlings at 15 days of age: A) body mass, B) T levels, C) CORT levels not corrected handling time; D) CORT levels corrected for handling time; E) cellular immune response to phytohemagglutinin. Data are least squares means (+1 SE) from the model.

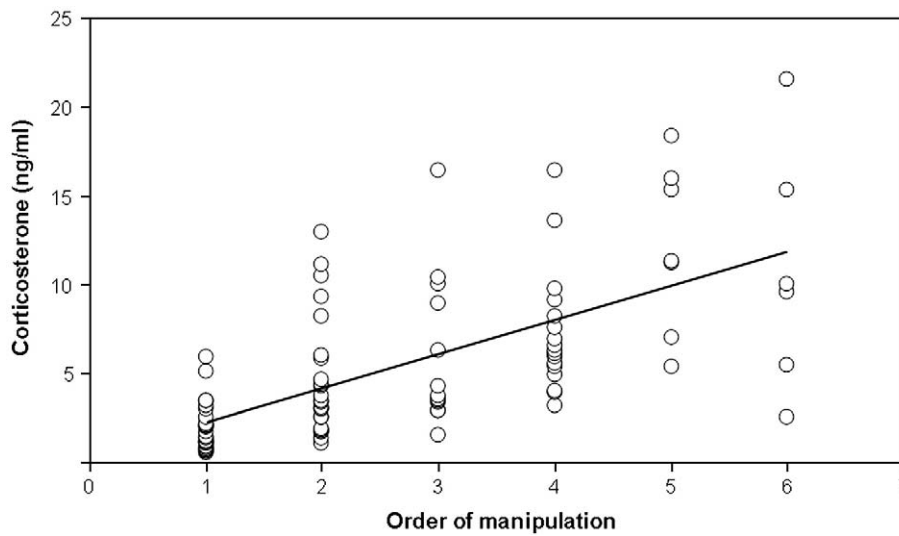


Fig. 2. Relationship between CORT plasma levels and order of nestling manipulation.

($F_{1,32}=0.05$; $P=0.8$), and was not considered in the model. Post-hoc tests showed that, contrary to predictions, T levels were higher in small and medium than in large broods (Post-hoc difference tests: small vs. medium: $P=0.85$; small vs. large, and medium vs. large: both $P<0.05$; Fig. 1B). CORT levels increased with increasing brood size (brood size: $F_{2,87}=8.08$, $P<0.001$; sex: $F_{1,87}=0.01$, $P=0.90$; brood size*sex: $F_{2,87}=0.86$, $P=0.42$; Fig. 1C). However, differences disappeared when we included in the model the effect of the differential handling time (brood size: $F_{2,75.9}=0.31$, $P=0.73$; sex: $F_{1,85}=0.12$, $P=0.72$; brood size*sex: $F_{2,82.4}=1.74$, $P=0.18$; order of manipulation: $F_{1,80.2}=40.89$, $P<0.001$; Fig. 1D): this is because CORT increased with order of manipulation (Fig. 2) and large broods obviously took longer to process. A similar absence of

relationship is found if the analysis is restricted to the first nestling that was manipulated in each brood (data not shown).

The final model for the cell-mediated immune response (smallest AIC) included the significant effects of manipulated brood size and T levels, as well as the non-significant effects of sex and sex*brood size interaction (brood size: $F_{2,45.7}=5.12$, $P<0.01$; sex: $F_{1,92.7}=0.05$, $P=0.83$; sex*brood size: $F_{2,86.2}=1.26$, $P=0.29$; testosterone levels: $F_{1,74.3}=4.40$, $P=0.03$). Immune response decreased with increasing brood size (Fig. 1E) and was negatively correlated with plasma T concentration (Fig. 3). This negative relationship was similar for all experimental groups, as shown by a non-significant interaction between experimental brood size and testosterone levels, which was removed from the model.

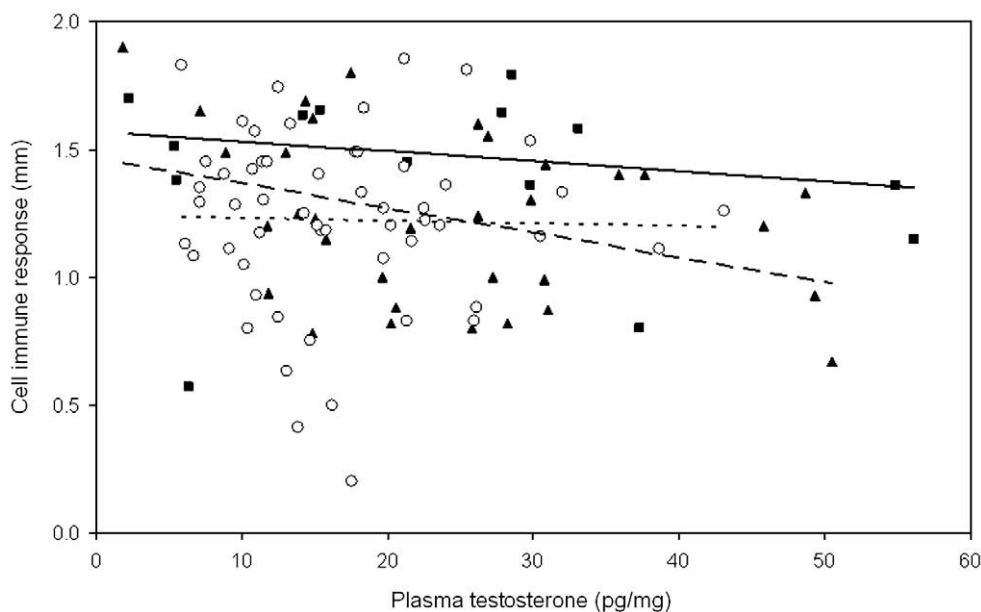


Fig. 3. Relationship between cellular immune response to phytohemagglutinin and T levels (small broods: squares and solid line, medium broods: triangles and dashed line; large broods: circles and pointed line).

Discussion

Our experiment aimed to test the relationship between T and CORT levels and sibling competition. To this end, we experimentally manipulated brood size, which is a standard way of modifying levels of competition in altricial birds (Stearns, 1992; Tinbergen and Boerlijst, 1990). Based on previous studies, we expected both steroids to increase with increasing levels of brood size manipulation (Goodship and Buchanan, 2006; Groothuis and Ros, 2005; Naguib et al., 2004; Quillfeldt et al., 2006; Saino et al., 2003).

The experiment was successful at inducing differences in size between nestlings, and by day 15 nestlings had become lighter and smaller with increasing brood size. Some tarsus length differences were still present in adulthood, although birds had compensated for body mass differences (Naguib et al., 2004). Previous experiments have shown that these size differences result in increased levels of begging and sibling competition at the nestling stage (Neuenschwander et al., 2003). Therefore, we assume that the intensity of sibling competition should covary with these differences in size and that nestlings in the larger broods would be exposed to the highest level of sibling competition.

Contrary to expectations, we found that T levels were higher in small than in large broods. This is in striking contrast to a similar previous experiment conducted in the zebra finch *Taeniopygia guttata*, where the opposite pattern was observed (Naguib et al., 2004), and to correlative data in other passerine, the pied flycatcher *Ficedula hypoleuca*, where begging rates were found to correlate with T levels (Goodship and Buchanan, 2006). Similar evidence has been found in several non-passerine bird species (Groothuis and Ros, 2005; Quillfeldt et al., 2006), although in several other cases T levels were not associated with increases in nestling competition (Nuñez de la Mora et al., 1996; Tarlow et al., 2001). The variety of results that can be observed in these studies most likely underlines species-specific differences in the action of steroid hormones.

In the case of CORT, the initial positive relationship that we observed with brood size was found to be a spurious result due to the varying handling time of the different brood sizes. Once this effect was removed, no differences between experimental groups were noted in basal CORT levels. This also contrasts with a previous study in another passerine species, the barn swallow (*Hirundo rustica*), where a decrease in nestling food availability led to an increase in CORT levels (Saino et al., 2003). Food restricted kittiwake chicks (*Rissa tridactyla*) also show an increase in their circulating CORT levels (Kitaysky et al., 2001a). Furthermore, CORT-implanted nestlings of the same species increase their begging rates (Kitaysky et al., 2001b). As in the case of T, our results are evidence of species-specific differences that could have resulted from the severity of the brood manipulation in our study species. It is also likely that the response of different species to food restriction may depend on the likelihood of such events in nature. We would expect seabirds (such as the kittiwake) and insectivorous birds that forage on the wing (such as the swallow) to be particularly exposed to food shortages and thus to have evolved response

mechanisms. In this respect, omnivorous species such as the starling might be less exposed to environmental perturbations, a difference that could explain the lack of CORT increase in response to the brood size manipulation.

We found a positive relationship between order of manipulation and circulating CORT. This pattern is evidence of a typical stress response, as shown in studies conducted in nestlings of a variety of species (Blas et al., 2006; Love et al., 2003; Sockman and Schwabl, 2001). However, these studies have also uncovered large species-specific differences in the magnitude of this response, as well as an age effect (Blas et al., 2006; Sims and Holberton, 2000). The magnitude of the stress response seems to be associated with certain developmental modes and age-stages in which the effects of CORT may mediate adaptive changes in responses to environmental perturbations (Blas et al., 2006; Kitaysky et al., 2003; Starck and Ricklefs, 1998).

Cell-mediated immune response decreased with increasing brood size, as found in many other previous studies (Fargallo et al., 2002; Hōrak et al., 1999; Naguib et al., 2004; Saino et al., 1997). The interpretation of this pattern is that food-restricted birds prioritise growth when confronted with the trade-off between allocation to growth or to costly immune defence, since poor growth would have the highest impact on survival. It has also been suggested that this trade-off is mediated by CORT (Saino et al., 2003), a hormone that would switch the organism into a catalytic state, promoting growth strategies towards immediate survival (Sapolsky, 1992).

We found that cell-mediated immune response was negatively related to T concentration, as predicted by the immunocompetence handicap hypothesis (Folstad and Karter, 1992), and shown before in other species at the nestling stage (Naguib et al., 2004). However, it is difficult to understand this pattern since we found that T levels and immune response followed a similar trend with respect to brood size (i.e. both higher in small broods). This pattern would lead us to expect a positive correlation between cell-mediated immune response and T levels. However, this correlation was consistently negative in all three experimental groups, as shown by the absence of a significant interaction with brood size. This apparently paradoxical result suggests that the effect produced by the brood manipulation on the immune response and T levels does not change the inherent negative relationship between these two components that predates the modification, and that could result from condition or genetic differences.

To summarise, we found that an experimental modification of sibling competition did not lead to a parallel response in CORT or T levels in nestlings of the spotless starling. Contrary to expectations we found a decrease in T levels with increasing brood size. This result adds to the increasing evidence of species-specific patterns of steroid responsiveness in birds (Goymann et al., 2007; Hirschenhauser and Oliveira, 2004), and calls for direct manipulative studies of steroid levels in nestlings (Fargallo et al., 2007; Kitaysky et al., 2001b).

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