Immune response covaries with corticosterone plasma levels under experimentally stressful conditions in nestling barn swallows (*Hirundo rustica*)

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Traits related to fitness are often pleiotropically linked or otherwise constrained in their expression. Organisms therefore trade between fitness components such as number and viability of their offspring. The physiological mechanisms mediating such trade-offs, however, have been poorly investigated. We manipulated brood size and satiation of nestling barn swallows, *Hirundo rustica*, to simulate the effect of two kinds of natural stresses, i.e., long-term intense competition in a large brood and acute food deprivation, and we measured their effect on body condition, T cell–mediated immune response, and corticosterone, the main hormone mediating the adrenocortical stress-response. Brood enlargement increased corticosterone levels compared with those for brood reduction, and brood enlargement depressed immune response, body mass, and condition. Corticosterone levels markedly increased after food deprivation. Immune response negatively covaried with corticosterone levels measured after long-term stress. Hence, living in a crowded nest and with food deprivation elicited a stress response mediated by corticosterone, and depressed an important component of offspring fitness such as T cell–mediated immunity. The negative covariation between circulating corticosterone and immunity suggests that the trade-off between offspring number and quality is mediated by variation in plasma levels of corticosterone, which has immunosuppressive effects. *Key words:* body mass, brood size, competition, corticosterone, food availability, *Hirundo rustica*, immunity, stress, trade-off. *[Behav Ecol 14:318–325 (2003)]*

A fundamental axiom of evolutionary theory is that life histories are shaped by natural selection, which promotes the evolution of strategies (i.e., the combinations of life-history traits) that maximize lifetime fitness (Roff, 1992; Stearns, 1992). However, constraints on the expression of fitness-related traits can cause negative covariation between fitness components, as when the traits compete for the same limiting resources (Lima, 1987; Slagsvold, 1984).

In altricial birds, the probability of offspring survival positively correlates with body mass at fledging (Lindén et al., 1992; Tinbergen and Boerlijst, 1990; review in Newton, 1989). However, offspring body mass and condition decrease with increasing size of the brood (e.g., Korpimäki and Hannu, 1996; Martin, 1987; Merilä and Wiggins, 1995; Saino et al., 1997, 2001; Sanz and Tinbergen, 1999). In addition, a positive association between brood size and nestling mortality has been demonstrated (Korpimäki and Hannu, 1996; Merilä and Wiggins, 1995).

Probably the most frequently invoked mechanism mediating the trade-off between offspring quality and number is competition for depreciable parental care (Clutton-Brock, 1991). However, the negative effect of brood size on survival may also be mediated by limitation of nutrients leading to weaker immune defense (Christe et al., 1998; Saino et al., 1997).

Recent studies have shown that nestlings in naturally large or experimentally enlarged broods have smaller T cell–mediated immune response compared with that of nestlings in naturally small or experimentally reduced broods (Hõrak et al., 1999; Saino et al., 1997, 2001). Acquired immunity is the main physiological mechanism of defense available to vertebrates against parasites (Pastoret et al., 1998). T lymphocytes play a central role in both cellular and humoral acquired immune response, and T cell–mediated immune response may thus strongly influence the general state and survival of offspring until independence (Christe et al., 1998; Hõrak et al., 1999). Competition for dietary resources may limit the availability of nutritional factors critical to immune system functioning, as shown in the barn swallow (*Hirundo rustica*) (Saino et al., 1997) and in other species in captivity (Chandra and Newberne, 1977; Dietert et al., 1994; Gershwin et al., 1985; Glick et al., 1981, 1983; Tsiagbe et al., 1987).

A partly alternative pathway of influence of a large brood on immunity is that food limitation or any other behavioral and/or parasitological factor varying between large and small broods act as a form of stress. Nestlings in large broods receive relatively small amount of food per capita (Saino et al., 1997, 2000, 2002b; unpublished data). The negative correlation between brood size and mean within-brood body mass corroborates the idea that food limitation increases with brood size (Martin, 1987). Nestlings in enlarged broods beg at higher rates and produce begging calls of different quality compared with those of nestlings in reduced broods (Kilner, 1995; Mondloch, 1995; Redondo and Castro, 1992; Sacchi et al., 2002; Saino et al., 2000; see also Lotem, 1998). Finally, nests with large broods contain disproportionately more ectoparasites (Saino et al., 2002a). Hence, a nest with a large brood is a relatively stressful environment.

In vertebrates, diverse types of stress—including those deriving from thermal and chemical conditions, food

deprivation, parental effort, and social interactions—can have serious negative effects on immunity (see Apanius, 1998; Bijlsma and Loeschcke, 1997; Wingfield, 1994; Wingfield et al., 1998). One consequence of stress is an increase in circulating levels of glucocorticosteroids—the so-called adrenocortical response—which eventually can lead to immune suppression (see Apanius, 1998; Wingfield et al., 1998). Hence, in a crowded brood, nestlings may be exposed to intense nutritional and/or behavioral stressors or to more intense parasitism, which may increase their circulating levels of glucocorticosteroids. This exposure can have negative effects on immune system functioning. However, corticosteroid levels in young birds under natural conditions have been poorly investigated (Kitaysky et al., 1999a,b; Schwabl, 1999), and we are unaware of field studies on the effect of stress on immunity.

The main aim of the present study was to analyze the effects of two forms of stress on levels of circulating corticosterone in nestling barn swallows, and to investigate covariation between corticosterone levels and major components of nestling general state, such as body mass and condition and an in vivo measure of T cell–mediated immune response. We manipulated brood size, thus determining either a relative abundance (reduced broods) or scarcity (enlarged broods) of food per individual nestling (Saino et al., 1997, 2000). We predicted that nestlings in enlarged broods had higher levels of corticosterone compared with those in reduced broods, because brood enlargement results in long-term stress, arising as a consequence of scrambling with nest mates to obtain food, struggling to out-compete begging by nest mates, and starving for relatively long periods compared with that of reduced broods.

Immediately after the first blood sampling, part of the nestlings in each brood were deprived of food for 2 h, thus mimicking naturally occurring periods of no food provisioning by parents during, e.g., spells of bad weather. We predicted that 2 h after the start of the food-deprivation procedure, corticosterone levels were larger in food-deprived compared with non–food-deprived nestlings. At the end of the food deprivation, we measured body mass and size and the lymphoproliferative response of T cells after mitogenic stimulation by phytohemagglutinin (Lochmiller et al., 1993). We predicted that immune response and body mass and condition negatively covaried with corticosterone levels, owing to the negative effects of corticosterone on growth and immunity.

MATERIALS AND METHODS

The barn swallow is a socially monogamous, semicolonial, insectivorous passerine. Females alone, in Palearctic populations, incubate the clutch of two to seven eggs for approximately 2 weeks. Both parents feed the offspring up to 1 week after fledging, which occurs 18–20 days after hatching (Møller, 1994).

We studied barn swallows breeding in two large colonies located in two farms southeast of Milano (northern Italy) in 2001. Nests were inspected every day to record breeding events. When we found two or more nests in which hatching was completed the same day in the same farm, we did an unbalanced cross-fostering of individually marked nestlings so that one randomly chosen brood in each pair of synchronous broods (hereafter, dyad) was enlarged by one nestling, whereas the other was reduced by one. Nestlings to be cross-fostered were chosen randomly. The number of cross-fostered nestlings depended on the original brood size and was established according to a predetermined scheme aimed at minimizing the difference between "resident" and "cross-fostered" nestlings present in each brood after manipulation (see Saino et al., 1997, 1999, 2000). However, when more than two broods were found that hatched synchronously, we matched preferentially those with a similar number of nestlings and placed in the same part or section of the stable. These decisions were adopted in order to minimize the effects of potentially confounding factors such as the difference in original brood size within dyads, reflecting parental quality, and any microenvironmental effects such as thermal conditions, disturbance by farmers attending the cows, or frequency of appearance of predators such as cats.

On day 12 after hatching, experimental nests were visited to collect a first blood sample and start the food-deprivation procedure. All nestlings of each brood were simultaneously extracted from the nest and subjected to a blood sampling (60–90 µl) by puncturing the brachial vein within 2.5 min of extraction by two experienced people. Nestlings were bled in a random sequence. No significant difference in time elapsed from extraction from the nest and bleeding of the last nestling of the brood existed between reduced and enlarged broods (t test for paired data: $t = 1.00$, df $= 18$, $p = .33$), mean difference in time until bleeding being approximately 0.12 (0.13 SE) min. Hence, relative corticosterone concentration of nestlings in enlarged versus reduced broods was not affected by different times of exposure to experimenter-induced stress before blood was sampled. This blood sample was used to measure the long-term effect of brood-size manipulation on circulating levels of corticosterone. Half the nestlings of each brood (or half the number + 0.5 in case of an uneven brood size) were assigned to a food-deprivation procedure by covering them with a thin net (Sacchi et al., 2002; Saino et al., 2000), allowing free nestling movements, whereas the others were allowed to continuously receive food from their attending parents. Individuals to be assigned to food-deprivation were chosen randomly within the groups of resident and cross-fostered nestlings. Two hours after the start of the food-deprivation procedure, nests were visited again to collect a second blood sample (70–90 µl) within 2.5 min of extraction from the nest. Again, bleeding sequence was random, and time taken to bleed all the nestlings did not differ between enlarged and reduced broods (t test for paired data: $t = 0.73$, df $= 19$, $p = .48$), with a mean difference of 0.08 (0.11 SE) min. The second blood sample was used to measure the cumulative effects of brood-size manipulation and short-term food deprivation on circulating corticosterone levels. Broods in a dyad were sampled in random order and in rapid sequence. All food-deprivation procedures started between 0900 and 1030 h to experimentally control for circadian variation in corticosterone levels. The timing of blood resampling was set at 2 h after the start of food deprivation because shorter food-deprivation periods (e.g., 1 h) may not result in a marked alteration of satiation status (personal observation), as judged from previous manipulations and observations of begging behavior, whereas longer time intervals might have resulted in a decline of corticosterone plasma levels after an initial increase.

After the second blood sample had been taken, we measured body mass (expressed in grams × 10), length of the right tarsus (considered a measure of body size to calculate a "body condition" index as body mass/tarsus length[3]), and started an in vivo test aimed at estimating the ability of nestlings to raise a T cell–mediated immune response. This test consists of a subcutaneous injection of 0.2 mg phytohemagglutinin (PHA), a lectin which is mitogenic to T lymphocytes, dissolved in 0.04 ml phosphate buffered saline (PBS) in the web of one wing, whereas the other wing web was injected with the same amount of the solvent (PBS) to serve as a control for the effect of the

injection per se. The thickness of both wing webs was measured by a pressure-sensitive micrometer before injection. Twenty-four hours after injection, the thickness was measured again to estimate the swelling of the PHA-injected wing web, while controlling for the effect of injection of PBS. According to Lochmiller et al. (1993) an index of wing web swelling was calculated as the difference (millimeters) in change in thickness of the PHA-injected web minus the change recorded for the PBS-injected control wing. Following Lochmiller et al. (1993) and several other later studies (Christe et al., 1998; Hõrak et al., 1999; Moreno et al., 1999; Saino et al., 1997, 1999; Sorci et al., 1997), this index was considered a measure of T cell–mediated immune response. Repeatability of wing web thickness measures taken with the same pressure-sensitive micrometers has been shown to be as high as 0.95 in previous studies of the same and other bird species (Saino et al., 1997; Sorci et al., 1997).

In a follow-up experiment with new birds, we repeated the same food-deprivation procedure, starting immediately after a blood sample had been taken. Half of each brood was assigned to a food-deprivation procedure. However, half of the food-deprived nestlings were subjected to blood sampling 1 h after the start of food deprivation, whereas the other half was bled 2 h after food deprivation, as in the main experiment. Nestlings were assigned randomly to either of the blood sampling protocols. These samples were used to analyze the average pattern of variation of corticosterone levels during the intermediate phases of the acute-stress episode resulting from food deprivation. We could not sample the same nestlings three times because this would probably have resulted in an excessive amount of blood being taken. In addition, taking blood three times would have confused the comparison of the results obtained on these nests and those in the brood-size manipulation experiment, because 2 h after the start of food deprivation, nestlings would have been sampled twice instead of only once, as in the brood-size manipulation experiment (see Discussion).

Corticosterone assays

Blood samples were collected in heparinized capillaries and taken to the laboratory for centrifugation (10 min at 11,500 r.p.m.). Plasma was then separated from blood cells by breaking the capillary well within the part occupied by plasma, to avoid contamination by blood cells, and frozen at −20°C for later corticosterone assay. Corticosterone concentration in the plasma was assayed by ^{125}I radioimmunoassay kit purchased from ICN Biochemical (Costa Mesa, California). Because we aimed at analyzing the effect of brood-size manipulation and food deprivation within dyads of cross-fostered broods, we analyzed all samples from each dyad within a single assay, thus analytically controlling for the effect of among-assays variation in corticosterone concentration estimates. Within-assay coefficient of variation of concentration estimates was relatively small (3.05% ± 0.37, $n = 12$ assays), as determined by assaying the same pool of barn swallow plasma in quadruplicate in each assay. For various reasons, including failure in obtaining an adequate amount of blood at either blood samplings, accidental breakage of the capillary containing the blood sample, and corticosterone concentrations falling below the detectability limit (3.1 ng/ml), corticosterone levels could not be measured in all nestlings (for sample sizes, see Results). However, in the analyses we always used the maximum sample available. We are confident that the 10% of the nestlings for which we could not measure corticosterone levels did not affect our results, because failure of experienced people in obtaining a blood

sample from barn swallow nestlings aged 12 days depends on random errors in puncturing the brachial vein, resulting in a small hematoma, independently of nestling size or mass under the constraining time limits imposed by the experimental protocol. In fact, there was no statistically significant difference in any morphological variable between nestlings that were or were not assayed for corticosterone (t test, $p > .4$ in all comparisons).

Statistical analyses

To analyze the effect of experimental manipulations on nestling body mass and condition, immunity, and corticosterone plasma levels, we adopted a fixed-effects general linear model in which we included brood-size manipulation (enlargement or reduction), food deprivation (yes or no), and dyad as factors. Brood-size manipulation and food deprivation were considered as fixed-effect factors because they were experimental manipulations specifically designed to assess the effect of alternative conditions on nestling phenotype. Dyad was also included as a fixed effect because the dyads we considered were not a random sample of all the possible dyads of broods existing in our population. In fact, dyads were chosen to meet several specific criteria, i.e., synchrony of hatching, similar original size, same original colony, and proximity within the colony. These criteria were adopted to minimize the difference in starting conditions between the two broods in each dyad, because the difference in original size may mirror differences in parental and egg quality, and different colonies or large reciprocal distance between nests in a colony are positively associated with differences in microecological conditions (see above). Because these criteria were obviously likely to reduced the within- relative to the among-dyads variance in the variables we measured, dyad was included as a fixed effect.

By including dyad as a factor in the analyses, we thus controlled for the effect of variance among dyads on nestling phenotype, and the effects of brood-size manipulation and food deprivation were thus estimated while controlling for the effect of variance among dyads. Similarly, in ANCOVA of nestling morphology and immunity in relation to corticosterone levels, dyad, brood-size manipulation, and food deprivation were entered as fixed-effect factors.

In ANOVA and ANCOVA, nestlings were therefore entered as independent observations. This is in keeping with several other studies in which nestlings were cross-fostered between pairs of broods, and nestling phenotype (including morphological, behavioral, and physiological traits) has been investigated (e.g., Christe et al. 2000; Merilä and Fry 1998; Sacchi et al., 2002; Saino et al., 1997, 1999, 2000; Sheldon et al., 1997; Smith and Wettermark, 1995; and references therein).

In ANOVA and ANCOVA, we initially entered all the main effects together with the interactions. We then progressively removed the nonsignificant terms from the models. At each step of this backward selection procedure, the nonsignificant term with the smallest associated effect size was eliminated. Main effects were eliminated only after that the relevant interactions had been removed. Power of the nonsignificant tests is reported as computed by the SPSS 10.1 package. The power associated to a particular term is that calculated at the step of the analysis when it was removed from the model, i.e., after the terms with smaller effect size had been removed. It must be emphasized that the final models could include different main effects and interactions, and this is the reason why the degrees of freedom associated to a particular term can vary between analyses, even if the size of the sample of nestling was the same.
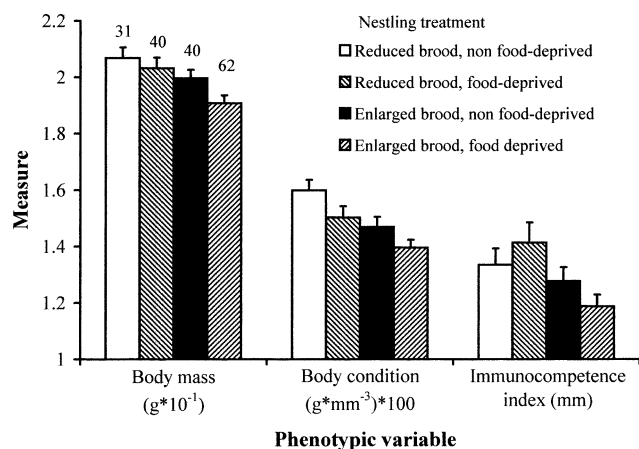
Figure 1
Mean (+SE) body mass (grams), body condition index (grams/millimeters^{-3}, body mass/tarsus length^3) and index of T cell–mediated immune response (millimeters, the swelling reaction to subcutaneous inoculation of phytohemagglutinin in the wing web) in relation to brood-size manipulation and food deprivation. Sample sizes, which were invariant for all variables, are shown. Measurement units of the variables have been adjusted to allow joint representation. Brood-size manipulation and food deprivation had a significant effect on body size and condition. Immune response was significantly reduced by brood enlargement.

RESULTS

In the present study, we considered 20 dyads of broods. On day 12, there were 74 nestlings in reduced broods and 102 in enlarged broods. For all these nestlings except two ($n = 174$), the measure of body mass, body mass corrected for body size, and immune response index were available. Corticosterone plasma concentration just before the start of the food-deprivation procedure could be measured in 159 out of the 176 nestlings (90.3%), whereas that at the end of the food-

deprivation procedure was measured in 158 nestlings (89.8%; see Methods).

Effect of experimental manipulations on nestling phenotype

Nestlings in enlarged broods and those subjected to food deprivation had smaller body mass and body condition index compared with those of nestlings in reduced broods and nestlings allowed to continuously receive food (Figure 1). In ANOVA, dyad, brood-size manipulation, and food deprivation had a significant effect on body mass (Table 1). The significant interaction between dyad and brood-size manipulation indicated that the difference in body mass between nestlings in enlarged versus reduced broods differed between pairs of cross-fostered broods (Table 1). However, the effect of the other interaction terms was not significant, and they were therefore excluded from the final model (effects of sequentially removed interactions: brood-size manipulation × food deprivation: $F_{1,113} = 0.79$, $p = .38$, power = 0.143; dyad × food deprivation: $F_{1,114} = 1.60$, $p = .07$, power = 0.915; see above). The analysis of body mass corrected for body size gave qualitatively similar results (Table 1), except for the lack of significant effect of the interaction between dyad and brood-size manipulation (effects of sequentially removed interactions: brood-size manipulation × food deprivation: $F_{1,113} = 0.25$, $p = .62$, power = 0.078; dyad × food deprivation: $F_{19,114} = 1.17$, $p = .30$, power = 0.774; dyad × brood-size manipulation: $F_{19,133} = 1.37$, $p = .15$, power = 0.863) (Figure 1).

The index of T cell–mediated immune response was significantly affected by dyad, and was significantly reduced by brood enlargement but not food deprivation (effects of sequentially removed interactions: brood-size manipulation × food deprivation: $F_{1,112} = 0.98$, $p = .32$, power = 0.166; dyad × food deprivation: $F_{19,113} = 1.49$, $p = .10$, power = 0.888; food deprivation: $F_{1,132} = 0.25$, $p = .62$, power = 0.079) (Table 1, Figure 1).

Corticosterone plasma level before food deprivation was significantly affected by dyad ($F_{19,138} = 5.99$, $p < .001$) and was

Table 1

ANOVA of nestling body mass, body condition index, and index of T cell–mediated immune response in relation to dyad and experimental manipulations

	Mean sum of squares	df	F	p
Body mass				
Dyad	2144.5	19,133	9.40	<.001
Brood-size manipulation	2590.2	1,133	11.35	.001
Food deprivation	2140.7	1,133	9.38	.003
Dyad × brood-size manipulation	496.5	19,133	2.18	.006
Error	228.2			
Body condition				
Dyad	0.197	19,152	5.90	<.001
Brood-size manipulation	0.442	1,152	13.25	<.001
Food deprivation	0.270	1,152	8.09	.005
Error	0.033			
T cell response				
Dyad	2616.3	19,133	2.59	.001
Brood-size manipulation	13653.8	1,133	13.49	<.001
Dyad × brood-size manipulation	1847.5	19,133	1.83	.026
Error	1011.8			

All main effects and interactions were initially included in the analyses. Nonsignificant terms were removed by backward selection. At each step, the term with the smallest effect size was eliminated (see Methods).
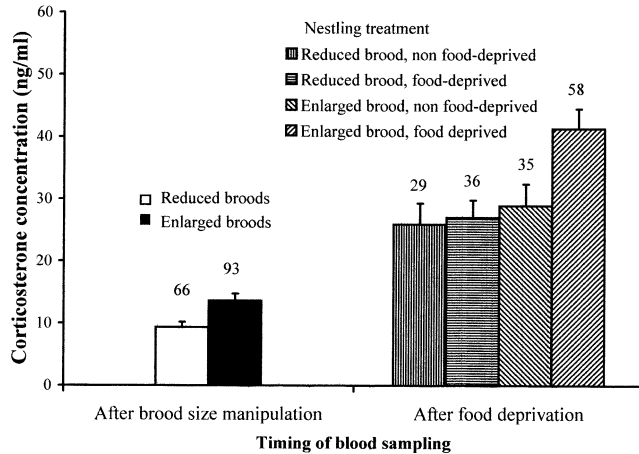
Figure 2
Mean (+SE) corticosterone levels (ng/ml) measured after brood-size manipulation but before food deprivation, food deprivation, and after food deprivation, in relation to experimental treatments of nestling barn swallows. Corticosterone levels before food deprivation were significantly larger in enlarged broods. Food deprivation determined and increase in corticosterone levels, but more so in enlarged compared to reduced broods. Sample sizes are shown.

Table 2
ANOVA of corticosterone plasma level at the end of the food-deprivation procedure in relation to dyad and experimental manipulations

	Mean sum of squares	df	F	p
Dyad	1032.7	19,117	3.76	<.001
Brood-size manipulation	2097.0	1,117	7.63	.007
Food deprivation	2000.7	1,117	7.28	.008
Dyad × food deprivation	726.6	19,117	2.64	.001
Error	274.9			

All main effects and interactions were initially included in the analyses. Nonsignificant terms were removed by backward selection. At each step, the term with the smallest effect size was eliminated (see Methods).

significantly larger in enlarged compared with reduced broods, as predicted ($F_{1,138} = 11.06$, $p = .001$) (Figure 2). The interaction term between dyad and brood-size manipulation had been removed by backward selection because its effect was not significant ($F_{19,119} = 1.40$, $p = .14$, power = 0.865). Corticosterone levels at the end of food deprivation were affected by dyad and were increased by brood enlargement and food deprivation (Table 2, Figure 2). In addition, the effect of food deprivation varied among dyads (Table 2). The effects of the other interactions were excluded from the final model because they were not significant (brood-size manipulation × food deprivation: $F_{1,97} = 2.29$, $p = .13$, power = 0.322; dyad × brood-size manipulation: $F_{19,98} = 1.05$, $p = .42$, power = 0.704).

A repeated-measures ANOVA on corticosterone levels measured before and after food deprivation was also performed to estimate the effect of experimental treatments. In this analysis, main between-subjects (dyad, brood-size manipulation, and food deprivation) and within-subjects (sampling before and after food deprivation) effects were initially entered together with all the interactions. Also in this analysis, nonsignificant terms were progressively removed by backward selection until a model containing only significant (or marginally nonsignificant) terms was obtained. Corticosterone levels at the end of food deprivation were larger than those before its start (within-subjects effect) (Table 3). Nestlings in enlarged broods and those subjected to food deprivation had larger corticosterone concentration than did nestlings in reduced broods or, respectively, those that were not subjected to food deprivation (Table 3, Figure 2). Change in corticosterone concentration during food deprivation and the effect of food deprivation per se varied among dyads (Table 3, interactions 1 × 3 and 1 × 4). The increase in corticosterone levels after food deprivation was larger in enlarged compared with reduced broods, although the effect was marginally nonsignificant (interaction 2 × 4, $p = .056$). However, the increase in corticosterone levels during food deprivation was significantly larger among food-deprived compared with non–food-deprived nestlings (interaction 3 × 4). Finally, the effect of food deprivation on change in corticosterone levels varied among dyads (interaction 1 × 3 × 4).

In a sample of nests ($n = 8$), in which brood size was not manipulated, we measured corticosterone concentration just before food deprivation ($n = 21$ nestlings) and concentration 1 h ($n = 11$ of the 21 nestlings) or 2 h ($n = 10$ of the 21 nestlings) after the start of the food-deprivation procedure. This group of experimental nests was established to investigate the temporal pattern of variation in circulating corticosterone at an intermediate stage of the food-deprivation protocol without inducing a long-term stress response by altering the number of nestlings. Mean corticosterone levels increased monotonically from basal levels to those recorded in nestlings that were bled 2 h after the start of food deprivation (mean basal concentration in all food-deprived nestlings, 8.21 [1.83 SE] ng/ml; concentration 1 h after the start of food deprivation, 21.97 [1.93]; concentration 2 h after start of food deprivation, 35.71 [5.56]). Mean corticosterone values computed across all food-deprived nestlings (basal values at time 0) and within the group of nestlings that were food deprived for 1 or 2 h, respectively, significantly increased with time (equations of the regression line for the linear term: $y = 13.64$ [2.46 SE] $x + 8.40$, $F_{1,22} = 30.81$, $p < .0001$, $R^2 = 0.583$). The increase in the variance explained by a model also including the quadratic term of time, which accounted for a nonlinear pattern of variation, was negligible (change in $R^2 < 0.01$). In addition, the relationship between corticosterone concentration and time was very far from deviating from linearity ($F_{1,21} = 0.001$, $p = .98$). All these analyses indicate that there was no absolute maximum in corticosterone concentration within the 2-h period of food deprivation.

Covariation between corticosterone levels, T cell–mediated immunity, and morphology

We first analyzed covariation of nestling body mass and condition, and immune response with corticosterone levels at the beginning and at the end of the food-deprivation period separately, while controlling for the effect of dyad, brood-size manipulation, and food deprivation and their interaction terms. Body mass did not significantly covary with corticosterone concentration after exclusion of nonsignificant terms from the ANCOVA model by backward selection ($F_{1,117} = 2.28$; $p = .13$, power = 0.332), and a similar result was obtained for body condition index ($F_{1,136} = 0.67$; $p = .41$, power = 0.129). The index of T cell–mediated immune response negatively covaried with corticosterone concentration before food deprivation ($F_{1,137} = 5.61$; $p = .019$, coefficient = −0.856 [0.361 SE]) (Figure 3) in a model in which only dyad and brood-size manipulation could be included as additional covariates.
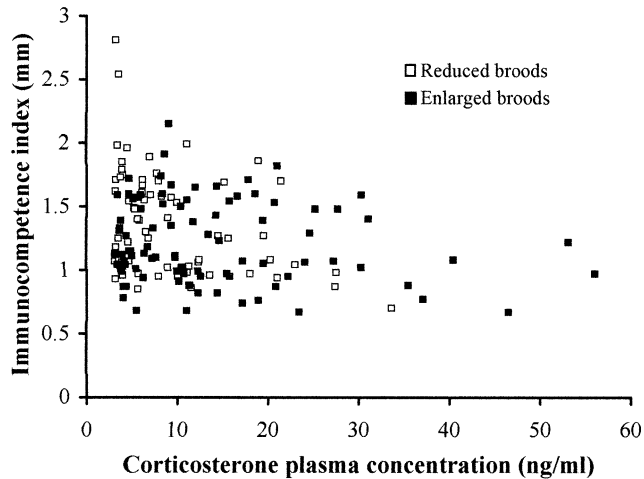
Figure 3
Negative covariation between index of T cell–mediated immune response (millimeters) and corticosterone levels (nanograms per milliliter) measured after brood-size manipulation but before food deprivation in barn swallow nestlings of reduced and enlarged broods.

No significant covariation between nestling morphology or immunity and corticosterone levels after the food-deprivation period existed (body mass: $F_{1,116} = 0.54$, $p = .46$, power = 0.113; body condition: $F_{1,135} = 3.63$, $p = .06$>, power = 0.473; T cell–mediated immune response: $F_{1,135} = 1.22$, $p = .27$, power = 0.195).

We repeated these analyses while simultaneously entering the two measures of corticosterone concentration as covariates, and dyad, brood-size manipulation, and food deprivation as factors. In these analyses, body mass did not significantly covary with corticosterone concentration before the start of food deprivation ($F_{1,102} = 0.35$, $p = .56$, power = 0.090) or after it ($F_{1,102} = 2.65$, $p = .11$, power = 0.364), and similar results were obtained for body condition index (corticosterone level before food deprivation: $F_{1,121} = 0.10$, $p = .75$, power = 0.062; after food deprivation: $F_{1,121} = 3.78$, $p = .054$, power = 0.090). However, T cell–mediated immune response index significantly and negatively covaried with corticosterone levels before the start of food deprivation, while controlling for the effect of corticosterone concentration at the end of food deprivation ($F_{1,121} = 5.70$, $p = .019$, coefficient = -0.902 [0.378 SE]), whereas the effect of corticosterone concentration at the end of food deprivation was not significant ($F_{1,121} = 0.35$, $p = .55$, power = 0.090).

In ANCOVA in which we controlled for corticosterone levels, the two experimental manipulations and dyad, immune response was found to positively covary with body mass ($F_{1,120} = 9.45$, $p = .003$, coefficient = 0.580 [0.189 SE]), whereas the covariation with body condition index was not significant ($F_{1,120} = 0.87$, $p = .35$, power = 0.154).

DISCUSSION

In the present study, we manipulated brood size and access to food by barn swallow nestlings to simulate two different kinds of naturally occurring stressful conditions. By either enlarging or reducing broods, we altered competition for food. Compared with nestlings in reduced broods, nestlings in enlarged broods had smaller body mass and index of T cell–mediated immune response and were in poorer condition, consistent with previous results (Saino et al., 1997, 1999, 2001). Food deprivation, simulating conditions of acute food stress, had negative effects on body mass and condition but

Table 3

Repeated-measures ANOVA of nestling corticosterone levels measured at two different times (after brood-size manipulation and after food deprivation) in relation to dyad and experimental manipulations

	Mean sum of squares	df	F	p
Main effects				
(1) Dyad	965.9	19,104	5.06	<.001
(2) Brood-size manipulation	2570.6	1,104	13.48	<.001
(3) Food deprivation	1833.8	1,104	9.62	.002
(4) Time of sampling	20007.3	1,104	132.74	<.001
Interactions				
1 × 3	367.4	19,104	1.93	.019
1 × 4	340.5	19,104	2.59	.001
2 × 4	557.4	1,104	3.71	.056
3 × 4	1234.9	1,104	8.19	.005
1 × 3 × 4	341.6	19,104	2.27	.005

All main effects and interactions were initially included in the analyses. Nonsignificant terms were removed by backward selection. At each step, the term with the smallest effect size was eliminated (see Methods). Interaction between brood-size manipulation and time of sampling was included because of its marginally nonsignificant effect.

not on immune response. In our experiment, we decided to extend the stress episode over 2 h because previous observations showed that a 1-h period does not modify begging behavior and thus could not have represented an intense stress (unpublished data). Feeding rates in this species are high, because individual nestlings can be fed several times per hour (Saino and Møller, 1995, Saino et al., 1997, 2000), but variance in feeding rates can also be high. Therefore, some nestlings may not receive food for several tens of minutes, and a food-stress response may not arise during short spells of food deprivation.

In a second experiment, we showed that 2 h after the start of food deprivation, corticosterone levels were larger than those recorded after 1 h, and the increase from basal levels had no intermediate maximum. This pattern could thus be expected on the basis of feeding behavior of barn swallows.

In our experiments, we allowed for perception of sibling and parental behavior by all nestlings, because all nestlings were left in their nest. This procedure enhanced the realism of the experiments because acoustic and visual communication among food-deprived and non–food-deprived nestlings was preserved. Increase in corticosterone levels of food-deprived nestlings was larger than in non–food-deprived ones, indicating that food deprivation can affect corticosterone levels. The increase in corticosterone levels by non–food-deprived nestlings may have occurred in response to social stress caused by intense begging behavior by food-deprived nestlings and in response to blood sampling, although bleeding was very short and may have had little effect on corticosterone levels 2 h later.

Both kinds of stress affected circulating levels of corticosterone, although to a different extent. Long-term stress determined by an increased brood size resulted in a small significant increase in corticosterone levels. This pattern was predicted because corticosterone concentration in peripheral plasma is expected to increase rapidly after acute stress and then to decline almost to basal levels, even when stressful conditions persist. Hence, the small difference in corticoste-

rone levels between nestlings in enlarged compared with reduced broods before food deprivation may reflect repeated episodes of behavioral and/or food stress.

Food-deprived barn swallow nestlings beg for food at higher rates than do non–food-deprived nest mates (Saino et al., 2000; see also Sacchi et al., 2002). In a recent study, Kitaysky et al. (2001) have shown that experimentally increased corticosterone levels in altricial nestlings of the kittiwake (*Rissa tridactyla*) cause increased solicitation of parents to provide food. Hence, the effect of food deprivation on begging behavior may be mediated by increased levels of corticosterone.

The observation that smaller T cell–mediated immune response in enlarged broods was associated with large corticosterone plasma levels, together with the negative covariation between immune response and corticosterone levels after long-term stress, corroborates the idea of an immunodepressive effect of corticosterone (see Apanius, 1998; Evans et al., 2000; Poiani et al., 2000; von Holst, 1998). The lack of significant covariation between immune response and corticosterone levels after food deprivation could be predicted because an increase in corticosterone levels results in depressed immune function within days, rather than within hours (Apanius, 1998).

From a functional perspective, two not mutually exclusive adaptive explanations can be invoked for the observation of negative covariation between corticosterone levels and immune response (see Apanius, 1998; Råberg et al., 1998; Saino et al., 2002b). When nutritional resources are severely limiting, downregulation of the immune system may serve to reduce allocation of resources to costly physiological functions, such as immunity, which may not be contingently limiting survival, in favor of competing activities such as body maintenance and somatic growth (Apanius, 1998; Råberg et al., 1998). At the mechanistic level, the main hormones mediating the response to stress, including corticosterone, may depress processes that do not contribute to survival to the current specific "stressor" and allow for larger allocation to activities more directly critical to survival (Sapolsky, 1987). This so-called nutritional limitation hypothesis (see Råberg et al., 1998) is not consistent with our observation of a positive covariation between immune response and body mass after controlling for the effect of corticosterone levels, because this suggests that no trade-off existed between these two traits. However, it should be emphasized that T cell—mediated immune response and body mass are just two of many components of offspring state. Hence, there is ample scope for the existence of trade-offs between immunity and other traits affecting nestling viability.

An alternative perspective is based on the observation that under stress animals are more exposed to the risk of incurring hyperactivation of the immune system against self, with potentially detrimental autoimmunopathological effects (see Apanius, 1998; Råberg et al., 1998; Saino et al., 2002b; and references therein). Depression of immune response under long-term nutritional and behavioral stress determined by brood enlargement may thus function as an adaptation to reduce the risk of immunopathology. Again, the hormonal mechanism producing immune depression would be mediated by corticosterone. In this case, a different kind of trade-off, between effective adaptive immune response against parasites and risk of autoimmune response, may have occurred.

Negative covariation between body mass and somatic growth, and corticosterone levels has repeatedly, although not invariably, been documented in adult birds (see Kitaysky, 1999b; Schwabl 1995; Silverin and Wingfield, 1998; Silverin et al., 1997; for reviews, see von Holst, 1998; Wingfield et al., 1998). In the present study, we found marginally nonsignif-

icant evidence for such negative covariation, in ANCOVA in which the power of the test of the effect of corticosterone concentration was rather low. Hence, we cannot exclude that lack of significant covariation was due to a type II statistical error.

In conclusion, we have shown that natural forms of stress of different kinds increase circulating corticosterone levels in barn swallow nestlings. Long-term, but not short-term, stress results in compromised T cell–mediated immune function, possibly because of depression of immune function determined by large corticosterone levels. The present study therefore suggests that the negative effect of a large brood size on immune response, documented in some bird species, may be mediated by corticosterone. Hence, corticosterone may play a central role in the evolution and expression of trade-offs between components of fitness such as offspring number and quality.

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