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## Vitamin E supplementation enhances growth and condition of nestling barn swallows (*Hirundo rustica*)

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**Abstract** To optimize fitness, organisms may have to trade the number and quality of individual offspring against their own condition and survival. Limiting micronutrients such as antioxidants may be crucial to this trade-off. We investigated whether vitamin E, a major antioxidant in the diet of vertebrates, is limiting to barn swallow (*Hirundo rustica*) nestlings. We manipulated brood size to alter the intensity of sib-sib competition and supplemented nestlings with two different physiological doses of vitamin E while establishing a control group. Treatment effects were measured on body mass and size, feather growth, T cell-mediated immune response and hematocrit. Supplementation with vitamin E at intermediate physiological doses improved nestling mass and condition and feather growth, whereas higher physiological doses did not enhance offspring quality compared to a control treatment. The positive effects of vitamin E on body mass and condition were only detectable from days 6 to 10 when maximum growth rate is attained. Experimental enlargement of broods reduced body mass and size and T cell-mediated immune response only during the late nestling period. The effect of vitamin E supplementation did not depend on brood size manipulation, as revealed by the nonsignificant statistical interaction. This result contradicts the hypothesis that availability of vitamin E depends on intrabrood competition and instead suggests that it depends on concentration of vitamin E in the insect prey of swallows. Thus, antioxidants may be available in limited amounts to

barn swallow nestlings and such limitation affects growth. In addition, present results confirm that barn swallow parents trade progeny number against growth and immunity of individual offspring.

**Keywords** Antioxidants · Brood size · Growth · Immunity · Survival · Vitamin E supplementation

### Introduction

Life history theory is founded on the principle of allocation, which states that an increase in the amount of limiting energy and materials devoted to one process must result in decrease in allocation to other processes (Roff 1992; Stearns 1992). Competition for limiting resources between different demands implies that individuals should trade the benefits of resource allocation to one component of fitness against the negative consequences of reduced allocation to other fitness components. Natural selection is expected to have favored the evolution of life history strategies wherein individuals allocate limiting resources to maximize their lifetime inclusive fitness (Saino et al. 1999).

In species where parents provide extensive care, reproductive success may depend on competition among offspring for limiting parental resources and the conflicting demands for parental maintenance (Stearns 1992). Under limiting nutritional conditions, the costs of provisioning offspring with food may thus mediate diverse trade-offs between components of parental fitness.

As there can be a high energetic cost of rearing a brood (e.g., Moreno et al. 1997; Verhulst and Tinbergen 1997), parents that raise a large number of high quality offspring may allocate relatively large amounts of time and energy to parental activities (Saino et al. 1999). There is typically an increase in feeding effort after an experimental enlargement of brood size (Lessells 1993), which is, however, less than proportional to the number of nestlings (Nur 1984; Saino et al. 1997), implying that average offspring quality declines with increasing brood size. Therefore, there is a clear trade-off between nestling quality and number (e.g., Nur 1984;

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Smith et al. 1989). For example, brood size enlargement has negative effects on growth rate and survival (Clark and Wilson 1981; Smith et al. 1989), and can depress T cell-mediated immunocompetence of the offspring (Saino et al. 1997).

Limitation of food may be the major mechanism mediating the trade-off between progeny number and quality in birds. The quality of nutrients is crucial for the proper development of the chicks. A large protein intake, for example, is important to sustain the rapid growth birds experience during their early posthatching period, and for the development of immunity (Glick et al. 1983; Gershwin et al. 1985; Lochmiller et al. 1993). Poor nutritional conditions in terms of protein intake can result in a reduced T cell-mediated immune response (Gershwin et al. 1985; Lochmiller et al. 1993), while protein supplementation has an opposite effect (de Neve et al. 2004). In addition, dietary deficiencies of particular amino acids and vitamins (e.g., antioxidants) reduce immune function (Tsiagbe et al. 1987; McWhinney et al. 1989; Chew 1996; Haq et al. 1996; Friedman and Sklan 1997).

In precocial birds, it was established that during early postnatal development chicks' tissues are vulnerable to lipid peroxidation (Surai et al. 1996), and physiological mechanisms have evolved to reduce oxidative stress generated by free radicals (Halliwell 1994). The antioxidant system of the newly hatched chicks includes fat-soluble antioxidants such as vitamin E and carotenoids (Surai et al. 1996; Surai and Speake 1998). Vitamin E was shown to be required for normal development and functioning of the immune system (see Tengerdy et al. 1984). However, only  $\alpha$ - and  $\gamma$ -tocopherol appear to be retained in animal tissues to any great extent (Rock et al. 1996).  $\alpha$ -Tocopherol is the most abundant form in nature (Sheppard et al. 1993) and has the highest biological activity (Weiser et al. 1996). Several studies showed that vitamin E supplementation increases immune response in diverse domestic mammal and bird species (e.g., Ferket et al. 1995; Meydani 1995; Finch and Turner 1996). Vitamin E influences the development of the immune system in chickens (Marsh et al. 1986) by modulating a variety of its components, including cell-mediated and humoral responses, macrophage function and phagocytosis, antibody production (e.g., Franchini et al. 1986; Haq et al. 1996; Friedman et al. 1998), and resistance to disease (Finch and Turner 1996).

To date, however, no field experimental study has addressed the issue of limitation of dietary vitamin E and the effect of enhanced availability of tocopherols in the diet. Ninni (2003) proposed that there may be constraints on foraging that prevent barn swallows from capturing insects with high concentration of antioxidants. They based their conclusion on the fact that adult barn swallows brought to their nest insect prey with lower concentrations of antioxidants ( $\alpha$ - and  $\gamma$ -tocopherols and carotenoids) than randomly selected insects collected in the field.

The main aim of this study was to investigate whether vitamin E is limiting in the diet of barn swallow (*Hirundo rustica*) nestlings. We manipulated brood size soon after

hatching to either decrease or increase the intensity of sib-sib competition for food, and we then supplemented part of the chicks in both types of broods with two different physiological doses of vitamin E while establishing a control treatment. The effects of brood size manipulation and vitamin E supplementation were investigated by measuring body mass and size, feather growth, T cell-mediated immune response, and hematocrit. We predicted that (1) brood size reduction and supplementation with vitamin E would result in enhanced phenotypic quality (i.e., increased body mass and size, body mass corrected for body size, feather growth, and T cell-mediated immune response; no prediction was made for hematocrit) of the nestlings compared to brood enlargement and supplementation with a control solution; and (2) the effect of vitamin E supplementation would increase with dosage. In addition, we predicted that supplementation with vitamin E would have greater effects on nestlings from enlarged broods compared to reduced broods because nestlings in enlarged broods should be farther away from optimal nutritional conditions, and the same increase in dietary availability of vitamin E should therefore result in a larger increase in phenotypic quality.

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## Materials and methods

The study was carried out from April to July 2004 in eight colonies 30 km east of Milano (northwestern Italy). The nests were visited every day to record breeding events and identify synchronous broods to be cross-fostered. Two broods were considered synchronous when they hatched the same day or when the first chick from the second nest hatched the same day of the last chick from the first nest. When more than two synchronous broods were available, we matched those with equal or similar brood size. The difference in premanipulation brood size between the two broods that were cross-fostered was always  $\leq 2$  chicks. Cross-fostering between the two nests in a pair (hereafter "dyad") occurred when nestlings were 2 days old. We reciprocally transferred an unbalanced number of nestlings between the two broods in a dyad so that the postmanipulation brood size was either increased or decreased by one nestling. The number of nestlings due to be transferred from each brood was decided according to a predetermined scheme that aimed at minimizing the difference in the number of "resident" and "cross-fostered" nestlings within each brood after manipulation. Treatment of each brood in a dyad was randomized, as were nestlings due to be transferred to the foster nest. As cross-fostering was reciprocal, both broods in each dyad contained nestlings from the two nests of that dyad (for further details see Saino et al. 1999). Chicks were marked on the tarsus with nontoxic markers before cross-fostering to allow individual recognition.

After brood size manipulation chicks were assigned to vitamin treatments according to a randomization procedure whereby each chick, independent of its origin, was assigned to supplementation with either 1 or 2 SD of the

mean estimated vitamin E daily intake of nestling barn swallows, or to a control treatment that consisted of supplementation with the solvent of vitamin E (also see below).

Vitamin E doses could not be decided based on previous studies because these were done on domestic birds (mainly Galliformes), mice, pigs, or humans; they often involved elevated doses and were carried out for different purposes. We rather aimed at supplementing nestlings with doses of the vitamin that elevated their intake within the natural range of variation, based on estimates of vitamin E content of insects on which barn swallows feed.

Thus, we first retrieved the data on vitamin E concentration in barn swallow chick food, kindly provided by P. Ninni (unpublished data) (also see Ninni 2003). According to these data, the mean (SD) concentration of tocopherols is:  $\alpha$ -tocopherol, 3.93  $\mu\text{g/g}$  (3.81);  $\gamma$ -tocopherol, 0.51  $\mu\text{g/g}$  (0.38);  $n=29$  feeding boli provisioned by parents to their chicks.

Secondly, we estimated the amount of insects that nestlings of this species should eat to satisfy their minimal requirements at different ages. It is rarely possible to directly measure daily food intake (DFI) in the field by monitoring focal individuals without substantially interfering with parental behavior, particularly in species in which offspring are provisioned up to several times per hour with relatively small amounts of food, like the barn swallow. Therefore, we decided to estimate the DFI based on the information on the energetic values of wildlife foods and the efficiency with which they are assimilated, combined with daily energy expenditure (DEE) data. We estimated DFI following Hart (2002) who based his estimations on studies using the doubly labeled water method. From an extensive review of the metabolic rates of free-living birds (and mammals), Hart (2002) generated a set of allometric equations linking body mass with DEE. Hart (2002) found a strong relationship between body mass and DEE with significant differences between taxonomic groups. The general form of the equation is:  $\log(\text{DEE}) = \log a + b \times \log \text{body mass}$ . They calculated a separate equation for insectivorous passerines, for which  $\log a=1.0017$  and  $b=0.7034$ . Consequently, we applied the algorithm  $\log(\text{DEE}) = 1.0017 + 0.7034 \times \log \text{body mass}$  using the mean body mass for every age of a large sample of chicks derived from our data collected in previous years in the same area.

DEE estimates were used to calculate the DFI based on the average energy and moisture content of the food type, correcting for the species' assimilation efficiency. Hart (2002) collated information on the energy and moisture contents of various wildlife foods and data on the efficiency with which free-living animals digest food. They determined that arthropods contain an average of 21.9 kJ/g dry weight and consist of 70.5% water. Therefore, arthropods contain 6.5 kJ/g fresh weight. On average, passerines only manage to make use of 76% of the energy in animal foods. Afterward, we made an adjustment for the necessities of altricial developing birds because they require additional energy to allocate to growth (Wijnandts

1984 in Klasing 1998), which decreases in relation to body mass as chicks age. Thus, our estimate resulted in a surplus of 79% in 2-day-old chicks, decreasing linearly to 37% in day 12. As an example, the mean mass of 2-day-old chicks is 3.42 g. Applying the algorithms described above yields 4.83 g, plus 79% to allocate to growth, results in a total of 8.65 g of insects.

Finally, we mixed two sources of tocopherols: Mixed Tocopherols 95 (Roche Vitamins, Parsippany, NJ, USA) and DL- $\alpha$ -tocopheryl acetate (F. Hoffmann, La Roche, Basle, Switzerland), reproducing the ratio of the SD between  $\alpha$ -tocopherol and  $\gamma$ -tocopherol in insects eaten by barn swallow chicks (3.81:0.38). As DL- $\alpha$ -tocopheryl acetate is a synthetic derivative composed of equal amounts of all the stereoisomers, it is less active. For that reason we made an adjustment to reach the biological activity of natural  $\alpha$ -tocopherol with DL- $\alpha$ -tocopheryl acetate, multiplying by 1.49 (following Weiser and Vecchi 1981) the amount required. We did not need any corrections for  $\gamma$ -tocopherol because it was obtained from a natural source.

Finally, we produced two solutions using corn oil as an excipient: 1 SD (9.29 ml tocopherols/1 l corn oil) and 2 SD (18.58 ml/l). We fed the chicks with predetermined volumes of either of these two solutions, or with solely the excipient oil as a control. Those quantities were established according to the estimated daily intake per age (see above). Thus, the amount of solution provided to the nestlings was 5  $\mu\text{l}$  at day 2, 8  $\mu\text{l}$  at day 4, 10  $\mu\text{l}$  at day 6, 11  $\mu\text{l}$  at day 8, 12  $\mu\text{l}$  at day 10, and 14  $\mu\text{l}$  at day 12. To allow comparison with other studies, the doses we administered as 1 SD at day 2 corresponded to 13.6 mg/kg of nestling mass. Vitamin E or the control solution was supplied every second day rather than every day to reduce the frequency of visits to the nests and thus disturbance.

We considered 54 dyads and 105 broods (3 broods were excluded because all nestlings died at very early life stages, apparently not because of the treatments). Morphological measurements at day 6 after hatching were available for a total of 452 nestlings (194 in reduced broods and 258 in enlarged broods): 152 of these nestlings were provisioned with oil, 151 with the 1 SD dose, and 149 with the 2 SD dose of vitamin E. At day 12 we had information on morphology of 180 nestlings in reduced broods and 245 nestlings in enlarged broods. Of these, 143 received the control solution, 142 the 1 SD dose, and 140 the 2 SD dose of vitamin E. Finally, at day 17 the sample consisted of 167 nestling in reduced broods and 230 nestlings in enlarged broods. Sample sizes according to vitamin E treatment were 129 in the control group, 136 in the 1 SD group, and 132 in the 2 SD group.

We checked whether vitamin E supplementation raised the levels of  $\alpha$ -tocopherol in the plasma of a subsample of 12-day-old chicks by comparing siblings exposed to different treatments but raised in the same nest. This approach has the advantage that all the nestlings compared were exposed to similar environmental conditions. The samples to be analyzed were chosen according to common parentage and availability of plasma. However, because not

all the groups of siblings raised in the same nest contained individuals assigned to all the experimental treatments, the comparisons between pairs of treatments are based on partly different broods.  $\alpha$ -Tocopherol concentration was assayed by high-performance liquid chromatography according to the methods outlined in Vuilleumier et al. (1983). The control nestlings had significantly smaller concentration of  $\alpha$ -tocopherol than the nestlings provisioned with the 1 SD dose [mean (SE) for controls: 2.20 (0.31)  $\mu\text{g/ml}$ ; 1 SD nestlings: 3.21 (0.39)  $\mu\text{g/ml}$ ;  $t$  test for paired data:  $t_{15}=2.83$ ,  $P=0.013$ , and  $n=16$  pairs of nestlings] or the 2 SD dose [mean (SE) for controls: 2.01 (0.13)  $\mu\text{g/ml}$ ; 2 SD nestlings: 2.67 (0.34)  $\mu\text{g/ml}$ ;  $t$  test for paired data:  $t_{14}=2.43$ ,  $P=0.029$ , and  $n=14$  pairs of nestlings]. However, there was no significant difference between nestlings from the 1 SD or 2 SD groups [mean (SE) for 1 SD: 2.54 (0.25)  $\mu\text{g/ml}$ ; 2 SD nestlings: 2.46 (0.28)  $\mu\text{g/ml}$ ;  $t$  test for paired data:  $t_{14}=0.29$ ,  $P=0.78$ , and  $n=14$  pairs of nestlings].

Thus, while supplementation with  $\alpha$ -tocopherol resulted in an increase in circulating levels of the vitamin, this increase was not dose-dependent. This suggests that either administration of the largest dose did not result in an increase in the amount of vitamin that was absorbed or that increased absorption resulted in larger accumulation in storage organs such as the liver (Surai 2003). Another nonexclusive explanation is that there seems to be a replacement of circulating  $\alpha$ -tocopherol so that when new vitamin E is absorbed, it displaces old vitamin E due to a rough homeostasis of this vitamin in plasma. As a consequence, higher doses of vitamin E produce relatively small increases in total tocopherols in plasma (see Burton et al. 1998).

### Morphological measurements

The chicks were weighed to the nearest 0.1 g (TANITA electronic balance) every second day from day 2, when cross-fostering occurred, to day 12 and then again on day 17. The birds were ringed on day 6. On day 6 and 12 both tarsi were measured using a digital caliper (approximation 0.01 mm). On day 12 and 17 the length of the eight primaries (descendant) and outermost tail feathers of both sides were measured with a ruler (approximation 0.5 mm). On day 12, T cell-mediated immune response was measured according to a standard *in vivo* test (Lochmiller et al. 1993; Saino et al. 1997). The thickness of both wing webs was first measured using a pressure-sensitive micrometer (approximation 0.01 mm). The web of the left wing was then injected with 0.05 ml of phytohemagglutinin (PHA) in phosphate-buffered saline (PBS) in a concentration of 0.5 mg PHA/0.1 ml PBS, while the right wing web was injected with the same amount of only PBS to serve as a control. After 24 h, the thickness of both wing webs at the inoculation sites was measured again. The difference between the change in thickness of the left (PHA) and the right (PBS) wing web was used as an index of T cell-mediated immune response. Fault bars in all tail

feathers were counted on day 13. Fault bars are the result of a variable time lag on the deposition of keratin (Murphy et al. 1989), producing absence or slimming of barbules appearing as narrow translucent bars across the feathers. They are considered to be symptoms of a variety of stressors, typically malnutrition (Slagsvold 1982; Newton 1986), experienced during feather formation. We measured chicks for the last time at day 17 because approaching nests after this critical age can increase mortality by inducing young to fly away when they are still not experienced and not able to return to the nest.

At days 12 and 17 a blood sample was taken in heparinized capillaries from all nestlings. The capillaries were centrifuged for 10 min at 11,500 rpm to measure hematocrit. Hematocrit was expressed as the proportion of blood volume occupied by packed cells.

Measures of bilateral characters were expressed as the mean of the two sides. To analyze body "condition," we used body mass data while entering tarsus length as an index of body size in analyses of covariance.

Because we measured every character only once, we cannot assess measurement repeatabilities. For this reason (and as the analysis were made entering the mean of both sides) we calculated repeatabilities comparing the two sides in the analysis. This procedure underestimates actual repeatabilities because they are confounded by fluctuating asymmetry. Repeatabilities (according to Lessells and Boag 1987) in morphological traits were high and the variance between subjects was significantly greater than the variance within subjects (one-way ANOVA SPSS 13.0) (tarsus day 6:  $n=303$ ,  $r=0.971$ ,  $F=140.03$ , and  $P<0.0001$ ; tarsus day 12:  $n=284$ ,  $r=0.904$ ,  $F=39.83$ , and  $P<0.0001$ ; rectrix day 12:  $n=284$ ,  $r=0.970$ ,  $F=130.55$ , and  $P<0.0001$ ; rectrix day 17:  $n=266$ ,  $r=0.983$ ,  $F=231.91$ , and  $P<0.0001$ ; wing day 12:  $n=283$ ,  $r=0.981$ ,  $F=208.38$ , and  $P<0.0001$ ; and wing day 17:  $n=271$ ,  $r=0.981$ ,  $F=203.83$ , and  $P<0.0001$ ). We do not provide repeatabilities for the thickness of the wing web. We measured very meticulously the wing web only once, because, being a soft tissue, it remains slightly deformed after the first time the micrometer is placed, subsequently resulting in lower values of thickness (personal observation).

### Statistical analyses

SAS software was used to perform nested ANOVAs through the general linear model procedure. Each model included treatments (three levels of vitamin E supplementation: control, 1 SD, and 2 SD, described earlier; and two levels of brood size manipulation: enlarged and reduced) as fixed effect factors with brood of origin as a random factor nested within dyads, and dyad as a random factor. The effect of dyad was tested against the error term of brood of origin nested within dyad, whereas the effect of brood of origin nested within dyad was tested against the residual error term, following Zar (1999, Appendix 7). Parsimonious models were obtained by a step-down procedure of exclusion of terms that did not significantly contribute to



the models. At each step, the term that gave the smallest contribution (largest  $P$  value) was excluded. Interactions were excluded before the relevant main effects.

Phenotypic values of traits that were measured repeatedly during nestling growth (e.g., body mass) were analyzed for each age separately, rather than using a repeated-measures design. The reasons for this are as follows. First, we did not know when the vitamin E treatment might affect nestling characteristics. Thus, including data on, for example, body mass or tarsus length measured soon after the start of the treatment could generate confusion in the analyses because the treatments were still ineffective. Secondly, we were particularly interested in phenotypic values at particular ages. For example, we were interested in analyzing body mass at day 12 when maximum mass is attained before mass recession starts and at day 17, just before fledging, because this may affect chances of survival of fledglings during the critical postfledging period. Similarly, we were mainly interested in body size at day 12 rather than day 6 because the former variable reflects final body size. Thus, even if we had adopted a repeated measures design, we would still have to run the analyses on particularly interesting focal ages.

We performed an analysis of offspring survival to check for bias in our results related to differential mortality among treatments. This analysis was performed using MLWIN 1.10.0006 with the same model structure as for the analyses of phenotypic data. Binary data were transformed by the logit link function and analyzed assuming binomial error distribution at the individual level. Model parameters were estimated by second order penalized quaslikelihood estimation. Significance was tested using the Wald statistic, which follows a  $\chi^2$  distribution.

The statistical significance level was set at  $\alpha < 0.05$ . In analyzing our data set, we ran multiple tests on different traits measured on the same nestlings. This may have inflated the risk of incurring type I statistical errors. However, it was shown that studies in the field of behavioral ecology are characterized by extremely low power of the statistical tests, resulting in increased probability of incorrectly accepting the null hypothesis when it is false, i.e., of incurring type II statistical errors (Jennions and Møller 2003). A commonly adopted procedure to reduce the risk of type I statistical errors is (sequential) Bonferroni correction, which basically consists of lowering the significance threshold according to the number of tests performed. It was pointed out that this can elevate the risk of type II statistical errors to unacceptably high levels, suggesting that Bonferroni correction should be avoided (Nakagawa 2004). We therefore decided not to apply Bonferroni correction, following Nakagawa (2004).

## Results

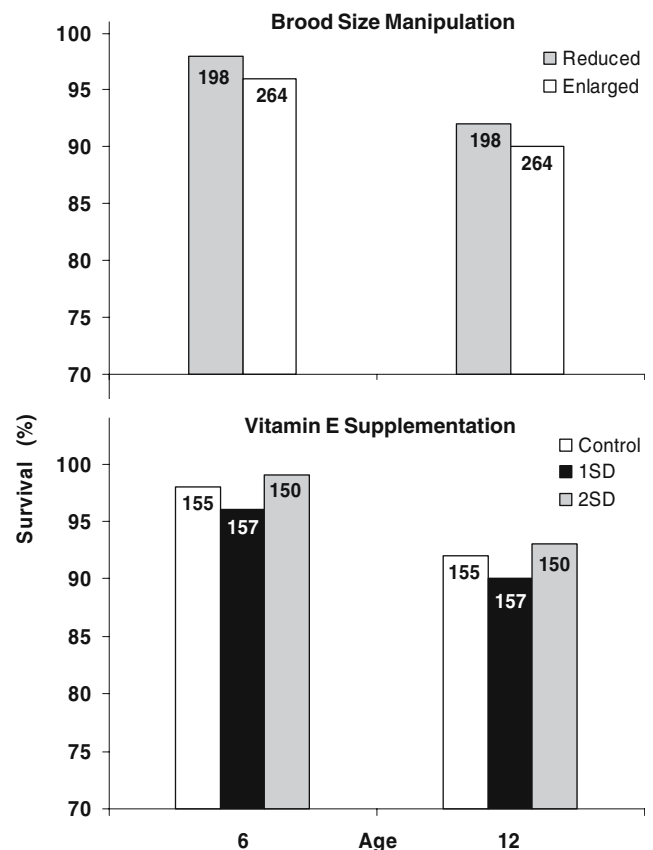
Nestling survival until day 6, 12, or 17 was not significantly influenced by brood size manipulation or vitamin E treatments (brood size manipulation: Wald  $\chi^2 < 2.34$  and  $P > 0.13$ ; vitamin E supplementation: Wald

$\chi^2 < 1.30$  and  $P > 0.25$ ). However, survival data until day 17 may have been partly confounded by early fledging particularly from reduced broods. For this reason, only survival data of days 6 and 12 are presented in Fig. 1.

All phenotypic characters, except body mass at days 4 and 6, were affected by the dyad (Tables 1, 2, and 3; see “Materials and methods” for sample sizes). All phenotypic values of the nestlings were significantly influenced by their origin, except T cell-mediated immune response (Tables 1, 2, and 3). In all analyses of phenotypic traits, the interaction between brood size manipulation and vitamin E supplementation was removed because it did not significantly contribute to step-down models.

### Body mass and condition

Brood size manipulation and vitamin E supplementation started on day 2. As expected, because chicks were randomly assigned to treatments, at that age there were no significant differences in body mass among experimental groups (vitamin E supplementation:  $F_{2,355} = 0.22$  and  $P = 0.80$ ; brood size manipulation:  $F_{1,355} = 0.24$  and  $P = 0.63$ ), implying that randomization actually produced



**Fig. 1** Percentage of nestlings that survived until ages 6 or 12 in relation of brood size manipulation (reduction or enlargement by one nestling) or vitamin E supplementation: 1 SD=9.29 ml tocopherols/1 l corn oil and 2 SD=18.58 ml/l (see “Materials and methods” for details). Numbers inside the columns denote the number of nestlings at the start of the experiment at day 2

**Table 1** Nested analysis of variance of body mass and condition

	Body mass			Condition		
	<i>F</i>	<i>df</i>	<i>P</i>	<i>F</i>	<i>df</i>	<i>P</i>
Day 4						
Dyad	1.23	50, 50	0.2373	3.22	50, 50	<0.0001
Nest of origin (dyad)	4.95	50, 353	<0.0001	4.17	50, 347	<0.0001
Tarsus length	–	–	–	535.78	1, 347	<0.0001
Day 6						
Dyad	1.54	50, 50	0.0646	5.48	50, 50	<0.0001
Nest of origin (dyad)	3.39	50, 351	<0.0001	2.96	50, 346	<0.0001
Vitamin E supplementation	–	–	n.s.	4.67	2, 346	0.01
Tarsus length	–	–	–	749.96	1, 346	<0.0001
Day 8						
Dyad	2.56	50, 50	0.0006	7.09	50, 50	<0.0001
Nest of origin (dyad)	2.30	50, 333	0.0001	2.08	50, 329	<0.0001
Vitamin E supplementation	3.32	2, 333	0.0375	4.92	2, 329	0.0078
Brood size manipulation	–	–	n.s.	5.01	1, 329	0.0259
Tarsus length	–	–	–	432.78	1, 329	<0.0001
Day 10						
Dyad	3.52	50, 50	<0.0001	3.74	50, 50	<0.0001
Nest of origin (dyad)	1.40	50, 327	0.046	1, 62	50, 320	0.0074
Vitamin E supplementation	–	–	n.s.	3.77	2, 320	0.0241
Brood size manipulation	24.68	1, 327	<0.0001	19.09	1, 320	<0.0001
Tarsus length	–	–	–	32.54	1, 320	<0.0001
Day 12						
Dyad	4.79	50, 50	<0.0001	5.00	50, 50	<0.0001
Nest of origin (dyad)	1.22	50, 323	0.1556	1.45	50, 321	0.032
Brood size manipulation	30.98	1, 323	<0.0001	25.86	1, 321	<0.0001
Tarsus length	–	–	–	47.43	1, 321	<0.0001
Day 17						
Dyad	2.98	49, 50	<0.0001	2.98	49, 50	<0.0001
Nest of origin (dyad)	1.88	50, 296	0.0007	1.88	50, 296	0.0007
Brood size manipulation	11.58	1, 296	0.0008	11.58	1, 296	0.0008

The step-down model including only significant terms. Tarsus length was entered as a covariate of body mass to analyze body condition. Tarsus length was not entered as a covariate in the analyses of body mass (dashes). See “[Materials and methods](#)” for sample sizes at ages 6, 12, and 17

**Table 2** Nested analysis of variance of tarsus, longest (eight) primary, and rectrix length

	Tarsus length			Wing feathers			Tail feathers		
	<i>F</i>	<i>df</i>	<i>P</i>	<i>F</i>	<i>df</i>	<i>P</i>	<i>F</i>	<i>df</i>	<i>P</i>
Day 6									
Dyad	3.50	50, 50	<0.0001	–	–	–	–	–	–
Nest of origin (dyad)	2.49	50, 349	<0.0001	–	–	–	–	–	–
Day 12									
Dyad	3.61	50, 50	<0.0001	2.16	50, 50	0.0037	1.92	50, 50	0.0115
Nest of origin (dyad)	2.64	50, 323	<0.0001	3.16	50, 321	<0.0001	3.28	50, 322	<0.0001
Vitamin E supplementation	–	–	–	3.15	2, 321	0.0441	3.50	2, 322	0.0314
Brood size manipulation	5.47	1, 323	0.02	–	–	–	–	–	–
Day 17									
Dyad	–	–	–	3.03	49, 50	<0.0001	2.16	49, 50	0.0038
Nest of origin (dyad)	–	–	–	2.07	50, 296	0.0001	2.85	50, 295	<0.0001
Brood size manipulation	–	–	–	–	–	–	4.44	1, 295	0.0359

The step-down model including only significant terms is reported. The spaces with a dash denote that the variable was removed from the model because it was not significant. See “[Materials and methods](#)” for sample sizes at ages 6, 12, and 17

**Table 3** Nested analysis of variance of hematocrit and T cell-mediated immune response

	Hematocrit			T cell-mediated immune response		
	<i>F</i>	<i>df</i>	<i>P</i>	<i>F</i>	<i>df</i>	<i>P</i>
Day 12						
Dyad	3.50	50, 50	<0.0001	6.98	49, 50	<0.0001
Nest of origin (dyad)	2.49	50, 349	<0.0001	–	–	–
Brood size manipulation	–	–	–	7.71	1, 321	0.006
Day 17						
Dyad	2.08	49, 50	0.006	–	–	–
Nest of origin (dyad)	2.62	50, 287	<0.0001	–	–	–
Brood size manipulation	10.38	1, 287	0.001	–	–	–

The step-down model including only significant terms is reported. See “Materials and methods” for sample sizes at ages 6, 12, and 17

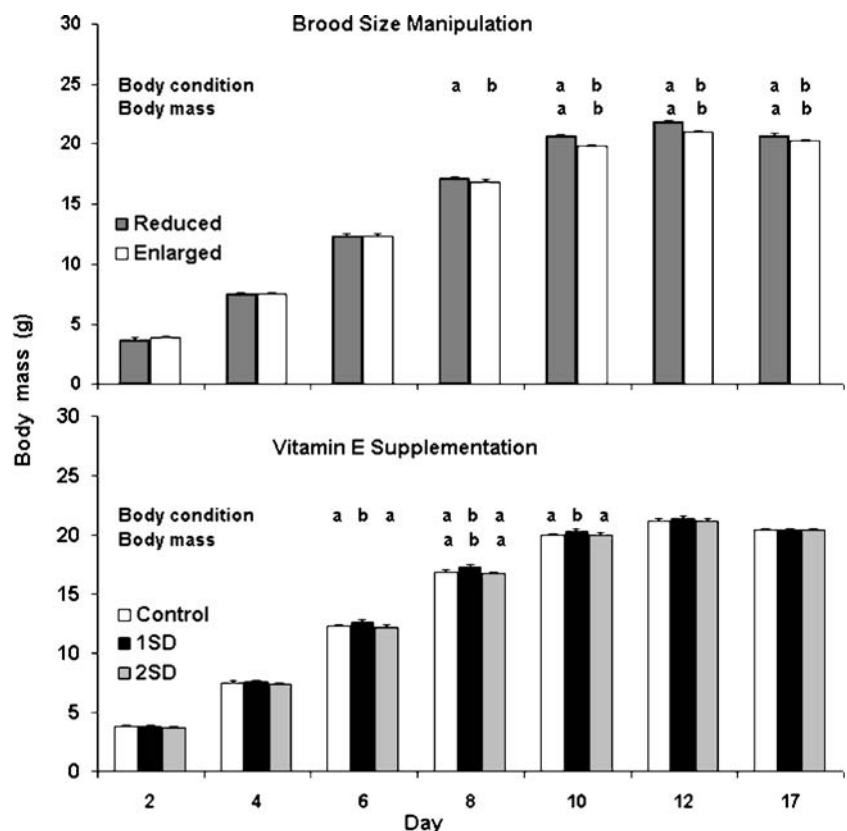
groups of nestlings that were homogeneous with respect to body mass.

Body mass was significantly affected by vitamin E supplementation only at day 8 (Table 1). However, a significant pairwise difference existed only between the 1 SD and the 2 SD groups with 1 SD nestlings being, on average, 3.0% heavier than 2 SD ones. Brood enlargement had a significant negative effect on body mass at all ages from day 10 (Table 1 and Fig. 2). At this age, nestlings from reduced broods had, on average, 4.5% larger body mass compared to those from enlarged broods but that gap decreased to 2.0% at day 17.

Body condition, expressed as body mass while controlling for tarsus length, was affected by vitamin E

supplementation at days 6, 8, and 10. Nestlings from the 1 SD group were invariably heavier than those from the control or 2 SD groups (Fig. 2). This enhancement in body condition was around 1.8% compared to controls or 2.8% compared to 2 SD nestlings. Body condition was not significantly affected by brood size manipulation until nestlings reached day 8, when nestlings from reduced broods were in 1.2% better condition than those in enlarged ones. Body condition was always significant until day 17 (Table 1 and Fig. 2). Nestlings from reduced broods attained the greatest advantage, compared with those in enlarged broods, at age 12 when they were in 3.3% better condition, dropping to 2.0% at day 17.

**Fig. 2** Mean (+SE) body mass of the nestlings for the brood size manipulation or vitamin E supplementation groups: 1 SD=9.29 ml tocopherols/1 l corn oil and 2 SD=18.58 ml/l (see “Materials and methods” for details). *Superscript letters* indicate significant differences between doses: groups not designated by the same *letter* differed significantly in body mass or body condition (body mass corrected for body size in ANCOVA; see “Materials and methods”) in Tukey pairwise tests. See “Materials and methods” for sample sizes at ages 6, 12, and 17. It should be noticed that the magnitude of the effects of vitamin E supplementation was very small



## Morphometric characters

Vitamin E supplementation had no obvious effect on tarsus length (Table 2 and Fig. 3). However, at day 12, when the tarsus is fully grown, brood size manipulation had a significant effect on tarsus length as nestlings from enlarged broods had shorter tarsi than those from reduced broods (Table 2 and Fig. 3).

At day 12, length of wing and tail feathers was not affected by brood size manipulation, whereas it significantly varied among vitamin E groups. Chicks supplemented with 1 SD dose had longer wing feathers and rectrices than those from the 2 SD and control groups (Table 2 and Fig. 4).

At day 17, the effects of vitamin E supplementation were no longer significant. However, nestlings in reduced broods had longer rectrices than those in enlarged broods, whereas there was no significant variation in wing length in relation to brood size manipulation (Table 2 and Fig. 4).

The number of fault bars on the rectrices did not vary in relation to experimental treatments. However, it varied among dyads ( $F_{49,50}=1.71$  and  $P=0.031$ ) and was significantly affected by the origin of the nestlings ( $F_{50,318}=1.83$  and  $P=0.001$ ). The count of fault bars can be affected by the absolute length of the feathers. However,

when we repeated this analysis while including rectrix length as a covariate we obtained qualitatively similar results (details not reported here).

## Hematocrit and T cell-mediated immune response

Brood size manipulation influenced hematocrit on day 17, when nestlings in enlarged broods, had lower values than those from reduced broods, but not on day 12, whereas vitamin E supplementation had no effect on hematocrit (Table 3 and Fig. 5). T cell-mediated immune response was measured at day 13 and was found to be larger in nestlings from reduced compared to enlarged broods, but we found no effect of vitamin E supplementation.

Body mass covaried positively with immune response and hematocrit when the chicks were 12 days old ( $F_{1,317}=6.11$ ,  $P=0.014$ ,  $B=0.0043$ , and  $SE=0.0174$ ;  $F_{1,320}=6.93$ ,  $P=0.009$ ,  $B=0.0036$ , and  $SE=0.0013$ ). However, there was no significant covariation between hematocrit and body mass on day 17 ( $F_{1,317}=6.11$  and  $P=0.24$ ).

## Discussion

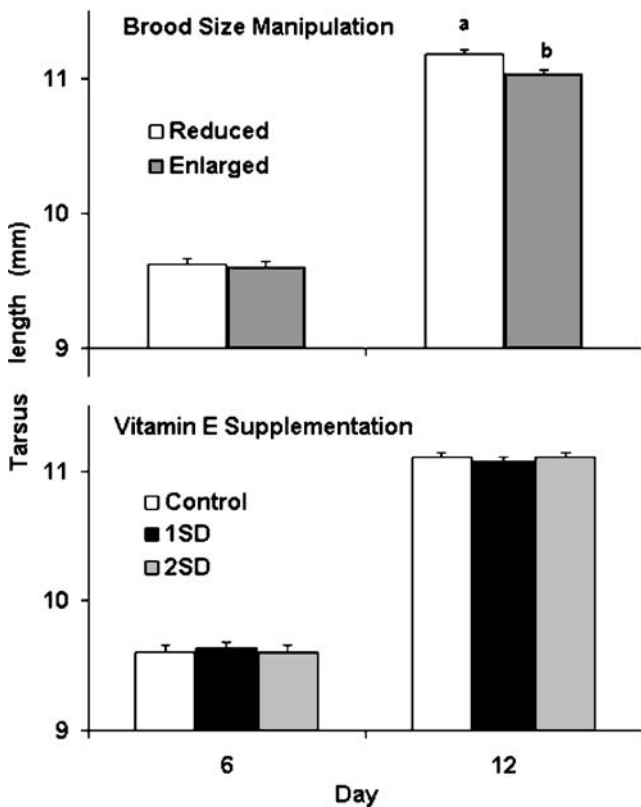
For the first time in any bird species under field experimental conditions, this study shows that body mass and condition and feather growth are influenced by availability of dietary vitamin E during the nestling period, suggesting that this important nutritional component is limiting to barn swallow nestlings. In addition, the present study confirms previous findings for the same model species that experimental manipulation of brood size affects morphology, physiology, and immune response of the nestlings.

Three aspects of the results from the vitamin E supplementation protocol deserve particular attention:

- 1) We expected a positive monotonic relationship between the physiological dose of vitamin E administered to the nestlings and the extent of its beneficial effects. Our results showed that vitamin E supplementation at 1 SD of the mean estimated daily intake enhanced the phenotypic values of the nestlings, whereas supplementation with 2 SD had no effect compared to the control treatment;
- 2) The positive effects of the intermediate dose were noticeable at day 6 and disappeared after day 10 for body mass and condition, and day 12 for feather growth;
- 3) The effect of vitamin E supplementation did not depend on that of brood size manipulation, as demonstrated by the lack of effect of the interaction between the two treatments.

Below we will discuss each of these findings.

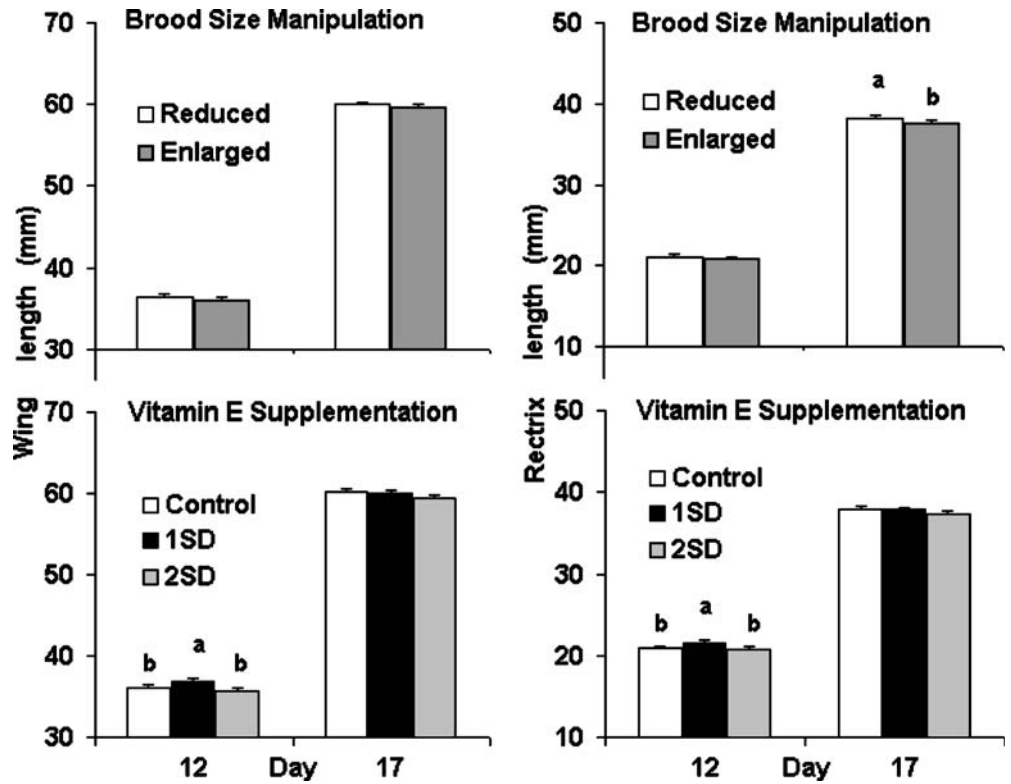
The nonlinear effects might be a consequence of interactions between vitamin E and other nutrients, especially vitamins. In birds, dietary levels of vitamins A and E significantly affect the utilization of dietary vitamin



**Fig. 3** Mean (+SE) tarsus length of the nestlings for the brood size manipulation or vitamin E supplementation groups: 1 SD=9.29 ml tocopherols/1 l corn oil and 2 SD=18.58 ml/l (see “Materials and methods” for details). *Superscript letters* indicate significant differences between doses: age groups not designated by the same *letter* differed significantly in Tukey pairwise tests. See “Materials and methods” for sample sizes at ages 6 and 12



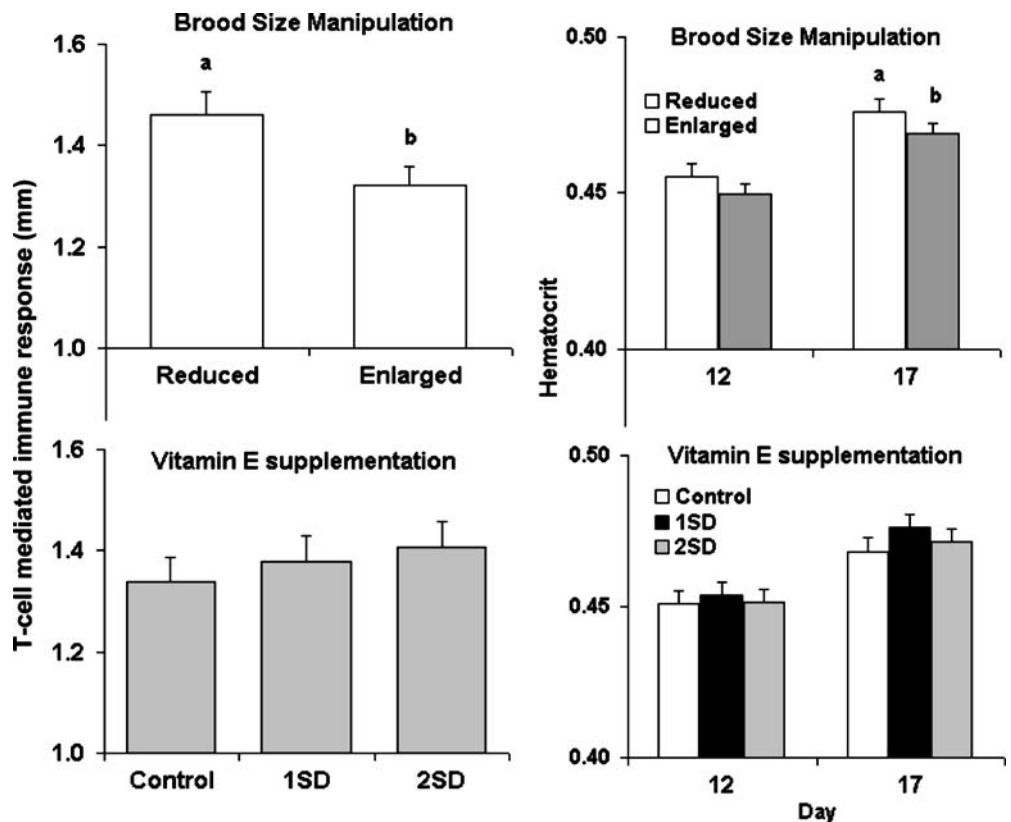
**Fig. 4** Mean (+SE) length of wing or tail feathers for the brood size manipulation or vitamin E supplementation groups: 1SD=9.29 ml tocopherols/1 l corn oil and 2SD=18.58 ml/l (See methods for details). Superscript letters indicate significant differences between doses: age groups not designated by the same letter differed significantly in Tukey pairwise tests. See “Materials and methods” for sample sizes at ages 12 and 17. Notice that y-axes do not start at zero



D3 with consequences, for example, on body mass (Murphy et al. 1981; Aburto and Britton 1998). High levels of vitamin E, A, or D3 affect growth and bone metabolism, weakening calcification (March et al. 1973).

In addition, high doses of vitamin E may displace other fat-soluble antioxidants in humans (Huang and Appel 2003), disrupting antioxidant systems and increasing vulnerability to oxidative damage (Miller et al. 2005). This alteration of

**Fig. 5** Mean (+SE) (expressed as the wing web swelling response; see “Materials and methods”) and hematocrit for the brood size manipulation or vitamin E supplementation groups: 1 SD=9.29 ml tocopherols/1 l corn oil and 2 SD=18.58 ml/l (See “Materials and methods” for details). Superscript letters indicate significant differences between doses: groups not designated by the same letter differed significantly in Tukey pairwise tests. See “Materials and methods” for sample sizes



the overall balance of antioxidants seems to be responsible for the impairments of different components of the immune system at high levels of vitamin E, although moderate doses improve immunity in chicks and turkeys (e.g., Friedman et al. 1998; Leshchinsky and Klasing 2001).

In addition, vitamin E was shown to enhance erythropoiesis. Rana et al. (1992) demonstrated that poultry chicks treated with vitamin E revealed lower depression in hematocrit values when exposed to gamma-irradiation, in comparison to those without vitamin E treatment. Vitamin E also has anticoagulant properties, possibly by interfering with vitamin K-dependent clotting mechanisms (Dowd and Zheng 1995). Nevertheless, we failed to find any significant effect in the physiological parameters measured as a result of vitamin E supplementation. In agreement with our findings, Tras et al. (2000) found no effects on any hematological parameters in broilers with vitamin E plus selenium supplementation treatment. In addition, we did not observe any symptoms revealing possible toxicity effects of vitamin E supplementation. The risk of adverse consequences from excess intake of vitamin E appears to be low, even at the highest experimental doses (Meydani et al. 1998).

Vitamin E supplementation was also effective only at intermediate stages during the nestling period. Growth of barn swallows, as in many other vertebrates, approximates a logistic pattern, at least until day 12 when pre fledging mass recession starts and skeletal growth is completed. Maximal growth rate is attained around day 8, i.e., when the effect of vitamin E was detected. Thus, additional vitamin E may have positive effects only during maximal growth, perhaps because metabolic rates and free radical production attain their highest level (Loft et al. 1994). Unfortunately, long-term consequences of the effects of vitamin E are hard to investigate because of low local recruitment in our study population.

Thirdly, we found no significantly larger effect of vitamin E supplementation on phenotypic values of nestlings from enlarged compared to reduced broods. Nestlings in enlarged broods are known to receive a smaller amount of food per capita from previous studies of the barn swallow (Saino et al. 1997, 2000) and of other passerines. In fact, nestlings in enlarged broods in the present study were lighter and had lower T cell-mediated immune response, suggesting that brood enlargement impaired nutritional conditions. It was shown that vitamin E is found at lower concentrations in the insect food of the barn swallows than in the aerial insect fauna of barn swallow habitats, suggesting that ecological or behavioral constraints prevent barn swallows from acquiring vitamin E-rich food (Ninni 2003). Thus, the lack of a differential effect of vitamin E supplementation on nestlings in enlarged vs reduced broods suggests that the low concentration of vitamin E in insects fed to nestlings limits growth and other physiological activities of all nestlings, regardless of brood size.

Brood size manipulation affected body mass and condition with nestlings in reduced broods being heavier and in better condition than those in enlarged ones, which is consistent with previous findings (Saino et al. 1997,

2002). The chicks reared in enlarged broods were therefore probably subjected to nutritional stress, which became apparent with time after day 8 for body condition and after day 10 for body mass, lasting at least until a few days before fledging when we measured the nestlings for the last time. Brood size manipulation also had an effect on morphometric characters, such as tarsus and rectrix length. Chicks from enlarged broods had shorter tarsi at day 12 when tarsus is fully developed in length and shorter rectrices at day 17. The effect of brood size manipulation on tarsus length is novel, as no significant effect of brood size manipulation with the same procedure adopted in the present study was found in previous studies of the same population (e.g., Saino et al. 1997). It is important to note that in our study population, tarsus length predicts the probability of local recruitment of both sexes (N. Saino, in preparation). Local recruitment into the breeding population, in turn, was often assumed to reflect viability of young passerines (e.g., Pettifor et al. 2001; Schmoll et al. 2003). Thus, the present study suggests that parent barn swallows trade offspring number with quality, in terms of body size, which predicts offspring viability.

Wing feathers, in contrast, were not affected by brood size manipulation. A possible explanation is that stressed individuals can shunt resources from traits that are less important to fitness than other features so that detrimental effects on overall fitness are minimized (Schew and Ricklefs 1998). Wing, rather than tail or skeletal, development may be more important for offspring viability in the post fledging period. Similar results were obtained by Searcy et al. (2004) in an experiment on song sparrows (*Melospiza melodia*) where the nutritional regime was manipulated.

In the present study, T cell-mediated immune response was also significantly smaller in nestlings from enlarged broods, consistent with previous findings on the barn swallow and other species from experiments of brood size manipulation (e.g., Saino et al. 1997, 1999; de Neve et al. 2004). Finally, hematocrit was negatively influenced by brood enlargement, suggesting that nutritional stress reduced erythropoiesis.

Neither of the treatments had an effect on the number of fault bars in the rectrices. The strong effect of dyad on the number of fault bars was probably due to weather conditions and/or seasonality. It is interesting to note that there was a strong and statistically significant effect of the nest of origin on this character, suggesting an innate component in this feature. Nevertheless, these growth abnormalities in the feathers are considered to be symptoms of some kind of stress such as malnutrition (Slagsvold 1982; Newton 1986), handling (King and Murphy 1984; Murphy et al. 1988), or other mechanical stress experienced during feather formation (Murphy et al. 1989; Negro et al. 1994). Fault bars were used as an index of "stress," "quality," or "body condition" in several studies (e.g., Bortolotti et al. 2002; Sodhi 2002).

Both nests in each dyad contained nestlings from each of the nests in that dyad. This allowed us to test for the effect of parentage, while controlling for the effects of variation among dyads, and variation due to experimental treatment

in a hierarchical analysis of variance. The effect of the nest of origin of individual nestlings, reflecting parentage and the effects of hatching environment, was significant for most traits with the exceptions of body mass and T cell-mediated immune response measured on day 12. Significant effects of nest of origin on nestling phenotypic values are probably due to the combination of additive genetic variation in the characters we considered, and to early maternal effects mediated, for example, by the quality and size of the eggs. We did not check for intraclutch variation in egg size and its potential effects on nestling phenotype, as we could not assign individual chicks to their original egg. Intraclutch variation might therefore have partly confounded the results by increasing the variance in chick phenotype. Body size and mass were repeatedly shown to exhibit a large component of variation due to genetic (e.g., Kinney 1969; Alatalo and Gustafsson 1988) and early maternal effects in cross-fostering experiments with a design similar to the one we adopted (e.g., Smith 1993; Saino et al. 2002). It is interesting to note that we found no evidence for variation in T cell-mediated immune response in relation to parentage. This result may suggest that heritable variation in this fundamental component of the acquired immune system is low, as could be predicted if strong selection exists for efficient antiparasite immune defense. In fact, T cell-mediated immune response measured *in vivo* by the same test we used here was shown to predict viability of the offspring in a number of studies (Møller and Saino 2004).

In conclusion, vitamin E appears to be limited for the growth of nestling barn swallows during the stage when growth rate is maximal. Only adults with a superior foraging efficiency may thus be able to assure a high quality diet to their offspring. Availability of quantitatively minor dietary components, such as antioxidants, may therefore play a major role in determining the phenotype of the offspring. However, availability of vitamin E in nestling barn swallow diet did not seem to be markedly affected by sib-sib competition, which itself affects morphology and immunity of the nestlings.

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