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Dietary antioxidants in life-history trade-offs: differential effects of a-tocopherol supplementation on blue tit *Cyanistes caeruleus* mothers and offspring during reproduction

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α-Tocopherol is assumed to be the most biologically active dietary antioxidant *in vivo*, but despite its potential importance little is known about its impacts on wild birds. Reproduction is presumed to be costly for parents through several routes, including increased oxidative stress, particularly for bird species producing large clutches. If dietary antioxidants can ameliorate oxidative stress associated with reproduction, mothers supplemented with dietary antioxidants are predicted to be in improved condition and/or invest more resources in reproduction than controls. We provided adult blue tit pairs with an α-tocopherol-enriched or control food supplement during nest building and egg laying, then cross-fostered half broods between treatment groups to test the theory that α-tocopherol-supplemented mothers would invest more in self-maintenance or reproduction than controls. We found that α-tocopherol supplementation had no effect on the maternal condition or reproductive investment. However, effects on nestlings were evident: nestlings from α-tocopherol-supplemented mothers were smaller at hatching. There was no effect on chick fledging mass, fledging success or lipid peroxidation, but the catch-up growth exhibited by chicks from α-tocopherol-supplemented parents may be considered costly. Thus, our results do not provide evidence for a benefit of maternal α-tocopherol supplementation at a biologically relevant dose on either themselves or their offspring. We discuss our findings in terms of ongoing research on the multifaceted roles that dietary 'antioxidants' can have *in vivo*, and the issues of disentangling their impacts on physiology and behaviour in the wild.

ADDITIONAL KEYWORDS: α-tocopherol – antioxidants – birds – growth – MDA – oxidative stress – reproduction – vitamin E.

INTRODUCTION

The availability of resources will determine how individuals balance investment in the current reproductive attempt against investment in self-maintenance, and future reproduction. There has been much interest in the role that antioxidants might play in underpinning such life-history trade-offs (Costantini, 2008; Dowling & Simmons, 2009; Metcalfe & Alonso-Alvarez, 2010). Reactive oxygen species (ROS) are naturally produced by the body during metabolism, immune responses and cell signalling. Although their production is unavoidable, and in some cases necessary, left unchecked these ROS will cause damage to lipids, muscle and DNA vital for physiological function

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(Finkel & Holbrook, 2000; Larcombe *et al.*, 2008, 2010a). Thus, all animals have evolved an endogenous antioxidant system, augmented by a potentially limited supply of dietary antioxidants, to remove excess ROS before damage accrues. At points in life where the endogenous antioxidant system may be operating at full capacity, these limited, dietary antioxidants may be especially important in resisting oxidative stress. Oxidative stress occurs where the production of pro-oxidants overwhelms the capacity to remove or neutralize them (Sies, 1991), and the ability to resist oxidative stress has been shown to boost survival and life expectancy in some wild populations, highlighting its importance to determining fitness (Alonso-Alvarez *et al.*, 2004; Bize *et al.*, 2008; Losdat *et al.*, 2012).

Breeding is a major life-history event that has been associated with increased oxidative stress through a variety of routes; thus, reproduction is used as a model to study the ecological and evolutionary impacts of physiological trade-offs involving antioxidants and oxidative stress (Alonso-Alvarez *et al.*, 2004; Monaghan, Metcalfe & Torres, 2009; Larcombe *et al.*, 2010b; Christe *et al.*, 2011; Metcalfe & Monaghan, 2013; Speakman & Garratt, 2014; Blount *et al.*, 2016). In birds, reproduction, egg formation, egg incubation and offspring rearing are all associated with increased metabolism (Hodum *et al.*, 1998; Weimerskirch *et al.*, 2003). Although the generality of the relationship between metabolic rate and oxidative stress has recently been questioned (Arnold *et al.*, 2007, 2015; Salin *et al.*, 2015; Speakman *et al.*, 2015), reproductive investment has been linked to a decrease in antioxidant defences (Alonso-Alvarez *et al.*, 2004; Losdat *et al.*, 2012). Reproduction is also linked to the magnitude of the physiological stress response in mothers (Romero *et al.*, 1997), and stress responsiveness and oxidative balance are likely to be associated (Sahin & Gümüşlü, 2007; Monaghan & Spencer, 2014). Given these proposed oxidative costs of breeding, the ability of individuals to resist oxidative damage might impact their ability to invest in the production of offspring (Speakman *et al.*, 2015). Since major breeding events are predicted to challenge the endogenous antioxidant system, the availability of dietary antioxidants could limit reproductive effort if they have an important *in vivo* role in free-radical quenching and prevention of oxidative damage.

Most studies investigating trade-offs between dietary antioxidants and reproduction have focussed on carotenoids, a class of lipophilic antioxidants that are also important in colour-based sexual/social signalling in birds and other animals. For example, experimental manipulations of carotenoid levels in eggs, either indirectly via the mother (Surai *et al.*, 2003; Biard, Surai & Møller *et al.*, 2005; Remes *et al.*, 2007; Berthouly *et al.*, 2008) or by direct injection into the yolk (Saino *et al.*, 2003; Marri & Richner, 2014), have shown that carotenoids can reduce oxidative susceptibility (Blount *et al.*, 2002) and improve offspring immunity (Saino *et al.*, 2003; Biard *et al.*, 2005; Leclaire *et al.*, 2015), body size (Biard *et al.*, 2005 but see Remes *et al.*, 2007) and fledging success (Marri & Richner, 2014). However, carotenoids may have multiple endogenous roles in addition to, or instead of, their putative role as free-radical scavenging antioxidants (Hartley & Kennedy, 2004). Therefore, these positive effects are not always necessarily attributable to antioxidant function. Indeed, carotenoids could be considered relatively minor antioxidants in birds (Costantini & Møller, 2008). Data on the impact of non-carotenoid antioxidants on breeding success and offspring development are more scarce, but potentially important. In this study, we provided birds with the antioxidant α-tocopherol, a biologically active form of vitamin E (Machlin, 1991; Sies & Murphy, 1991; Costantini, 2008).

α-Tocopherol is suggested to be the major lipophilic antioxidant involved in membrane defence (Tappel, 1962). Deficiencies in vitamin E are associated with a range of disorders in many taxa (Zingg, 2007), effects generally attributed to its antioxidant properties specifically (Traber & Atkinson, 2007). α-Tocopherol can play a role in mediating gene expression (Azzi & Stocker, 2000; Azzi *et al.*, 2004) and immune processes (Leshchinsky & Klasing, 2001; Wintergerst, Maggini & Hornig, 2007), but is most commonly researched for its potential as an important antioxidant. Data from poultry science suggest widespread beneficial effects of supplementary vitamin E for birds (Surai, 2002). Although data for non-commercial species are less common, it has been shown that provision of vitamin E can reduce oxidative damage in adult house finches *Haemorhous mexicanus* (Giraudeau *et al.*, 2013) and reduce parasite burden in adult ring-necked pheasants *Phasianus colchicus* (Orledge *et al.*, 2012). Although supplementation to nestlings directly improved growth, tarsus length and the fledging success of some bird species (Matrková & Remeš, 2014; Maronde & Richner, 2015), other studies have found no impact on oxidative damage or the immune system in nestling tits (Larcombe *et al.*, 2010b; Marri & Richner, 2015). To our knowledge, the impacts of supplementing vitamin E to wild adult birds on their reproductive success and offspring development have not been tested.

In birds, maternal nutritional status has been shown to affect egg size (Nager, Monaghan & Houston, 2000) and the deposition of substances within the egg such as antibodies, lipids, proteins and hormones (Blount *et al.*, 2002; Gasparini *et al.*, 2007; Siitari *et al.*, 2015). These, in turn, can influence offspring phenotype (Navara, Hill & Mendonca, 2006; Giraudeau, Ziegler & Tschirren, 2016). Adequate antioxidant deposition into yolk is vital to ensure normal development of nestlings, particularly since antioxidant levels cannot be adjusted until after hatching. Furthermore, antioxidant concentration in

egg yolk may have a significant bearing on levels of antioxidants in tissues such as blood, brain and livers (Surai, Noble & Speake, 1996; Surai *et al.*, 1998). By allocating extra antioxidants into yolk, a female may improve or alter the health or condition of her nestlings post-hatching. Antioxidants that are deposited into egg yolks are often dietary acquired, including carotenoids and vitamin E (Deeming & Pike, 2013). This suggests another trade-off between dietary antioxidants and reproductive effort if egg quality is limited by the availability of these dietary antioxidants before egg laying.

In this study, we assessed the effects of biologically relevant α-tocopherol supplementation of parents during nest building and egg laying, on maternal condition, reproductive effort pre- and post-hatching, and offspring development and phenotype in a wild population of blue tits, *Cyanistes caeruleus*. By cross-fostering partial broods, we specifically tested whether compared with a control, α-tocopherol supplementation impacts: (1) maternal body condition or parental investment; (2) clutch size and quality; (3) development or oxidative damage levels of offspring or (4) reproductive success.

METHODS

Study site

The study was conducted in spring 2006 in an established nest box-breeding population in predominantly Oak Woodland at the Scottish Centre for Ecology and the Natural Environment (SCENE), Rowardennan, Loch Lomond, UK (56080N, 4370W).

Ethical statement

This research adhered to the Association for the Study of Animal Behaviour Guidelines for the Use of Animals in Research, the legal requirements of the UK and all institutional guidelines.

Nest building and egg laying: dietary manipulation and clutch size

Dietary antioxidant levels were manipulated from mid-nest building until clutch completion. Nest boxes were visited every 2 days until nests were one-quarter constructed (a ring of moss but with the nest box floor centre still bare). The next day, an empty 130 × 130 × 50 mm green mesh suet feeder (Haiths, Cleethorpes, UK) was installed on a branch, sapling or trunk within 3 m (but usually less than 1.5 m) of that nest box, to habituate parent birds to their presence. Visits continued every 2 days until nests were half built (having a visible but unlined nest cup), at which point feeders were stocked with ~125 g of either control lard or α-tocopherol-enriched lard. All food supplements were prepared the night before use, by melting lard and pouring into foil-lined moulds. For the α-tocopherol treatment, the lard was cooled, and 250 mg of α-tocopherol acid succinate (Sigma, Poole, UK) was added and evenly mixed to 1 kg of cooled lard. All food was stored in a freezer overnight. The method of α-tocopherol supplement delivery was based on methods established at the site (Ramsay & Houston, 1998) and designed to provide a biologically relevant dose of 0.37-mg additional α-tocopherol (or an increase of ~30% of normal daily intake) to supplemented birds (see Supporting Information, S1 for further details). Supplements were replaced every 2 days to ensure freshness and α-tocopherol stability. This assumption was tested in a later experiment, where we found only a small decrease in detectable vitamin E in lard after 48 h (maximum decrease of 25% from starting concentration at the point that new feeders were provided: see Supporting Information). Feeders were removed when incubation commenced, and no new eggs had been laid for 2 days, after which nests were undisturbed for 10 days during incubation. Consequently, the duration of supplementation varied with nest building rate and clutch size, but the duration of supplementation did not vary between treatment groups [GLM, total treatment duration: $F_{1,69} = 0.55$, $P = 0.855$; treatment duration before first egg: $F_{1,69} = 0.259$, $P = 0.613$; mean total treatment durations (days ± SE): control 15.09 ± 0.57, α-tocopherol 15.31 ± 0.72; mean treatment duration before first egg (days ± SE): control 4.91 ± 0.61, α-tocopherol 4.47 ± 0.61], and duration of supplementation was included in analyses (see Statistical Methods). A total of 94 blue tit pairs (47 control and 47 α-tocopherol) were randomly assigned to the feeding trial. After accounting for nests that were unsuitable for cross-fostering due to failure to find treatment/hatch date/clutch size matches, we had a sample of 24 cross-fostered broods.

When laying commenced, eggs were numbered daily with non-toxic, permanent ink to identify lay order. The fifth laid egg from each nest was removed on the day it was laid for antioxidant analysis, replaced with a dummy egg to prevent females from laying a replacement. The egg was kept chilled and taken immediately to a freezer where it was stored at −40 °C until analysis. We used clutch size as a measure of female reproductive effort. In addition, the lengths and widths of all eggs were measured using vernier callipers to within 0.05 mm. Egg volume was calculated using the equation $V = 0.51 \cdot LB^2$ (Hoyt, 1979). Total and mean egg volumes were used to assess maternal investment in terms of clutch quality.

Day 3: cross-fostering and initial nestling measurements

We performed a cross-fostering trial to separate the effects of the manipulation on 'egg effects' (i.e. egg

quality and incubation environment, along with genetic inheritance) from the effects of the rearing environment. After hatching day 0 (the day on which more than half of eggs within a clutch hatched), broods were undisturbed until the cross-fostering when nestlings were 3 days old. Half broods were swapped between dyads of supplemented and control-treated parents. Nests were paired according to feeding treatment, brood size (± 1 nestling) and exact hatching date. We did not cross-foster any nests that did not hatch on the same day. Before cross-fostering, each nestling was individually marked with a unique colour combination on the three patches of down on their heads using non-toxic ink. The nestlings were weighed, and half were randomly selected using a coin toss for fostering. We did not know the laying order of the chicks, but given the large brood sizes and randomized cross-fostering with respect to size (day 3), it is highly unlikely that laying order systematically effected results. While cross-fostered nestlings were transported to their new nest box in a heated box, their siblings were also kept out of the nest in a heated box to control for the disturbance involved in cross-fostering. Cross-fostering was accomplished within 30 min. For broods with no suitable nest pairing for cross-fostering, all nestlings were marked and measured at the nest site and returned to their own nest.

Nests were visited on days 5, 7, 9 and 11 to remark as necessary, with non-toxic ink and, from day 9, using a unique combination of toenail clips. At day 14, they were ringed, blood sampled (see below) and left to fledge naturally.

Female condition measurement

To investigate the effects of treatment on female condition, adult females were caught by nest box traps, blood sampled and measured when their nestlings were 5–6 days old. Following blood sampling, we measured females' tarsus length and weight (to within 0.1 g). For each bird, condition was calculated as the residuals from the regression of Ln (mass) on 3*Ln (tarsus). Physiological condition indices [blood glucose level and heterophil to lymphocyte (H/L ratio)] were also measured but not included in the main text (see Supporting Information, S2).

Egg yolk antioxidant analysis

We used the fifth laid egg from each nest to perform antioxidant concentration analysis. This egg was chosen to allow the maximum time for supplementary α-tocopherol to be incorporated into eggs while also maximizing sample size (most females lay at least five eggs in our population). We measured carotenoid and α-tocopherol content using high-performance liquid chromatography (HPLC). Eggs were frozen at –40 °C until extraction took place. Eggs were removed from the freezer, and their shells were removed with tweezers. The egg was then left to thaw until the albumen around the yolk had melted, leaving a frozen yolk. A dissecting needle was used to impale the yolk, which was then rubbed over tissue paper until all albumen was removed. The yolk was weighed to the nearest 0.001 g, placed in an Eppendorf, an equal volume distilled water was added to each, and they were then homogenized. Antioxidant extraction on 200 μL of yolk water solution using previously outlined methods (Larcombe *et al.*, 2008). HPLC and data analysis were conducted as previously described (Arnold *et al.*, 2010a).

Parental investment

To determine whether differences in incubation were mediated by our supplement, we calculated incubation duration as the number of days elapsed between incubation commencing and the first egg hatching.

To examine the effect of the manipulation on adult provisioning behaviour, we collected videos of parent visitation to the nest box on the day after cross-fostering, when nestlings were 4 days old. Video cameras (50 × 50 × 20 mm) were attached to the inside of the nest box back wall, facing the entrance hole to capture parents' entrances during peak provisioning from 0600 to 1200 h. The cameras were connected to a VCR in a waterproof box that was camouflaged with forest litter. The video recording equipment was installed the day before filming to allow adults to habituate, and the nest boxes were not disturbed on day 4. The time of each visit and, where possible, the contents of the adult beak were recorded. Food was assigned to the following categories: (1) caterpillar, (2) spider, (3) non-caterpillar (definitely prey, not a caterpillar or spider), (4) unknown (did not resemble a typical prey item) and (5) not visible.

Offspring development and fledging success

To examine the effect of adult treatment on nestling morphology and condition at fledging, we measured nestling weight on day 13 and oxidative damage levels, morphology and plumage coloration on day 14, just prior to fledging. The growth rate was calculated for each bird between days 3 and 13 as: (mass day 13 – mass day 3)/10, giving a rate of daily body mass gain in gram/day. On day 14, half of a brood was transported to SCENE in a heated bag. On arrival, nestlings were removed from the bag one at a time and blood sampled immediately by venipuncture of the wing vein. One drop of blood was put in ethanol for subsequent molecular sexing (Griffiths *et al.*, 1998;

Arnold *et al.*, 2007). The remaining blood was collected in 75 μL heparinized capillary tubes. The capillary tubes of blood for malondialdehyde (MDA) analysis were centrifuged, and haematocrit readings were taken from each, before these were stored at –20 °C. After blood sampling, wing length and tarsus length were measured. Finally, a spectrophotometer (Ocean Optics S2000) was used to collect reflectance readings (see Supporting Information, S3). Birds were removed from their nests for no longer than 1 h. Fledging success was recorded; we checked nest boxes when the nestlings would have been 25 days old, after fledging. The identity of any dead nestlings in the nest box was noted. We attempted to assess recruitment of adult and juvenile birds from this study in the breeding season of 2007, but the sample size was too small to make robust conclusions (see Supporting Information, S4).

Nestling oxidative damage analyses

In order to assess the effect of supplemental feeding treatment on oxidative stress, MDA, a by-product of lipid peroxidation, was quantified in the plasma of a subsample of nestlings. Owing to the relatively large volume of plasma (50 μL) required for these analyses, not all birds could be measured. Instead, we analysed plasma samples from at least one nestling of each sex, per treatment per brood. This meant a final sample size of 90 samples (~50% of all cross-fostered nestlings). MDA analysis was performed according to a standard method (Young & Trimble, 1991) with the modifications outlined previously (Larcombe *et al.*, 2015).

Statistics

Since we ended up with a lower sample size of cross-fostered nests than anticipated from the 94 starting nests, for the analyses that did not involve offspring or parent condition and phenotype we performed statistical tests on birds from the cross-fostered nests alone (n = 184), and then with data from all nests (n = 417) to augment the sample size. This only applies where we had reasonable grounds to assume the cross-fostering would have no effect and is reported in the results where applicable.

Measures of female condition, reproductive output and yolk antioxidant concentrations were analysed using general linear models in SPSS v14 (SPSS Inc., Chicago, IL, USA). Dependent variables were as follows: female body condition, clutch size, yolk mass (fifth egg), antioxidant concentrations, total egg volume and fledging success. Treatment was entered as a fixed factor and hatching date as a covariate in every model. Since birds varied in nest building rate and latency to begin egg laying, the number of days of supplementation was entered as a covariate in models. Initially, the interactions treatment × hatching date and treatment × treatment duration were included in all models to account for potential date and treatment duration effects, respectively. These terms were not significant and excluded from final models. Yolk antioxidant concentrations were analysed using GLMs with total yolk carotenoid and total yolk tocopherol concentration as dependent variables. Reproductive output (clutch size egg volumes, antioxidant concentrations) was modelled with female body condition as an additional covariate.

Data on nestling growth, size and oxidative stress were analysed using general linear mixed models (GLMM) in SAS v8 (SAS Institute Inc., Cary, NC, USA). Response variables were body mass day 3, body mass day 14, growth rate, MDA, tarsus length, and condition. Identity (ID) of egg parent's nest and identity (ID) of rearing parents nest were added as random factors in each model, to control for non-independence of nestlings of the same origin and hatching environment, or rearing environment, respectively. Initial models included brood size as a covariate, but this was never significant and subsequently removed. Sex, parental treatment, rearing treatment and all possible two-way interactions were added as fixed factors into each model. MDA was modelled including growth rate as an additional covariate as our previous work suggests growth rate is a strong determinant. Models were simplified by dropping non-significant terms from the model, starting with non-significant interactions, until only factors significantly contributing to the model remained. In the results below, non-significant values are provided at the point the term was omitted from the model, and only significant interaction terms are reported. Means ± 1 SE are reported throughout the results.

RESULTS

Maternal condition

There was no significant difference in female body condition between α-tocopherol (0.12 ± 0.25) and control-fed birds (–0.21 ± 0.25), when nestlings were 5 days old (univariate GLM, $F_{1,32}$ = 1.538, P = 0.224). There was no significant relationship between female condition and hatching date (P > 0.1).

Clutch size and quality

There were no differences in the clutch size (eggs laid), nor the total clutch volume between control (clutch size 10 ± 0.48; clutch volume 1468.4 mm^3 ± 18.82) and α-tocopherol (clutch size 10.77 ± 0.41; clutch volume 1468.2 mm^3 ± 22.64) supplemented birds (multivariate

GLM, $F_{2,28} = 0.151$, $P = 0.861$). Clutch size and total clutch volume were positively correlated with female body mass (multivariate GLM, $F_{2,31} = 3.531$, $P = 0.041$). There was no effect of hatching date on volume of eggs laid ($P > 0.4$). There were no differences in the average egg volume or yolk volume between control or α-tocopherol-supplemented birds (multivariate GLM, $F_{2,23} = 0.218$, $P = 0.806$). There was no effect of female mass, or condition on egg volume or yolk volume ($P > 0.203$ in all cases). Comparing only cross-fostered nests, there were no significant differences in total clutch volume (α-tocopherol: $n = 12$, 15 953.15 mm^3 ± 828.73; control: $n = 12$, 1563.15 mm^3 ± 901.72, GLM $F_{1,23} = 0.09$, $P = 0.79$) or average egg volume (α-tocopherol: $n = 12$, 1445.85 mm^3 ± 22.47, control $n = 12$, 1463.20 mm^3 ± 27.79, GLM $F_{1,23} = 0.278$, $P = 0.62$) between the treatment groups, so this is not due to systematic biases in egg exchanges.

In the fifth laid eggs, there were differences in the yolk mass attributable to treatment. Although overall α-tocopherol-treated females had fifth eggs with bigger yolks (means: control 0.2456 g ± 0.0038; α-tocopherol 0.2539 g ± 0.0053), there was a treatment × clutch size interaction (GLM $F_{1,28} = 7.49$, $P = 0.01$). Figure 1 shows a positive linear relationship between clutch size and yolk mass in control birds, but not in α-tocopherol-treated birds; the impact of α-tocopherol on yolk mass was stronger in birds with smaller clutches than those with larger clutches. There was a marginal trend for heavier females to lay fifth eggs with larger yolk mass (GLM $F_{1,28} = 3.61$, $P = 0.068$). Despite this, there were no differences in the concentrations of α-tocopherol (GLM $F_{1,25} = 1.01$, $P = 0.314$) and total carotenoids (GLM $F_{1,25} = 0.238$, $P = 0.793$) between treatments. The analyses accounted for differences in the duration of treatment (days treatment before egg laid: tocopherol concentration $F_{1,29} = 0.0$, $P = 0.99$; carotenoid concentration $F_{1,29} = 0.0$, $P = 0.99$). The small difference in yolk mass between birds was insufficient to change the total antioxidant content of yolks (rather than concentrations). Mean concentrations of antioxidants in the yolks of all eggs were α-tocopherol: control treatment ($n = 12$) 232.88 ± 21.97 μg/mL, α-tocopherol treatment ($n = 14$) 224.37 ± 26.42 μg/mL, total carotenoids: control treatment ($n = 12$) 76.94 ± 10.24 μg/mL, α-tocopherol treatment ($n = 14$) 82.59 ± 11.69 μg/mL. There was no effect of female mass or total clutch volume on concentrations of yolk antioxidants ($P > 0.19$ in both cases). However, Figure 1 shows a significant negative relationship between maternal body condition and yolk α-tocopherol (GLM $F = 6.398$, $P = 0.026$) and yolk carotenoid concentrations (GLM $F = 9.613$, $P = 0.009$). There was no difference in hatching success between treatment groups, and no effect of female condition, or date on hatching success or fledging success (GLM, $P > 0.345$).

Parental investment

Feeding treatment did not affect incubation duration (means: control treatment 14.96 ± 0.33 days, α-tocopherol treatment 14.84 ± 0.31 days, univariate

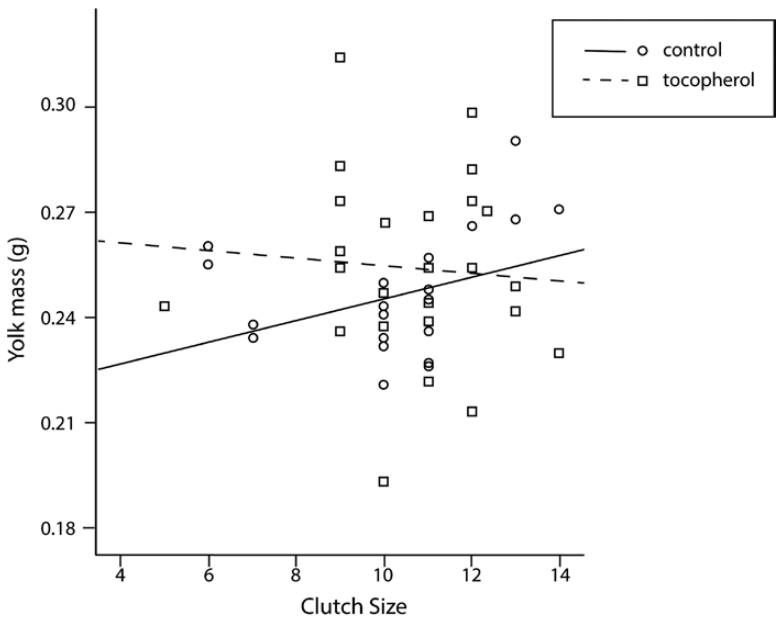

Figure 1. (a) Concentration of yolk α-tocopherol and (b) total carotenoid decreased with maternal body condition [residuals of ln (mass) on 3*ln (tarsus)].

GLM $F_{1,30}$ = 0.001, P = 0.97). There was no effect of total clutch volume, female condition or date on duration of incubation (GLM P > 0.3 in all cases).

No aspect of nestling provisioning between 06:00 and 08:00 was affected by dietary treatment. Using data only from a subset of cross-fostered that were filmed (n = 16), there was no difference in number of feeds per brood (GLM, $F_{1,15}$ = 0.719, P = 0.411) or number of feeds per nestling in the 2-h observation (GLM, $F_{1,15}$ = 1.68, P = 0.215: α-tocopherol: n = 12, mean 7.03 feeds ± 1.16; control: n = 17, mean 5.76 feeds ± 0.25, GLM, $F_{1,28}$ = 0.39, P = 0.54). There was a non-significant trend for the proportion of caterpillars provided to decline with date (GLM $F_{1,28}$ = 3.35, P = 0.07). Thus, parents from different treatments did not vary in the amount or type of prey provided to nestlings. Including data from non-cross-fostered nests to enhance the sample size (n = 29) did not change the results (feeds per 2 h; α-tocopherol: mean 56.14 ± 9.28; control: mean 46.67 ± 1.33, GLM, $F_{1,28}$ = 0.103, P = 0.751 or proportion of caterpillars GLM $F_{1,28}$ = 0.005, P = 0.94, proportion caterpillar α-tocopherol: mean 0.87 ± 0.03; control: mean 0.87 ± 0.04).

Offspring development

At 3 days old (prior to cross-fostering), nestlings from α-tocopherol-treated parents weighed significantly less than those from control-treated parents (GLMM, $F_{1,188}$ = 24.28, P < 0.0001; Fig. 2a). Mass gain between days 3 and 13 was then faster for these nestlings, than nestlings whose egg parents received control treatment (see Fig. 2b; Table 1), and by day 14, there was no longer a significant effect of egg parent' feeding treatment on mass (GLMM $F_{1,38.1}$ = 0.69, P = 0.41). These results for growth rate and body mass day 14 indicate an impact of the treatment on patterns on development, but do not allow us to determine whether development in the nest is directly altered by parents' treatment, or whether patterns of development are an

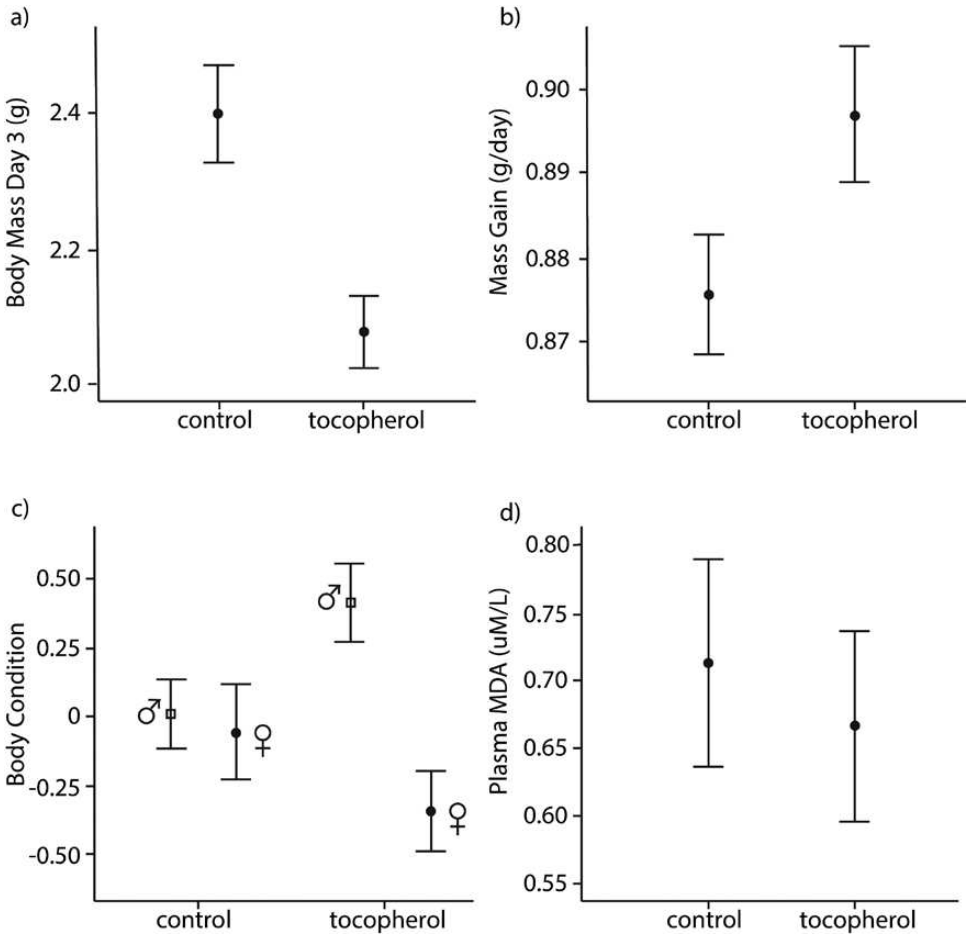

Figure 2. Mean (± 1 SE) differences between nestlings from eggs laid by females that had received either α-tocopherol or control diet in: (a) mass of nestlings age 3 days; (b) mass gain per day between days 3 and 13; (c) body condition of nestlings aged 14 days [residuals of ln (mass) on 3*ln (tarsus)] and (d) MDA concentration.

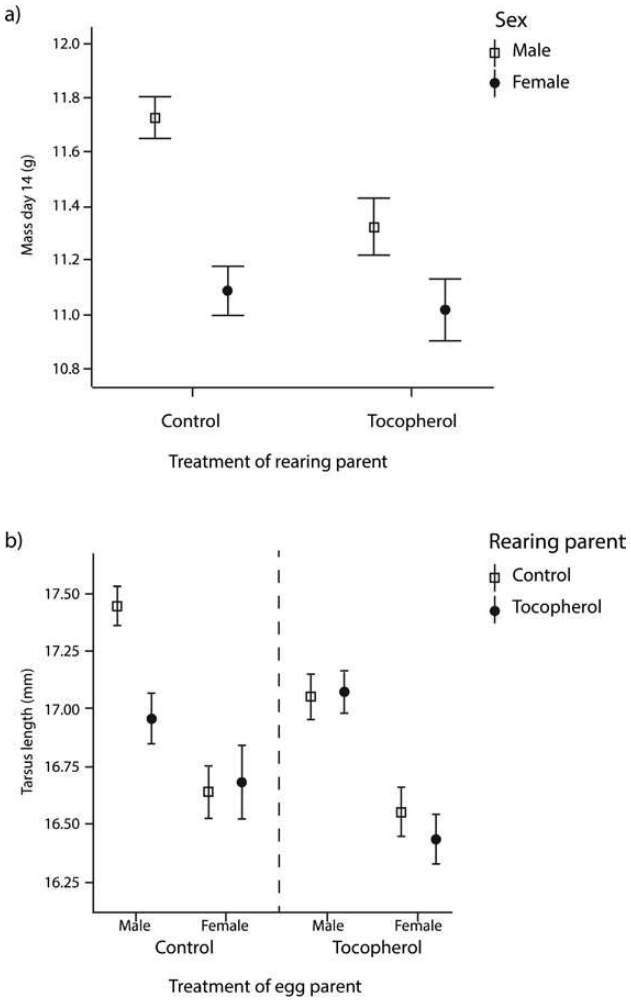

Figure 3. Differences (mean ± 1 SE) between nestlings. (a) Mass aged 14 days of male and female nestlings, reared by parents from different treatment groups. (b) Tarsus length nestlings, laid by either α-tocopherol or control-treated parents and reared by either α-tocopherol or control-treated parents.

indirect side effect of differences in mass at hatching. We re-ran the models for body mass and growth rate including the interactions of mass day 3 × treatment of rearing parent and mass day 3 × treatment of egg-laying parent to account for these possibilities. None of these interactions were significant (body mass day 14: mass3 × rearing treatment $F_{1,86.4} = 0.9$, $P = 0.35$; mass3 × egg treatment $F_{1,158} = 0.9$, $P = 0.36$; growth rate 3–13: mass3 × rearing treatment $F_{1,92.3} = 0.88$, $P = 0.35$; mass3 × egg treatment $F_{1,84.3} = 0.74$, $P = 0.39$). From this, we suggest that egg effects as a result of the treatment resulted in smaller nestlings, and smaller nestlings always engage in catch-up growth regardless of treatment. In contrast, feeding treatment of rearing parent had no effect on the rate of mass gain (GLMM $F_{1,15.1} = 0.48$, $P = 0.50$). However, nestlings raised by control-fed adults were of greater mass at day 14 than those raised by α-tocopherol-fed adults (Table 2; Fig. 3a). The identity of both rearing parent and egg parent explained variance in mass gain between days 3 and 13, indicating that growth rate is determined both by genetic, maternal and early rearing effects and by provisioning by rearing adults (Table 1). In these models, there were no sex differences in body mass at day 3 (GLMM, $F_{1,192} = 0.019$, $P = 0.66$), but males gained more mass than females between the ages of 3 and 14 days ($F_{1,160} = 23.56$, $P < 0.0001$). There was no significant interaction between sex and either treatment of egg ($F_{1,174} = 1.71$, $P = 0.193$) or rearing parents (GLMM $F_{1,174} = 1.81$, $P = 0.179$; Table 2).

With regards to body size, however, at 14 days of age, nestlings from α-tocopherol-supplemented egg parents had smaller tarsi than nestlings from control eggs (Table 3). There was also a significant interaction between treatment of rearing parents and sex on tarsus length (Table 3). While in general males had longer tarsi than females (means: males 17.14 ± 0.05 mm, females 16.57 ± 0.06 mm), male nestlings raised by control-treated adults had longer tarsi than male nestlings raised by tocopherol-treated adults (Fig. 3b). The identity of egg parent significantly explained some variance in tarsus length, but identity of rearing parent did not (random factors: egg parent $Z = 1.57$, $P = 0.058$; rearing parent $Z = 0.76$, $P = 0.224$).

There was a non-significant trend for nestlings from eggs laid by α-tocopherol-fed parents to be in better condition at fledging (greater mass for skeletal size) than birds from control-fed egg parents ($P = 0.071$; Fig. 2c; Table 3). As body mass was not impacted by egg parents' treatment, although tarsus length was, this result is probably driven by the smaller tarsi in the nestlings from eggs laid by tocopherol-treated parents. There was no significant effect of treatment of rearing adults (GLMM $F_{1,20.9} = 0.97$, $P = 0.34$) or offspring sex (GLMM $F_{1,184} = 2.61$, $P = 0.11$) on condition (Table 3). As with most morphometric measures, there was a variance in offspring condition was significantly attributable to identity of egg parents, but not to identity of rearing parents (random factors: egg parent $Z = -2.54$, $P = 0.011$, rearing parent $Z = 2.21$, $P = -0.902$).

In spite of the differences in nestling mass and growth between treatment groups neither genetic nor rearing parent treatment had a significant effect on plasma levels of MDA (GLMM: parents treatment, $F_{1,79.7} = 0.35$, $P = 0.55$; rearing treatment, $F_{1,19.4} = 0.19$, $P = 0.67$) (Fig. 2d). There were no sex differences in MDA (GLMM $F_{1,80.5} = 0.29$, $P = 0.59$). In contrast to morphometric measures, variance in MDA was not significantly explained by identity of rearing parent ID or egg parent ID (random factors: egg parent $Z =$

Table 1. Output from GLMM testing effects of feeding treatments and sex on growth rate (mass gain per day) of nestlings between days 3 and 13

Random factor	Estimate	Wald's Z	P
Egg parent ID	$1.13 \times 10^{-3} \pm 6.2 \times 10^{-4}$	1.85	0.033
Rearing parent ID	$6.9 \times 10^{-4} \pm 4.3 \times 10^{-4}$	1.61	0.054
Residual	$3.4 \times 10^{-3} \pm 4.1 \times 10^{-4}$	8.44	< 0.0001
Main effects		$F_{\text{d.f.}}$	P
Egg treatment		$10.33_{1,25.2}$	0.0036*
Rearing treatment		$0.48_{1,15.1}$	0.499
Sex		$23.56_{1,160}$	< 0.0001*
Egg treatment × rearing treatment		$0.70_{1,154}$	0.403
Sex × egg treatment		$2.78_{1,160}$	0.0976
Sex × rearing treatment		$1.28_{1,152}$	0.260

Non-significant interactions shown below were removed from the model in stepwise fashion and values are given at point of removal. 'Egg treatment' and 'egg parent ID' refer to the biological parents, and 'rearing treatment' and 'rearing parent' refer to the treatment groups to which each nestling was cross-fostered.
*Significant main effects.

Table 2. Output from GLMM testing effects of feeding treatments and sex on mass in nestlings aged 14 days

Random factor	Estimate	Wald's Z	P
Egg parent ID	0.1285 ± 0.053	2.43	0.0076
Rearing parent ID	0.020 ± 0.025	0.83	0.203
Residual	0.301 ± 0.034	8.93	< 0.0001
Main effects		$F_{\text{d.f.}}$	P
Egg treatment		$0.69_{1,38.1}$	0.410
Rearing treatment		$4.78_{1,12.6}$	0.048*
Sex		$38.47_{1,183}$	< 0.0001*
Egg treatment × rearing treatment		$0.55_{1,167}$	0.460
Sex × egg treatment		$1.71_{1,174}$	0.193
Sex × rearing treatment		$1.81_{1,174}$	0.179

Non-significant interactions shown below were removed from the model in stepwise fashion and values are given at point of removal.
*Significant main effects.

0, P = n.a.; rearing parent Z = 1.17, P = 0.12; residual Z = 5.51, P < 0.0001). We added growth rate as an additional covariate in the model explaining lipid peroxidation and found faster growth was associated (if not significantly) with increased MDA (GLMM $F_{1,73.8}$ = 3.83, P = 0.054). It is notable that in spite of more rapid increase in body mass in nestlings from eggs laid by tocopherol-treated mothers that there was no treatment effect on MDA. It should be noted that MDA was only measured in a subset of nestlings (n = 90), where mass and growth rate were calculated for every bird (cross-fostered n = 184; all birds n = 417), and this might reflect an insufficient sample size. Alternatively, nestlings from eggs laid by tocopherol-treated mothers might have been better able to resist oxidative damage, although the interaction term, growth rate × egg parent treatment, was not significant when added to the model suggesting that the slope of the growth rate–MDA relationship did not differ among treatment groups.

During the course of the experiment, only 5 nestlings out of 203 from fostered nests died post-hatching, precluding an analysis of mortality in relation to treatment.

Note on multiple comparisons

Multiple testing was a necessary part of our experiment to uncover the impacts of supplementation of vitamin E on a wide range of behavioural, physiological and developmental traits. We made the decision not to adjust P-values for multiple comparisons in

Table 3. Output from GLMM testing effects of feeding treatments and sex on tarsus length in nestlings aged 14 days

Random factor	Estimate	Wald's Z	P
Egg parent ID	0.129 ± 0.051	2.54	0.0111
Rearing parent ID	–0.0012 ± 0.0098	–0.12	0.902
Residual	0.185 ± 0.020	9.07	< 0.0001
Main Effects		$F_{\text{d.f.}}$	P
Egg treatment		$8.24_{1,8.21}$	0.0063*
Rearing treatment		$7.03_{1,11.4}$	0.022*
Sex		$67.63_{1,172}$	< 0.0001*
Egg treatment × rearing treatment		$0.62_{1,163}$	0.431
Sex × egg treatment		$0.03_{1,171}$	0.858
Sex × rearing treatment		$4.41_{1,172}$	0.0372*

Non-significant interactions shown below were removed from the model in stepwise fashion and values are given at point of removal.
*Significant main effects.

Table 4. Output from GLMM testing effects of feeding treatments and sex on body condition in nestlings aged 14 days

Random factor	Estimate	Wald's Z	P
Egg parent ID	$2.9 \times 10^{-4} \pm 1.9 \times 10^{-4}$	1.57	0.058
Rearing parent ID	$8.1 \times 10^{-5} \pm 1.1 \times 10^{-5}$	0.76	0.224
Residual	$1.1 \times 10^{-3} \pm 1.2 \times 10^{-5}$	8.92	< 0.0001
Main Effects		$F_{\text{d.f.}}$	P
Egg treatment		$3.69_{1,17.3}$	0.071
Rearing treatment		$0.97_{1,20.9}$	0.335
Sex		$2.61_{1,184}$	0.108
Egg treatment × rearing treatment		$0.27_{1,168}$	0.601
Sex × egg treatment		$1.79_{1,181}$	0.183
Sex × rearing treatment		$0.013_{1,179}$	0.721

Non-significant interactions shown below were removed from the model in stepwise fashion and values are given at point of removal.

our analyses. The different responses we compared were planned and used to test scientifically credible hypotheses, given background literature on the effects of vitamin E/antioxidants. Furthermore, many of the individual response variables were correlated (e.g. body mass, growth rate, tarsus length and body condition, or clutch size, total egg volume, average egg volume and yolk volume), which effectively reduces the overall number of tests. We did, however, compare several traits at once in our two sets of analyses, which can increase the incidence of type 1 errors (false positives). Rather than reducing the number of tests, and missing potentially important but varied biological impacts of the supplement, or increasing the likelihood of a type II error although correction for multiple testing, we have interpreted all of our statistical outputs cautiously. We note that if we had chosen an extremely conservative Bonferroni transformation (with a P-value of 0.0083), the most important results of the study would have been upheld regardless.

DISCUSSION

In this experiment, we tested the impact of the varying availability of a dietary antioxidant during egg laying on maternal condition, parental investment, clutch size and quality, and offspring development and survival. We predicted that any effect of vitamin E would be most likely to reflect the benefits of antioxidant function specifically since α-tocopherol has a proven role as an antioxidant *in vivo*. We found no evidence for any benefit of the vitamin E supplement on female condition. Although the clutch size, clutch volume, incubation and feeding rates did not differ between treatment groups, there was an impact of vitamin E

supplementation on yolk mass in fifth laid eggs. The yolks of α-tocopherol-treated females were of greater mass, especially in females with smaller clutches, than those of controls. Female body condition was actually negatively correlated with yolk levels of vitamin E regardless of treatment. The supplementation also had a significant effect on the pattern of developmental rates of offspring, although in a manner that does not fit a clear prediction of a benefit to the supplement.

Our results showed that despite female and male breeding birds willingly consuming the food supplement there was no effect on reproductive output in terms of a total number of eggs or offspring fledged or on their body condition. We also assessed blood measures of physiological stress (glucose levels and heterophil/lymphocyte ratio) in females, and these were similarly unaffected by our treatment (see Supporting Information, S2). We are confident that our treatment was successful insofar as providing enhanced vitamin E to mothers: birds willingly consumed the supplement; the vitamin E was largely stable; and supplementing parents with α-tocopherol had significant impacts (regardless of their potential benefits or otherwise) on yolk mass and growth of resultant offspring. Paradoxically, although yolk mass was generally greater in α-tocopherol-treated females (at least in the fifth eggs) prior to cross-fostering, 3-day-old nestlings from eggs laid by α-tocopherol-treated females were significantly smaller than nestlings from control eggs. Reasons for this apparent contradiction are discussed below. Nestlings from eggs laid by α-tocopherol-treated females grew faster than nestlings from eggs laid by control females, but by day 14, there was no significant difference in mass mediated by treatment of egg-laying parents, indicating this was probably catch-up growth, as is often seen in smaller birds at hatching. Patterns of growth and development have been linked to vitamin E in wild birds before (de Ayala, Martinelli & Saino, 2006; Matrková & Remeš, 2014). In chickens, it has also been demonstrated that faster-growing breed lines have a higher demand for vitamin E than slower growing lines (Surai *et al.*, 2002) and α-tocopherol appears capable of preventing oxidative stress-induced growth retardation in chicken embryos (Satiroglu-Tufan & Tufan, 2004). Vitamin E deficiency in last laid eggs also limits the growth of yellow-legged gull chicks (Parolini *et al.*, 2015). In a study of great tits, nestlings from carotenoid fed mothers gained more mass between days 9–14 than nestlings from control parents (Berthouly *et al.*, 2008) although the difference only became visible at 14 days old. These studies suggest that vitamin E or other dietary antioxidants might be predicted to promote faster growth (and greater eventual size) or ameliorate growth-related costs in neonates. However, in our study, the faster-growing nestlings from α-tocopherol eggs weighed less on day 3 than nestlings from control eggs and caught up rather than attaining a larger size at fledging. It is difficult to see this as advantageous to the chicks and certainly does not indicate a demonstrable benefit, even if despite growing faster the nestlings from eggs laid by α-tocopherol-treated mothers did not have increased lipid peroxidation. There is often assumed to be a cost to 'catch-up' growth, potentially paid later in life (Metcalfe & Monaghan, 2001, 2003). This catch-up growth may be considered a cost rather than benefit of the treatment, although in terms of MDA, it is also possible that parental supplementation allowed chicks to resist this cost. We attempted to quantify survival costs for nestlings and their parents in this study, but recapture rates were too low to be conclusive (see Supporting Information).

We calculated growth rate from the change in mass between days 3 and 13. This captures variation in mass gain, but is only an approximation of the actual growth rate per day in terms of skeletal size. For example, nestlings from eggs laid by control females had longer tarsi prior to fledging than nestlings from eggs laid by α-tocopherol female. As eggs did not differ in any measured antioxidant markers, such a difference in offspring size/development cannot be explained by a negative physiological effect on the young birds (e.g. vitamin E toxicity at high doses). A possible explanation is that the supplement had some impact on the reproductive physiology or behaviour of the adult birds receiving the treatment (see below).

The shorter tarsus length we found was in contrast to a study of collared flycatchers in which vitamin E supplementation increased nestling tarsus size but not body mass (Matrková & Remeš, 2014). In addition, we found male nestlings raised by α-tocopherol-treated parents had significantly shorter tarsi than males raised by control-treated birds, regardless of origin. In blue tits, it has been suggested that tarsus length is a good measure of body condition and rearing conditions (Senar *et al.*, 2002). Our results could indicate that rearing conditions were poorer, at least for males, in the nests of α-tocopherol-treated adults. In part, as a result, nestlings from eggs laid by α-tocopherol-supplemented parents were in 'better condition' on day 14. Condition scores based on relationships between skeletal size and body mass are used to assess rearing conditions and survival probability in a range of bird species, but in one blue tit population, the survival probabilities of nestlings were shown to be dependent on body mass, and only indirectly by tarsus length (Råberg, Stjernman & Nilsson, 2005). Without further information on adult survival and fitness, we cannot conclude whether greater skeletal size or greater body mass per skeletal size is better. Thus, we have no clear evidence of a direct benefit to nestlings of parental α-tocopherol supplementation.

Our results may alternatively be explained by an unanticipated treatment effect on parent investment strategies. The hypothesis underlying our experiment was that, if reproduction and oxidative stress are linked, then reproductive investment will be shaped by current levels of dietary antioxidants. However, by providing a vitamin E supplement near to the nest site to manipulate these levels, it is also possible that we provided cues that mismatched perceived and true environmental quality. Although this is unmeasurable, it may explain some of our seemingly contradictory results, as both own state and perceived environmental quality may mediate investment decisions, especially in a trade-off between chick rearing and self-maintenance (for survival and future reproduction), but in different directions. Yolks, for example, were generally larger in supplemented than control mothers' fifth eggs, which is consistent with a straightforward positive effect of supplementation on investment. In contrast, supplemented parents produced smaller 3-day-old chicks, sustained lower growth rates in their own chicks than those achieved by control foster parents and produced fledglings with smaller tarsi than controls. If environmental quality were overestimated, then reduced provisioning effort may occur on the expectation of environmental compensation, in terms of prey quality over quantity. Although if so, at 4 days old, we found no such evidence of a treatment group difference in nestling provisioning rate or in proportion of caterpillars provided. Alternatively, supplemented parents may have invested more into clutch size than could ultimately be sustained by their immediate environment, as the supplements were removed just after egg laying. This is similar to a recent study on canaries *Serinus canaria* where a manipulation of antioxidant levels in parents prior to breeding influenced their timing of breeding, without benefit to reproductive success (Costantini *et al.*, 2015). An omission in our study was more detailed analysis of incubation behaviour, falling in the period between the end of the supplementation and chick data collection, when the mismatch of artificial and true environmental conditions occurred. While total incubation duration did not differ between treatment groups, incubation is costly to parents (Gorman & Nager, 2004) and incubation conditions known to play a role in determining embryonic growth and subsequent hatching mass (Kim & Monaghan, 2006). It is possible that knock on effects occurs at later reproductive stages, for example depositing more yolk, investing fewer resources in incubation or provisioning immediately post-hatching and allowing rapid catch-up growth in offspring (while investing more in self-maintenance), could represent an adaptive strategy in these perceived early-season conditions. While we are not able to determine the mechanisms involved, we do show that a manipulation of antioxidant availability at a critical stage of reproduction can have impacts within and among different stages of reproduction.

Egg effects (ID of genetic parents) explained some variance in all of our morphometric measures, where rearing environment did not. This, together with the pervasive impact of the feeding treatment of parents on their offspring development even in foster nests, suggests that some aspect of egg or nestling development was 'programmed' or manipulated prior to the cross-fostering. In chickens, carotenoid content in egg yolk is more important in determining circulating levels in chicks than the carotenoid content of their neonatal diet (Karadas *et al.*, 2005) and the effect of early antioxidant levels on antioxidant assimilation in later life has also been demonstrated in zebra finches *Taeniopygia guttata* (Blount *et al.*, 2003). Therefore, maternal allocation of antioxidants in eggs may be an adaptive strategy, improving the oxidative status of nestlings, regardless of post-hatching diet. In other studies of Parids, females supplemented with carotenoids increased carotenoid concentration in egg yolk, leading to a range of benefits for nestlings (Biard *et al.*, 2005; Helfenstein *et al.*, 2008). We found no treatment difference in tocopherol or carotenoid concentrations in yolks of fifth laid eggs (although the yolk was generally larger). However, yolk antioxidants may have been different in other eggs, especially since antioxidant levels in yolk can increase or decrease across the laying sequence and clutch sizes are highly variable in tit species (Hõrak, Surai & Moller, 2002; Biard *et al.*, 2005; Török *et al.*, 2007). Alternatively, other yolk constituents that impact size and development, for example hormones (Verboven *et al.*, 2003), may have been modified by females in response to α-tocopherol supplementation. In this study, we aimed to examine impacts on chick development, but a companion study sampling antioxidants and other constituents in the complete clutch would help to interpret our results.

We found no difference in MDA levels between nestlings from eggs laid by parents receiving the tocopherol and control treatments. If dietary antioxidants are limiting for reproducing birds, provision of the free-radical scavenging antioxidant, α-tocopherol, was predicted to allow increased investment in reproduction or lower oxidative costs for parents and their offspring. It is worth considering why this prediction was not clearly upheld. First, as suggested for carotenoids, perhaps benefits of supplementation of α-tocopherol may be attributed to functions unrelated to antioxidant action (Hartley & Kennedy, 2004). While a strong *in vivo* free-radical scavenger, α-tocopherol has received increasing attention for in its roles in immune responses and gene expression. How these may have been altered by our supplementation is impossible to conclude from our results, although we stress that these other

putative proximate mechanisms of tocopherol action would still be predicted to benefit the recipient birds. A further possibility is the idea that α-tocopherol was not limited in the natural diet of blue tits. Our previous results have shown that relatively high levels of α-tocopherol present in caterpillars in this population (Arnold *et al.*, 2010b). The high fledging and hatching success in the study year suggest high caterpillar densities. Repeating the experiment in more adverse conditions might have improved the ability to detect impacts of vitamin E. Indeed, it is worth noting that antioxidant defences, in general, are considered to have low energetic costs (Speakman & Garratt, 2014); thus, nutrition alone may not be limited to the prevention of oxidative damage in many contexts. Lastly, as proposed above, it is possible that the provision of extra antioxidants shifted the balance in the trade-off between current and future reproductive effort, if females receiving α-tocopherol invested in self-maintenance rather than the current reproductive output. Concentrations of other important yolk constituents, such as antibodies, are found not simply to reflect a passive correlation with maternal circulating levels at the time of deposition, but vary between mothers and with their condition and context. If such maternal investment is possible with antioxidants too, then, whether our manipulation enhanced mothers' immediate perception of the provisioning environment, or her own perceived longer-term prospects, or both, then it may have altered her investment into her current brood. Fitting this possibility, control parents invested most into the current brood, achieving greater hatching and fledging mass than date-matched supplemented parents. The unexpected negative correlation between maternal body condition and egg α-tocopherol levels, independent of treatment, may reflect similar trade-offs. Although the sample size was too small for a robust analysis, we did find an indication that α-tocopherol-treated birds may have survived better to reproduce in future years.

Supplementation with α-tocopherol, the putative membrane-bound, free-radical scavenging antioxidant, did not result in a demonstrable benefit for the parents receiving the supplement. Thus, our study did not find support for the idea that dietary antioxidants are limiting in reproducing blue tits in our population or that dietary antioxidants aid reproduction. Nevertheless, we found clear differences in the patterns of offspring growth attributable to the dietary treatment. These results also failed to support the idea that α-tocopherol offers a substantive benefit for the offspring of α-tocopherol-treated parents. Our results add to the growing recognition that the roles of dietary acquired antioxidants are complex and that attributing their benefits to particular physiological functions is a challenge for future research.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

S1. Supplementary food, calculation of a-tocopherol concentration, confirmation of vitamin E retention in lard.
S2. Physiological condition 95 measures.
S3. Nestling plumage 159 coloration.
S4. Recruitment of breeding 222 and juvenile birds.