



Vitamin E improves growth of collared flycatcher *Ficedula albicollis* young: a supplementation experiment

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In altricial birds, the quantity and quality of food provided by parents is a crucial determinant of nestling performance. Vitamin E is an important micronutrient with various physiological functions, including a positive role in the antioxidant system. Sufficient intake of vitamin E has been shown to condition normal avian development in poultry, yet, our knowledge of the role of vitamin E in free-living birds is limited. Thus, we experimentally examined the effects of vitamin E on nestling development in the collared flycatcher *Ficedula albicollis*. We supplemented nestlings with vitamin E and evaluated their growth and survival till fledging. Increased availability of vitamin E did not affect body mass, wing length or survival, but improved tarsus growth. The effect of supplementation on tarsus length changed over season and with initial body mass. Supplemented nestlings that were smaller at hatching and those that hatched later in the season grew longer tarsi compared to the control. Our results suggest that 1) vitamin E may be limiting for the development of collared flycatcher nestlings, 2) seasonal changes of vitamin E availability may affect breeding success of collared flycatchers, and 3) increased income of vitamin E may improve growth of nestlings with bad start in life.

In altricial birds, nestling performance greatly depends on the care provided by parents. One of the most important parts of parental care is food provisioning (Bernardo 1996, Price 1998). Nestling growth is determined by both the quantity and quality of food they receive (Perrins 1976, Martin 1987, Massias and Becker 1990, Johnston 1993, Saino et al. 1997, Wright et al. 1998, Romano et al. 2006). Besides energetic content and macronutrients, micronutrient composition is an important aspect of the quality of food provided by parents.

Vitamin E is an important micronutrient with various physiological functions. It is considered the most important antioxidant in cell membranes, where it prevents oxidative damage by scavenging free radicals and thus breaks the chain propagation of lipid peroxidation (Surai 2002). Apart from its antioxidant role, vitamin E has also been suggested to participate in the regulation of enzymes and gene expression, signalling and membrane processes (Brigelius-Flohé 2009). Sufficient intake of vitamin E conditions normal avian development, functional immune system and reproduction (Surai 2002, Catoni et al. 2008).

Growing chicks are more likely to develop diseases related to vitamin E deficiency than adult birds. When faced with low vitamin E content in food, adult birds can prevent depletion of their existing vitamin E pool by recycling vitamin E molecules (Surai 2002). In contrast, growing birds must gradually build up their vitamin E reserves from food intake, which might be only partly offset by vitamin E

supply in the egg yolk. Moreover, antioxidant function of vitamin E is probably particularly important for chicks during the period of rapid growth, when consumption of oxygen is high and production of cell-damaging free radicals greater (Rollo 2002, Alonso-Álvarez et al. 2007, Kim et al. 2011, but see Garratt and Brooks 2012). This is supported by studies showing that increased intake of vitamin E in growing chicks may lower oxidative damage (Sodhi et al. 2008, Noguera et al. 2011, Orledge et al. 2012) and improve growth performance (de Ayala et al. 2006, Noguera et al. 2011). Hence, vitamin E might be limiting for growing chicks.

The necessity of vitamin E in reproduction and chick development has been firmly established in poultry (Surai 2002). However, studies on free-living birds are rare and their results are ambiguous. Vitamin E supplementation improved nestling growth in some studies (de Ayala et al. 2006, Noguera et al. 2011), but in other studies no such effect was found (Larcombe et al. 2010, Noguera et al. 2010). Vitamin E supply in natural food, and consequently the potential for vitamin E limitation, can differ among species with differing food ecology and foraging behaviour. To broaden the spectrum of species studied, we experimentally studied the effect of vitamin E availability on the performance of collared flycatcher *Ficedula albicollis* nestlings. Collared flycatchers are forest-dwelling songbirds, which hawk aerial prey as one of their primary foraging techniques and thus differ from other

common forest songbirds, (e.g. titmice, *Parus*, *Cyanistes*; Perrins 1979). We supplemented nestlings with vitamin E and evaluated their growth and fledging success. We expected nestlings provided with extra vitamin E to grow faster and survive better than control nestlings.

Material and methods

General fieldwork

This experiment was conducted on three adjacent nest-box plots (188 nest-boxes in total) in a deciduous forest near Grygov (49°31'N, 17°19'E) in eastern Czech Republic. The forest was dominated by lime *Tilia* spp. and oak *Quercus* spp. with interspersed ash *Fraxinus excelsior*, common alder *Alnus glutinosa* and common hornbeam *Carpinus betulus*. Nest-boxes were placed about 1.5 m above ground. Design of nest-boxes is described in more detail in Lambrechts et al. (2010), study site Olomouc. In addition to collared flycatchers, the nest boxes were inhabited by great tits *Parus major*, blue tits *Cyanistes caeruleus*, and nut-hatches *Sitta europaea*. Field work was carried out in 2008 from late April until June. We checked the nest-boxes daily to record laying of the first egg and clutch completion. At the end of incubation, we visited nests once a day to record hatching dates. The day when the first nestling hatched was considered as day 0 of brood age.

At a brood age of two days we marked the nestlings by clipping down feathers on their head and back for further individual recognition. When the young were eight days old, we ringed them with aluminium rings. To assess the growth of nestlings, we weighted them at day 2 and 14 of brood age (referred below as day 2 and 14, respectively) on a digital balance to the nearest 0.05 g. At day 14, we also measured their wing and tarsus length. Tarsus length was measured with a digital calliper to the nearest 0.01 mm and wing length with a ruler to the nearest 0.5 mm. We measured nestlings at day 14 because pre-fledging offspring size is an important predictor of survival after fledging in collared flycatchers (Merilä et al. 2001) and other bird species (Gebhardt-Henrich and Richner 1998). We kept the handling of nestlings to a minimum. To assess fledging success, we noted each dead nestling found during supplementation and checked nest-boxes for dead nestlings after fledging.

Vitamin E supplementation

We used a control treatment and two dosages of vitamin E (see below). We randomly divided nestlings within each nest into three groups with the same number of nestlings, or, if not possible (i.e. if the number of nestlings was not divisible by 3), kept the difference in the number of nestlings to one nestling only. Each of the groups was then randomly assigned to one of the three treatments. In this way, we ensured that all three treatments were represented in each brood and at the same time the number of nestlings in each treatment was maximally balanced within a nest. At the start of the experiment, the mean (SD) number of nestlings per nest was 1.6 (0.71) for the control and E1, and 1.7 (0.69)

for E2 (mean brood size = 4.9, SD = 1.69, n = 17). We supplemented nestlings every third day: at day 2, 5, 8 and 11. To reduce the variability in the age of supplementation and to avoid supplementation of very small nestling, we performed the first supplementation at day 2 in late afternoon (start of supplementation at 5:17 pm \pm 1 h 41 min [mean \pm SD]). For logistical reasons, time of supplementation was more variable in older nestlings.

Vitamin E includes several forms of tocopherols and tocotrienols. Of these, α -tocopherol was repeatedly shown to have the highest biological activity (Surai 2002) and generally dominates over other forms of vitamin E in avian tissues (Surai and Speake 1998, Karadas et al. 2005, Ewen et al. 2006). However, the physiological role differs between each of the diverse forms of tocopherol. Other forms of tocopherol and/or a mixture of several tocopherols provide stronger specific health effect than α -tocopherol alone (Jiang et al. 2001, Hensley et al. 2004, Saldeen and Saldeen 2005). Therefore, supplementation with different tocopherols may result in different biological effects. Thus, we decided to provide nestlings with a mixture of tocopherols rather than α -tocopherol alone.

Vitamin E dosage was based on the amount of tocopherols presumably obtained by collared flycatcher nestlings from natural diet at a given age. The concentration of tocopherols in collared flycatcher diet or in the diet of any other passerine with similar ecology was unknown at the beginning of this study. Hence, we used the concentration in insects provided by barn swallows to their nestlings: 3.93 $\mu\text{g g}^{-1}$ of α -tocopherol and 0.51 $\mu\text{g g}^{-1}$ of γ -tocopherol (de Ayala et al. 2006). The amount of insects eaten by the nestling was calculated as in de Ayala et al. 2006. As we had no information on nestling body growth in our flycatcher population, we used the growth curve from a nearby population (Krist et al. 2004). As the basis for our calculations, we used original data kindly provided by M. Krist (the data are now available in their full form at Dryad Digital Repository, doi: 10.5061/dryad.1758).

Based on these calculations, we estimated the daily food intake per nestling as 7.1, 13.8, 18.0 and 18.1 g of insects at days 2, 5, 8 and 11, respectively. We prepared two concentrations of tocopherols. The lower dose (E1) was equal to the estimated daily amount of α - and γ -tocopherol received in food, i.e. we multiplied estimated food provisioned per nestling by the concentrations of tocopherols in barn swallow diet. The concentration of the higher dose (E2) was double that of E1. A caveat of this approach is that more recent studies indicated that tocopherol content in the diet of collared flycatcher nestlings might be higher than we estimated originally. Arnold et al. (2010) showed that some caterpillars contain from about 10-fold (*Erannia* larvae) to 30-fold (*Operophtera* larvae) more α -tocopherol than aerial insects fed by barn swallow parents to their nestlings (de Ayala et al. 2006, Arnold et al. 2010). However, for two reasons we still do not know the true vitamin E content of collared flycatcher nestlings' diet.

First, despite decades of research, our knowledge of the collared flycatcher nestlings' diet is limited (for review see Cholewa and Wesolowski 2011). Moreover, this diet may greatly vary among years, habitats, early vs late broods, with the age of chicks, and the actual availability of prey types

(Löhr 1957, Bureš 1986, 1995, Török 1986, Török and Tóth 1988, Krištín 1992). For example, the proportion of caterpillars in nestling diet in these studies ranges from 3.6 to 47%; most often caterpillars form 20 to 30% of the number of food items. Second, there has been so far only one study of tocopherol content in the diet of nestlings in a forest passerine (Arnold et al. 2010). That unique study was performed in a different latitude and habitat and was limited to five weeks in one year, three groups of invertebrates, and one form of tocopherol. In addition, the concentration of tocopherol greatly varied among the three tested groups and even between two groups of caterpillars.

Based on the limited knowledge, we guessed that the diet of collared flycatcher nestlings could contain about 2- to 13-fold (on average 7-fold) more α -tocopherol than the diet of barn swallow nestlings. Our estimate was based on the following assumptions about nestling diet: 1) the diet contains 4 to 50% of caterpillars (on average 25%); 2) caterpillars are the main source of tocopherols, 3) caterpillars contain about $100 \mu\text{g g}^{-1}$ α -tocopherol with remaining diet items contain $3.93 \mu\text{g g}^{-1}$, and 4) α -tocopherol is the predominant form of tocopherol.

To prepare the supplement, we used Aqua-E (Yasoo Health, Johnson City, TN, USA). Aqua-E contains 13.4 mg ml^{-1} of natural form α -tocopherol, d- α -tocopherol, together with 12 mg ml^{-1} of γ -tocopherol and 4.6 mg ml^{-1} of other tocopherols and tocotrienols. To reach the α : γ tocopherol ratio found in the insects eaten by barn swallow nestlings (7.71:1; de Ayala et al. 2006), we further used a microemulsion of dl- α -tocopheryl acetate in water (prepared by DSL Food, Olomouc, Czech Republic). As dl- α -tocopheryl acetate is considered to have lower biological activity than d- α -tocopherol, we multiplied the amount of added dl- α -tocopheryl acetate by 1.49 (based on international units, United States Pharmacopeia 1980). Thus, the final mixture consisted of 79% of dl- α -tocopheryl acetate, 10% of d- α -tocopherol, 8% of γ -tocopherol and 3% of other tocopherols and tocotrienols. Average mass-specific dosage decreased with age. Dose E1 ranged from 25.0 mg of total tocopherols and tocotrienols per one kg of nestling body mass at day 2 (SE = 1.22, n = 25) to 13.4 mg kg^{-1} at day 11 (SE = 1.17, n = 21). Dose E2 was from 53.0 mg kg^{-1} at day 2 (SE = 3.27, n = 27) to 24.7 mg kg^{-1} at day 11 (SE = 0.54, n = 22).

Vitamin E supplements were prepared by dissolving the two sources of vitamin E in water. Because both our sources of tocopherols were water-soluble, we assured the supplement was homogenous. Thus, all nestlings within a treatment received the same concentration of vitamin E, whereas control nestlings received water only. Fresh solution was prepared in the evening prior to supplementation and kept in dark glass bottles at 6°C overnight. Every morning, the solutions were shaken and poured off into 1.5 ml eppendorf tubes for field supplementation. In the field, eppendorf tubes were kept in black plastic boxes filled with polystyrene to avoid light and thermal degradation. Feeding supplementation was performed by pipetting one of the two vitamin E dosages or the control into each nestlings' mouth. Nestlings received 8, 15, 20 and $20 \mu\text{l}$ of solution at age 2, 5, 8 and 11 d, respectively. Due to extremely slow growth in cold and rainy weather, six nestlings in two nests

were first supplemented at the age 3 d. To avoid cross-infection, the micropipette tip was never reused among broods.

Statistical analysis

To identify potential effects of vitamin E supplementation on nestling growth and survival, we used linear mixed models (LMM). The dependent variables were Body mass, Tarsus and Wing at day 14 (continuous variable) and Survival till fledging (categorical variable with two levels). To control for the effect of common origin, Nest was entered as a random factor. Our experimental treatment was represented by a fixed factor Vitamin E. Vitamin E was entered as a categorical variable with three levels – control, dose E1 and E2. All models included Hatching date to control for the effect of season, and Initial body mass (body mass at the start of the experiment, i.e. day 2) to control for size differences among nestlings at the start of the experiment. Initial body mass did not differ significantly between treatment groups ($R^2 < 0.01$, $F = 0.19$, $p = 0.83$, $n = 79$). All models also included Brood size at day 8 to control for the intensity of sibling competition. In smaller broods, sibling competition is usually reduced (Ricklefs 2002).

Further, we tested the interaction of Vitamin E with these three covariates. The interaction Vitamin E \times Hatching date may be relevant, as α -tocopherol content in caterpillars increases over the course of the nesting season, but overall availability of caterpillars decreases over the season (Arnold et al. 2010, our unpublished data). Caterpillars form an important part of collared flycatcher nestlings' diet (Cholewa and Wesolowski 2011). Thus, we expected that the effect of supplementation might change over the season. We included the interaction Vitamin E \times Initial body mass because nestling body mass varied (Initial body mass: mean 2.80 g , SE 0.08 , range 1.3 to 4.3 g , $n = 82$). The dosage of vitamin E was held constant irrespective of nestling size. Thus, smaller nestlings received relatively larger doses than larger nestlings within the same treatment group. The interaction was fit to account for this difference. The interaction Vitamin E \times Brood size was included to test whether the effect of supplementation depended on the level of sibling competition. We kept an interaction in the model only if it was statistically significant. In the model with Body mass as a dependent variable, we also included Tarsus as a covariate. This approach tested nestling condition (i.e. body mass controlled for tarsus length). However, we note that results of the model without Tarsus (i.e. testing body mass per se) were similar and are not presented here.

We started supplementation of the whole flycatcher population in our nest-boxes in 2008, with 83 nestlings in 17 broods. Sample size was lowered due to nestling mortality, technical problems and missing data. The final sample size was 15 broods with 63 nestlings for models with body size and 74 nestlings for survival model. Descriptive statistics of nestlings included in our study are provided in Supplementary material Appendix 1, Table A1.

We fitted LMMs using SAS software. Continuous dependent variables were tested in the procedure

MIXED. Covariance parameters were estimated by REML (restricted or residual maximum likelihood). We used the COVTEST statement to produce asymptotic standard errors and Wald Z-tests for covariance parameter estimates of random effects. We tested survival in the GLIMMIX procedure with Survival as a binomial dependent variable. We used WALS statement to produce Wald Z tests of estimates and asymptotic standard errors of random effects. Where appropriate, variables were checked for normal distribution. Residuals from each model were checked to conform to the requirements of normal distribution, equal variance, and linearity. In order to be able to interpret tests of main effects in the model with significant interactions (Tarsus length model), we centred all continuous input variables prior to statistical analysis by subtracting the mean.

We tested potential effects of vitamin E supplementation on several traits of the same nestlings. Such multiple testing with a constant significance level (here at $\alpha = 0.05$) increases the probability of type I error, i.e. rejecting the null hypothesis when it is true. To reduce this error, statistical corrections of p-values such as Bonferroni correction are often recommended. However, we decided to avoid these corrections. As we expected only small treatment effects and performed rather large number of tests, the Bonferroni correction would lead to low statistical power. Instead, we report effect sizes with their standard errors and original p-values. We provide results of full models including nonsignificant variables (except nonsignificant interactions). Based on this information it is possible to interpret biological relevance of our results (Moran 2003, Nakagawa 2004, Forstmeier and Schielzeth 2011).

Results

Tarsus length at day 14 was affected by vitamin E supplementation and this effect changed both with the season and with body mass at hatching (Table 1, Fig. 1). Although at the beginning of the season control nestlings grew longer tarsi than nestlings supplemented by the dose E1, towards the end of the season the tarsus of an E1 supplemented nestling was predicted to be 1.6 mm longer than the tarsus of a control nestling (Fig. 1A). At the same time, initially smaller nestlings grew longer tarsi when supplemented compared to control ones. The model predicted that among initially smallest nestlings E1 chicks would grow 1.5 mm longer tarsi than control chicks (Fig. 1B). In both these cases, tarsus length changed in response to vitamin E supplementation similarly irrespective of the dose (E1 vs E2). Besides interactions, overall effect of vitamin E supplementation on tarsus length approached statistical significance, with dose E1 being strongly significant (Table 1). To make sure that our effects were not driven by interactions, we re-ran the Tarsus model without them. Results were similar: overall effect of vitamin E supplementation approached significance ($p = 0.054$) with dose E1 being strongly significant ($p = 0.017$, effect size 0.546, SE 0.222). These findings confirm a beneficial effect of vitamin E supplementation on tarsus growth.

Nestling body mass, wing length at day 14 and survival till fledging were unaffected by vitamin E supplementation.

However, nestlings with greater body mass at hatching grew faster and survived better. Neither growth nor survival of nestlings changed over the season (except in the case of tarsus length described above). Growth and survival of nestlings did not depend on brood size either. The only exception was nestling body mass. Here, nestlings in larger broods were in worse condition than nestlings in smaller broods: the model predicted a body mass decrease of 0.57 g per sibling when a positive correlation with tarsus length was controlled for.

Nest identity explained a significant part of variability in wing length and body mass (66.1 and 62.9%, respectively). Tarsus length and survival till fledging were not significantly affected by nest identity.

Discussion

We supplemented collared flycatcher nestlings with vitamin E and evaluated their subsequent growth and fledging success. Our treatment affected tarsus length, but did not alter nestling body mass, wing length or survival. Tarsus length changed with increased vitamin E availability in a season-dependent way and the change depended also on initial nestling body mass. Supplementation was most beneficial (in terms of longer tarsi at the age of 14 d) for initially smaller nestlings and nestlings that hatched later in the season. Thus, we showed that at least in some aspects vitamin E is limiting in the development of collared flycatcher young.

Our supplementation affected tarsus length, but did not affect body mass or wing length. We measured both body mass and tarsus at brood age of 14 d. In the case of body mass, this might have been too late, because nestling collared flycatchers may reach their asymptotic mass earlier (Bureš and Weidinger 2003). Hence we cannot rule out that the increased vitamin E intake affected the body mass of nestlings earlier during growth. For example, we were not able to detect whether nestlings from one of the treatment groups reached their asymptotic mass earlier than nestlings in other groups. This would agree with vitamin E supplementation in barn swallows, where the positive effect of vitamin E supplementation was restricted to a short period of maximal growth (day 6 to day 12; de Ayala et al. 2006).

Inconsistent effects of vitamin E on different phenotypic traits obtained here were found in previous experiments on free-living birds too. In barn swallows, the situation was the opposite of our findings: body mass, condition, and feather length were affected by vitamin E supplementation, while tarsus length was not (de Ayala et al. 2006). In yellow-legged gull chicks, a beneficial effect of vitamin E supplementation on chick body mass was also confirmed, but only at the age of 8 d, not at younger age (Noguera et al. 2010, 2011). In titmice, vitamin E supplementation did not alter nestling growth and survival, regardless of nestling age and morphometric traits in question (Larcombe et al. 2010, Matrková et al. unpubl.). Thus, the effect of increased vitamin E intake differs among species and depends on the trait examined and the age of nestlings.

The positive effect of vitamin E on nestling growth presented here and in the barn swallow (de Ayala et al. 2006) contrasts with the absence of any effect in titmice

Table 1. The effect of vitamin E supplementation on growth and survival of collared flycatcher nestlings.

| Tarsus* | Fixed effect | | Estimate | SE | DF | F | p |
|-----------|-------------------------------|----------|----------|---------|-------|----------|-----------------|
| | Intercept | | -0.166 | 0.1540 | | | |
| | Initial body mass | | 0.821 | 0.2141 | 52.4 | 4.6 | 0.04 |
| | Hatching date | | -0.162 | 0.0950 | 10.6 | 0.8 | 0.39 |
| | Brood size | | -0.072 | 0.0839 | 18.1 | 0.7 | 0.40 |
| | Vitamin E | | | | 43.9 | 3.0 | 0.06 |
| | Vitamin E × Hatching date | | | | 44.8 | 6.8 | <0.01 |
| | Vitamin E × Initial body mass | | | | 45.3 | 4.5 | 0.02 |
| | | Category | Estimate | SE | DF | t | p |
| | Vitamin E | 1 | 0.459 | 0.1892 | 44.9 | 2.4 | 0.02 |
| | | 2 | 0.223 | 0.1826 | 43.6 | 1.2 | 0.23 |
| | Vitamin E × Hatching date | 1 | 0.372 | 0.1036 | 45.0 | 3.6 | <0.01 |
| | | 2 | 0.287 | 0.1026 | 47.7 | 2.8 | <0.01 |
| | Vitamin E × Initial body mass | 1 | -0.737 | 0.3091 | 47.1 | -2.4 | 0.02 |
| | | 2 | -0.797 | 0.2955 | 44.7 | -2.7 | <0.01 |
| | Random effect | | Estimate | SE | % Var | Wald's Z | p |
| | Nest | | 0.081 | 0.0773 | 19.8 | 1.1 | 0.15 |
| | Residual | | 0.329 | 0.0732 | 80.2 | 4.5 | |
| Wing | Fixed effect | | Estimate | SE | DF | F | p |
| | Intercept | | 1.809 | 4.3862 | | | |
| | Initial body mass | | 0.352 | 0.0366 | 50.9 | 92.5 | <0.01 |
| | Hatching date | | 0.015 | 0.0314 | 11.0 | 0.2 | 0.65 |
| | Brood size | | 0.014 | 0.0359 | 13.6 | 0.1 | 0.71 |
| | Vitamin E | | | | 45.7 | 0.8 | 0.46 |
| | | Category | Estimate | SE | DF | t | p |
| | Vitamin E | 1 | 0.054 | 0.0456 | 46.4 | 1.2 | 0.25 |
| | | 2 | 0.010 | 0.0437 | 45.2 | 0.2 | 0.82 |
| | Random effect | | Estimate | SE | % Var | Wald's Z | p |
| | Nest | | 0.037 | 0.018 | 66.1 | 2.0 | 0.02 |
| | Residual | | 0.019 | 0.004 | 33.9 | 4.7 | |
| Body mass | Fixed effect | | Estimate | SE | DF | F | p |
| | Intercept | | -13.685 | 24.2191 | | | |
| | Initial body mass | | 0.515 | 0.2227 | 51.4 | 5.4 | 0.02 |
| | Hatching date | | 0.148 | 0.1736 | 12.4 | 0.7 | 0.41 |
| | Brood size | | -0.575 | 0.1999 | 15.5 | 8.3 | 0.01 |
| | Tarsus | | 0.437 | 0.1720 | 48.2 | 6.5 | 0.01 |
| | Vitamin E | | | | 46.5 | 0.3 | 0.75 |
| | | Category | Estimate | SE | DF | t | p |
| | Vitamin E | 1 | -0.202 | 0.2865 | 47.4 | -0.7 | 0.48 |
| | | 2 | -0.164 | 0.2632 | 45.9 | -0.6 | 0.54 |
| | Random effect | | Estimate | SE | % Var | Wald's Z | p |
| | Nest | | 1.095 | 0.5178 | 62.9 | 2.1 | 0.02 |
| | Residual | | 0.646 | 0.1369 | 37.1 | 4.7 | |
| Survival | Fixed effect | | Estimate | SE | DF | F | p |
| | Intercept | | 98.316 | 79.9224 | | | |
| | Initial body mass | | -4.884 | 2.0638 | 68.0 | 5.6 | 0.02 |
| | Hatching date | | -0.609 | 0.5510 | 33.5 | 1.2 | 0.28 |
| | Brood size | | -1.041 | 0.4863 | 48.7 | 4.6 | 0.04 |
| | Vitamin E | | | | 68.0 | 1.5 | 0.23 |
| | | Category | Estimate | SE | DF | t | p |
| | Vitamin E | 1 | 3.057 | 1.8960 | 68.0 | 1.6 | 0.11 |
| | | 2 | 2.480 | 1.6628 | 68.0 | 1.5 | 0.14 |
| | Random effect | | Estimate | SE | | Wald's Z | p |
| | Nest | | 1.533 | 2.0476 | | 0.8 | 0.23 |

*Centred data (subtracted mean prior to statistical analysis).

Linear mixed model. For fixed effects, type 3 tests and denominator DF are presented, numerator DF = 2 for Vitamin E and its interactions, numerator DF = 1 in all other cases. For random effects, covariance parameter estimates are presented (REML method), % Var = % of explained variability. Body mass [g], tarsus length [mm] and wing length [cm] were measured at the brood age of 14 d (hatching day = day 0). Response variable Survival is binomial, results for non-survived nestlings (13 out of 74) are presented. Sample size was 63 and 74 nestlings from 15 nests, for the models of growth and survival, respectively.

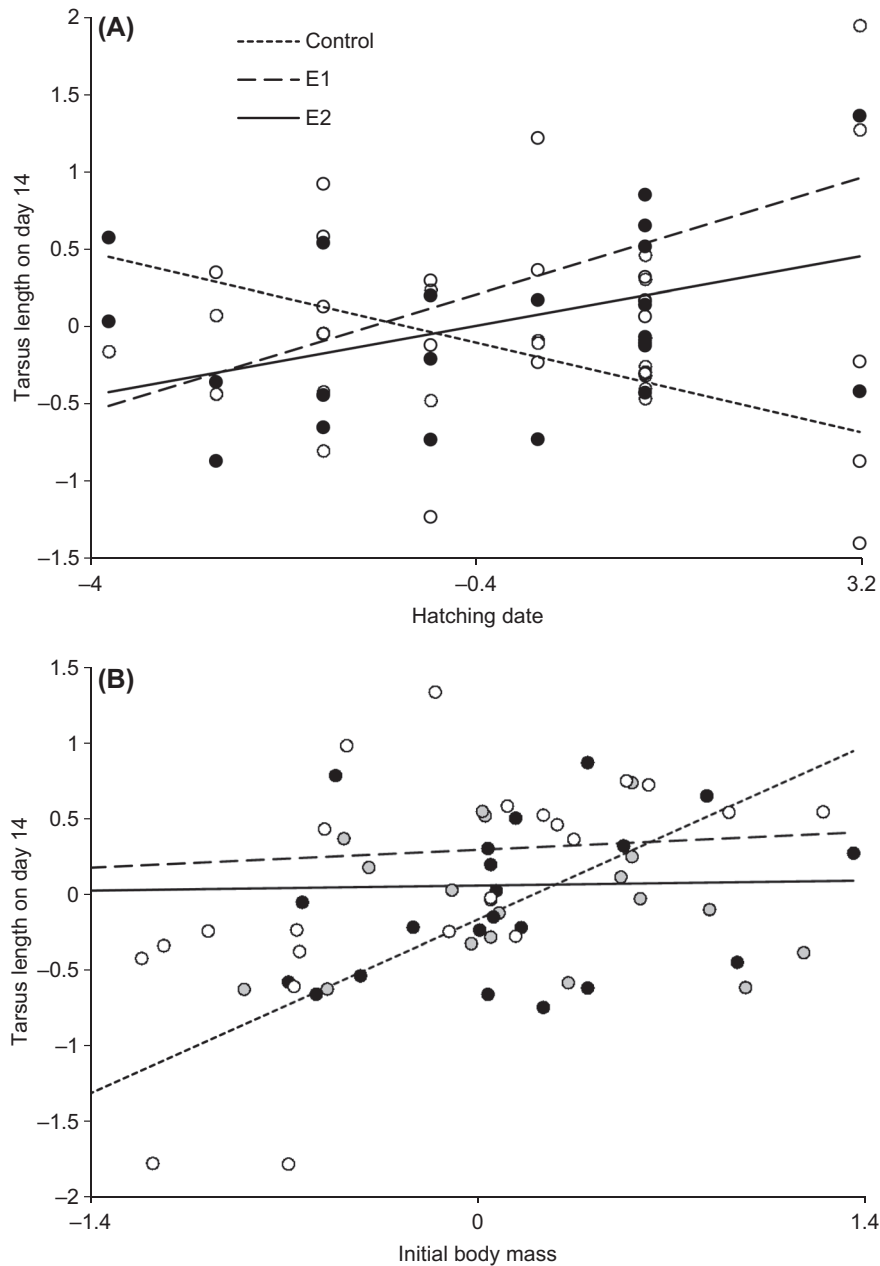


Figure 1. Effect of vitamin E availability on tarsus length. Effect of vitamin E supplementation on collared flycatcher nestling tarsus length at the age of 14 d (in mm) in relation to season (in days, A) and body mass at the start of experiment (in g, B). Depicted are regression lines and residuals; white, grey and black points represents the control, dose E1 and E2, respectively. Continuous variables were centred prior to statistical analysis by subtracting mean. In both cases, the slope of nestlings receiving doses E1 and E2 significantly differed from the slope of control nestlings. For details, see Table 1.

(Larcombe et al. 2010, Matrková et al. unpubl.). The difference in reactions of great tit vs collared flycatcher nestlings was found despite carrying out both studies in the same forest. We think that this difference stems from different nestling diet and different timing of breeding of these species. Caterpillars are estimated to contain more α -tocopherol than aerial insects fed by barn swallow parents to their nestlings (de Ayala et al. 2006, Arnold et al. 2010). Caterpillars dominate both great and blue tit nestling diet (Cholewa and Wesołowski 2011, Remeš and Matysioková 2013), but their share of collared flycatcher nestling diet is lower (Cholewa and Wesołowski 2011; see also Material and

methods). Second, while tocopherol content of caterpillars increases over season, availability of tocopherol-rich caterpillars sharply declines towards the end of season (Arnold et al. 2010). Thus, caterpillars are less available for flycatchers, as flycatchers in our study site breed ca. two weeks later than titmice (our unpublished data). Correspondingly, caterpillars are replaced by other food items in late broods (e.g. ants and flies, Löhrl 1957, 1976). This fits into our results, where only nestlings from late broods benefited from vitamin E supplementation. Thus, we suggest the observed seasonal change in response to vitamin E supplementation is likely to reflect the shift in

nestling diet, which leads to lower vitamin E availability for late broods. Alternatively, but not exclusively, nestling demand for tocopherol may increase over season (e.g. due to increasing temperature or lower food quality – Perrins 1976).

We were surprised to find comparable effects of the two doses of vitamin E (E2 was twice as concentrated as E1). One explanation might be that E1 already provided an optimal level of vitamin E supply and E2 was beyond the saturation threshold (i.e. beyond the threshold where the effect of vitamin E is dose-dependent). However, why would not nestlings then suffer from an excess of vitamin E in the E2-supplemented group? One possible explanation is that the collared flycatcher is a generalist, capable of using different foraging techniques and providing nestlings with a variable diet. Depending on the immediate food supply, parents can switch to different prey types (Bureš 1986, 1995, Török 1986; see also Material and methods). As there is a large difference in tocopherol content among different groups of invertebrates (de Ayala et al. 2006, Arnold et al. 2010), it is likely that the vitamin E content of nestling diet varies considerably even within the same habitat. It is thus possible that collared flycatcher nestlings are adapted for variable tocopherol supply stemming from variable diet supplied by parents. They might thus have evolved a better mechanism to deal with excess supply of vitamin E, which may not be absorbed or may be excreted (Bramley et al. 2000).

Initially smaller nestlings benefited from vitamin E supplementation, while initially larger nestlings did not. At the same time, initial body mass was a strong determinant of body mass in older age. Smaller nestlings may suffer competitive disadvantage from their larger siblings, as parent collared flycatchers do not allocate food according to nestling size or condition, but according to begging intensity (Rosivall et al. 2005). Recently, Moreno-Rueda (2007) suggested that begging may increase oxidative stress. This idea was supported by an experiment in yellow-legged gulls, where vitamin E supplementation increased begging intensity, especially in smaller chicks (Noguera et al. 2010). Noguera et al. (2010) suggest that vitamin E alleviates the oxidative cost of begging, which may enhance solicitation and increase parental feeding effort, resulting in increased growth in supplemented chicks. As at the same level of need smaller chicks are more compromised in survival than larger ones, they should consequently benefit more from more intensive begging (Noguera et al. 2010). The need of antioxidants may be also higher in smaller nestlings, as catch-up growth may increase oxidative damage (Mangel and Munch 2005, Alonso-Álvarez et al. 2007, De Block and Stoks 2008). Consistently with this notion, supplementation of dietary antioxidants in free-living birds reduced oxidative damage only in nestlings limited in their growth by poor nutrition (Hall et al. 2010, Noguera et al. 2011).

Increased availability of vitamin E improved growth of some flycatcher nestlings. Body size at around the time of fledging is an important predictor of future fitness in birds (Gebhardt-Henrich and Richner 1998). In the collared flycatcher, a positive directional selection on tarsus length was found (Kruuk et al. 2001). Thus, although our supplementation did not affect fledging success, higher vitamin E

intake during early life may have resulted in higher post-fledging survival and/or fertility of the young.

To conclude, vitamin E is limiting for collared flycatcher nestlings during development. Experimentally increased intake of vitamin E had a beneficial effect on tarsus growth in late broods and in initially smaller nestlings. Our data thus suggest that increased vitamin E availability may help to compensate for initial disadvantages among nestlings. Future studies would benefit from studying bird species with diverse food preferences and foraging techniques and from including estimates of long-term offspring performance.

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Supplementary material (Appendix JAV-00368 at < www.avianbiology.org/readers/appendix >). Appendix 1.