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Differential effects of α-tocopherol supplementation on blue tit *Cyanistes caeruleus* mothers and offspring

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Running head: Vitamin E affects blue tit reproduction

Abstract

Oxidative stress may be a cost of reproduction. If dietary antioxidants can ameliorate oxidative stress, mothers supplemented with dietary antioxidants are predicted to be in improved condition and/or invest more antioxidant resources in reproduction than controls. Increased investment of antioxidants in eggs could also protect embryos or neonates from the damaging effects of reactive oxygen species (ROS). α-tocopherol is the most biologically active dietary antioxidant *in vivo*, but little is known about its impacts on reproduction in wild birds. 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. α-tocopherol supplementation had no effect on maternal condition or reproductive investment. However, effects on nestlings were evident: nestlings from α-tocopherol supplemented mothers were smaller at hatching but grew faster, and did not pay a cost of increased lipid peroxidation. This indicates a treatment effect on the supplemented parents, in terms of reproductive physiology and/or maternal investment, but no effect on chick fledging mass or success. Our results are congruent with research suggesting dietary antioxidants at biologically relevant doses might have multifaceted roles not limited to ROS-scavenging, and highlight the need for further research to disentangle the impacts of antioxidants and oxidative stress in reproductive events.

Key words:

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 and oxidative stress 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. Though 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 (Finkel & Holbrook, 2000; Larcombe et al., 2010a; Larcombe et al., 2008). Thus all animals have evolved an endogenous antioxidant system, augmented by a potentially limited supply of dietary antioxidants, to remove excess ROS before damage can accrue. Oxidative stress occurs where the production of pro-oxidants overwhelms the capacity to remove or neutralise 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, leading to the use of reproduction as a model on which to study the ecological and evolutionary impacts of physiological trade-offs involving antioxidants and oxidative stress (Alonso-Alvarez et al., 2004; Christe et al., 2011; Larcombe et al., 2010b; Metcalfe & Monaghan, 2013; Monaghan et al., 2009; Speakman & Garratt, 2014). However, despite the burgeoning interest in this field, to date the results of field studies demonstrating oxidative costs of reproduction have been equivocal and there remains a relative lack of experimental tests (Isaksson et al., 2011; Metcalfe & Monaghan, 2013; Speakman & Garratt, 2014; Stier et al., 2012).

The associations between breeding, antioxidants and oxidative stress can arise through different mechanisms. In birds, reproduction, egg formation, egg incubation, and offspring rearing (Hodum et al., 1998; Weimerskirch et al., 2003) are all associated with increased metabolism (Alonso-Alvarez et al., 2004) and although the generality of the relationship between metabolic rate and oxidative stress has recently been questioned (Arnold et al., 2015; Arnold et al., 2007; Salin et al., 2015; Speakman et al., 2015) increased metabolic rate has long been linked to accumulated oxidative damage. Reproduction may

also increase levels of physiological stress suffered by mothers (Romero et al., 1997), and the stress response and oxidative balance are likely to be associated (Monaghan & Spencer, 2014; Sahin & Gumuslu, 2007). Given the major oxidative costs that are suggested to be associated with 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.

Oxidative damage in reproducing animals can have impacts beyond limiting the number of offspring produced. While reproductive effort and oxidative damage may be correlated, in a meta-analysis breeders showed overall lower levels of oxidative damage than non-breeders (Blount et al., 2015). The hypothesized explanation for this seemingly paradoxical result is that the deleterious effects of oxidative damage in mothers on their offspring's health and development are so strong that an overall decrease in maternal oxidative damage is necessary as an "oxidative shield" for their offspring. Developing embryos and neonates will be especially vulnerable to oxidative damage as rapid growth produces high quantities of ROS that potentially damage tissue (Tsunekage & Ricklefs, 2015), and egg yolk is particularly high in polyunsaturated fatty acids (PUFAs) that are highly susceptible to attack by ROS (Cherian and Sim, 1997). This oxidative shielding might therefore be considered a maternal effect: an impact of parental phenotype on the offspring's phenotype, holding genetic sources of variation constant (Kirkpatrick & Lande, 1989). Maternal effects are of great interest given growing understanding of the pervasive effects of early development on long term fitness (Lindstrom, 1999) including oxidative stress (Costantini et al., 2012). In birds, maternal nutritional status has been shown to affect egg size (Nager et al., 2000) as well as 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 (Giraudeau et al., 2016; Navara et al., 2006). 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 like blood, brain and livers (Surai et al., 1998; Surai et al., 1996). By allocating extra antioxidants into yolk a female may improve or alter the health or condition of her nestlings even post-hatching. Antioxidants that are deposited into egg yolks

are often dietary-acquired; including carotenoids and vitamin E (Deeming & Pike, 2013), offering another trade-off between dietary antioxidants and reproductive effort if egg quality is limited by the availability of these dietary antioxidants before egg laying.

Most studies investigating trade-offs between dietary antioxidants and reproduction have focussed on carotenoids, a class of lipophillic antioxidants. For example, experimental manipulations of carotenoid levels in eggs, either indirectly via the mother (Berthouly et al., 2008; Biard et al., 2005; Remes et al., 2007; Surai et al., 2003) or by direct injection into the yolk (Marri & Richner, 2014; Saino et al., 2003), have shown that carotenoids can reduce oxidative susceptibility (Blount et al., 2002) as well as improving offspring immunity (Biard et al., 2005; Leclaire et al., 2015; Saino et al., 2003) 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, especially when oxidative stress or damage has not been measured directly. Indeed, carotenoids could be considered relatively minor antioxidants in birds (Costantini & Møller, 2008). In order to elucidate the role of antioxidant availability specifically, supplementing a nutrient with a proven role in antioxidant defence *in vivo*, and no direct role in pigmentation or cell signalling is valuable. In this study we provided birds with the antioxidant α-tocopherol, a biologically active form of vitamin E (Costantini, 2008; Machlin, 1991; Sies & Murphy, 1991). Vitamin E is the major lipophillic antioxidant involved in membrane defence (Tappel, 1962), deficiencies in vitamin E are associated with a range of illnesses and disorders in many taxa (Zingg, 2007), and its effects are attributed to its antioxidant properties specifically (Traber & Atkinson, 2007) . Data from poultry science suggest widespread beneficial effects of supplementary vitamin E for birds (Surai, 2002). Though 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). Though supplementation to nestlings directly improved growth rate of barn swallows *Hirundo rustica* and tarsus length of collared flycatchers *Ficedula albicollis* (Matrková & Remeš, 2014) , as well as the fledging success of great tit *Parus major* (Maronde & Richner, 2014), it has previously been shown to have 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 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 nestbox-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 two 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 130x130x50mm green mesh suet feeder (Haiths, Cleethorpes, UK) was installed on a branch, sapling or trunk within 3m (but usually less than 1.5m) of that nest box, to habituate the parent birds to the

presence of feeders. Visits continued every two days until nests were half built (having a visible but unlined nest cup), at which point feeders were stocked with approximately 125g 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 a-tocopherol supplement delivery was based on methods established at the site (Ramsay & Houston, 1998) and designed to provide a biologically relevant dose of 0.37mg additional α-tocopherol (or an increase of ~30% of normal daily intake) to supplemented birds (see supplementary material S1 for further details). Supplements were replaced every 2 days to ensure freshness and that the α-tocopherol did not oxidize. Feeders were removed when incubation commenced, and no new eggs had been laid for two 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 1st egg: $F_{1,69} = 0.259$ $p = 0.613$. Mean total treatment durations (days ± *S.E.*): control 15.09 ± 0.57 α-tocopherol 15.31 ± 0.72 Mean treatment duration before 1st egg (days ± *S.E.*): 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 -20 ºC until analysis. To assess female reproductive effort we counted all of the eggs in the nest. 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 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 had hatched), broods were undisturbed until the cross-fostering when nestlings were three 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. Whilst 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 minutes. For broods with no suitable nest pairing of 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, 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 nestbox 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 0.1g). 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 ESM S2).

Egg yolk antioxidant analysis

We used the 5th 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 whilst also maximising sample size (most females lay at least five eggs in our population).We measured carotenoid and α-tocopherol content using HPLC. Eggs were frozen at -40 ºC 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 0.001g, then placed in an eppendorf and an equal volume distilled water was added to each and they were then homogenised. Antioxidant extraction was then achieved using previously outlined methods though substituting 200µl of yolk water solution for plasma.(Larcombe et al., 2008). HPLC and dat analysis were then 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. Black and white video cameras (50x50x20mm) 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 1200hrs The cameras were connected to a videocassette recorder (VCR) in a waterproof box that was camouflaged with forest litter to reduce disturbance around the nest area. 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 parental 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 colouration on day 14, just prior to fledging. 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 g/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 (Arnold et al., 2007; Griffiths et al., 1998). The remaining blood was collected in 75 µl heparinised capillary tubes. The capillary tubes of blood for 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 ESM S3). Birds were removed from their nests for no longer than one hour. 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 also 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 so is not reported in the main text (see ESM S4)

Nestling oxidative damage analyses

In order to assess the effect of supplemental feeding treatment on oxidative stress, malonidialdehyde (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. 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 we had anticipated from the 94 starting nests, for the analyses that did not involve offspring or parent condition and phenotype we performed statistical tests on the cross-fostered nests alone, and then with data from all nests to augment the sample size. This only applies where we had reasonable grounds to assume the cross-fostering would have no effect (i.e. pre cross-fostering procedures like clutch size, egg volume and incubation) 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: female body condition, clutch size, clutch mass, total egg volume and fledging success. Treatment was entered as a fixed factor model, and hatching date as a covariate in every model. Since some birds took less time than others to complete nest building and begin egg laying following the treatment, the number of days of treatment before egg laying commenced was entered as a covariate in models. Initially, the interactions treatment*hatching date and treatment*treatment duration were included to account for treatment differences at different points in the breeding season, or after receiving the treatment for longer. However these terms were never significant and therefore were not included in final models. Yolk antioxidant concentrations were analysed using GLMs with total yolk carotenoid and total yolk tocopherol concentration as dependent variables. Measures of reproductive output (clutch size egg volumes, antioxidant concentrations) were 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). 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. 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 standard error 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) or 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, 15953.15 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, prior to cross fostering highlighting the suitable matching of for every pair.

In fifth eggs, there were differences in the mass of yolks attributable to treatment. Although overall α-tocopherol treated females had 5th 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 that there is 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 marginally non-significant trend for heavier females to lay 5th 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. 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 there was a significant relationship between yolk antioxidants and female body condition. Figure 1 shows that there was a 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 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 (GLM, $F_{1, 15} = 1.68$, $p = 0.215$). 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. Also, there were no treatment differences in number of feeds per nestling (feeds per nestling per 2 hours: α-tocopherol: n = 12, mean 7.03 ± 1.16; control: n = 17, mean 5.76 ± 0.25, GLM, $F_{1, 28} = 0.39$, $p = 0.54$) in the two hour period they were observed. Including data from non-cross fostered nests to enhance the sample size (n = 29) did not change the results

(Feeds per 2 hours. 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, Figure 2 a). Mass gain between days 3 and 13 was then faster for these nestlings, than nestlings whose egg parents received control treatment (see Figure 3.1b; 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 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; Figure 3a). The identity of both rearing parent and egg parent explained variance in mass gain between days 3-13, indicating that growth rate is determined both by genetic 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 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). Whilst 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 (Figure 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, Figure 3.1c; Table 3). As body mass was not impacted by egg parents' treatment, though 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). 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 = 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 with increased MDA (GLMM $F_{1, 73.8} = 3.83$, p = 0.054). It is notable that in spite of faster growth 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, where mass and growth rate were calculated for every birds 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, though the interaction term growth rate*egg parent treatment was not significant when added to the model suggesting 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.

Discussion

In this experiment, we tested the impact of 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 reflect the benefits of antioxidant supplementation specifically, since α-tocopherol has a more limited role as a strict antioxidant *in vivo* than carotenoids; another class of lipophilic dietary antioxidants with widespread reported benefits and multifaceted physiological roles. We found no evidence for any benefit of the vitamin E supplement on female condition. Although 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 pre- and post-hatching.

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 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 ESM S2). We are confident that our treatment was 'successful', as birds consumed the supplement, and supplementing parents with α-tocopherol during egg laying had significant impacts on the yolk size, and growth and body condition of resultant offspring, compared with controls. Paradoxically, though 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. Patterns of growth and development have been linked to vitamin E in wild birds before (de Ayala et al., 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) though 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. In our study the faster growing nestlings from α-tocopherol eggs weighed less on day 3 than nestlings from control eggs, rather than attaining a larger size at fledging. However, despite growing faster the nestlings from eggs laid by α-tocopherol treated mothers did not pay an increased cost in terms of lipid peroxidation: MDA levels were identical between treatment groups, despite a link between faster growth and increased MDA levels in blue tit nestlings in this and other experiments in the population (Larcombe et al. 2010). This might indicate a protective effect of the treatment though it is difficult to determine whether the greater rate of growth of nestlings from eggs laid by α-tocopherol supplemented birds was advantageous to these nestlings or their parents. There is often assumed to be a cost to "catch-up" growth (Metcalfe & Monaghan, 2001; Metcalfe & Monaghan, 2003) but it is also possible that higher quality individuals can resist these costs. We attempted to quantify survival costs for nestlings and their parents in this study but re-capture rates were too low to be conclusive (see ESM).

We inferred growth rate from differences in mass between days 3-13. Although this means nestlings differed in their mass gain per day, this only offers an approximation of actual growth rate. Indeed, nestlings from eggs laid by control females had longer tarsi prior to fledging than nestlings from eggs laid by α-tocopherol female. This is in contrast to a study of collared flycatchers in which vitamin E supplementation to nestlings increased tarsus size but not body size (Matrková & Remeš, 2014). In addition, 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. However, despite being skeletally smaller, 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 have also been 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 (Raberg et al., 2005). Since benefits or otherwise of greater skeletal size vs greater body mass per skeletal size are difficult to interpret, it is unclear whether skeletally smaller birds with higher condition scores were actually in better condition or not. Certainly, any prediction of a clear benefit for nestlings from eggs laid α-tocopherol treated parents was not confirmed. However, results for body mass and growth indicate that some aspect of nestling development was strongly altered by our feeding treatment suggesting an impact of tocopherol on reproductive physiology or behaviour of the adult birds receiving the treatment.

The hypothesis underlying our experiment was that, if reproduction and oxidative stress are linked, then reproductive investment will be shaped by current levels of oxidative damage and/or 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. This 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 straight forward 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. Though 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. Whilst 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 occur 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. Whilst 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 (though the yolk were generally larger). It should be stressed that the absence of an effect in 5th laid eggs does not certify that yolk antioxidants were identical between treatment groups: it is impossible to perform a non-destructive assay of yolk contents for entire clutches, and 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 (Biard et al., 2005; Hõrak et al., 2002; Török et al., 2007). Yolk α-tocopherol may still have been responsible for the differences in growth rate and size observed in this experiment. Alternatively, other yolk constituents that impact size and

development e.g. hormones (Verboven et al., 2003) could have been modified by females in response to α-tocopherol supplementation. Unfortunately, not all aspects of egg composition can be measured at once, and we can only speculate as to whether eggs in this study varied in any unquantified way.

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. Firstly, 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 are present in caterpillars in this population (Arnold et al., 2010b). The high fledging and hatching success suggest a relatively good year for the breeding population, which in turn could be evidence of high caterpillar densities. Repeating the experiment in more adverse conditions might have improved the ability to detect impacts of vitamin E when existing dietary availability may have been more limiting. Secondly, for taxa with diets that are relatively abundant in dietary antioxidants, the contribution of these nutrients to the overall oxidative balance may be low. Indeed, it is worth considering that antioxidant defences in general are considered to have low energetic costs (Speakman & Garratt, 2014) such that nutrition alone is unlikely to be limiting in terms of the prevention of oxidative damage. We predicted that our experiment would allow an assessment of the benefits of antioxidant protection specifically. Perhaps many of the benefits previously attributed to putative antioxidants, such as vitamin E or carotenoids, were unrelated to a strict antioxidant function (Hartley & Kennedy, 2004). The links between the immune system, metabolism, physiological and oxidative stress, and reproduction are likely to be extremely complex and multifaceted and the mechanisms by which individual dietary-acquired nutrients alter them may be equally complex. 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, in contrast, invested most into the current brood, achieving greater hatching and fledging mass than date-matched supplemented parents. Similarly, we could speculate that the unexpected negative correlation between maternal body condition and egg α-tocopherol levels, independent of treatment, reflects natural variation in this trade off, with mothers in better condition more likely to survive to the following breeding season and those in poorer condition investing into current brood. We did attempt to assess the survival and breeding effort of α-tocopherol and control treated adult birds in the following breeding season (ESM). Although the sample size was too small for a robust analysis, we did find some indication that α-tocopherol treated birds survived better to reproduce in future years.

Supplementation with α-tocopherol, the principal membrane-bound, free radical scavenging antioxidant, did not result in a demonstrable benefit for the parents receiving the supplement. Thus, overall our study did not find support for the idea that dietary antioxidants are limiting in reproducing blue tits in our population. Nevertheless, we found clear differences in the patterns of offspring growth attributable to the dietary treatment, indicating the treatment was 'successful'. These result supports previous suggestions that the oxidative status of adult birds might impact reproductive decision making or physiology, and of an important role for α-tocopherol in the development of neonatal birds. 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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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)).

Figure 2 Mean (± 1 S.E.) 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 3-13; **c)** Body condition of nestlings aged 14 days (residuals of ln (mass) on 3*ln (tarsus) **d)** MDA concentration.

Figure 3 Differences (Mean ± 1 S.E.) 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 α-tocopherol, or control treated parents

Table 1 Output from GLMM testing effects of feeding treatments and sex on growth rate (mass gain per day) of nestlings between days 3-13. 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' refers to the biological parents and 'rearing treatment' and rearing parent' refers to the treatment groups to which each nestling was cross-fostered. Significant main effects are marked *.

Random factor	Estimate	Wald's Z	P
Egg parent ID	$1.13x10^{-3} \pm 6.2\ x10^{-4}$	1.85	0.033
Rearing parent ID	$6.9\ x10^{-4} \pm 4.3\ x10^{-4}$	1.61	0.054
Residual	$3.4\ x10^{-3} \pm 4.1\ x10^{-4}$	8.44	<0.0001
Main Effects		$F_{d.f.}$	*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 x Rearing treatment		$0.70_{1,154}$	0.403
Sex x Egg treatment		$2.78_{1,160}$	0.0976
Sex x Rearing treatment		$1.28_{1,152}$	0.260

Table 2 Output from GLMM testing effects of feeding treatments and sex on mass in nestlings aged 14 days. Non-significant interactions shown below were removed from the model in stepwise fashion and values are given at point of removal. Significant main effects are marked *.

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		$F_{d.f.}$	*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 x Rearing treatment		$0.55_{1,167}$	0.460
Sex x Egg treatment		$1.71_{1,174}$	0.193
Sex x Rearing treatment		$1.81_{1,174}$	0.179

Table 3 Output from GLMM testing effects of feeding treatments and sex on tarsus length in nestlings aged 14 days. Non-significant interactions shown below were removed from the model in stepwise fashion and values are given at point of removal. Significant main effects are marked *.

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		$F_{d.f.}$	*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 x Rearing treatment		$0.62_{1,163}$	0.431
Sex x Egg treatment		$0.03_{1,171}$	0.858
Sex x Rearing treatment		$4.41_{1,172}$	0.0372*

Table 4 Output from GLMM testing effects of feeding treatments and sex on body condition in nestlings aged 14 days. Non-significant interactions shown below were removed from the model in stepwise fashion and values are given at point of removal. Significant main effects are marked *.

Random factor	Estimate	Wald's Z	P
Egg parent ID	$2.9x10^{-4} \pm 1.9 x10^{-4}$	1.57	0.058
Rearing parent ID	$8.1 x10^{-5} \pm 1.1 x10^{-5}$	0.76	0.224
Residual	$1.1 x10^{-3} \pm 1.2 x10^{-5}$	8.92	<0.0001
Main Effects		$F_{d.f.}$	*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 x Rearing treatment		$0.27_{1,168}$	0.601
Sex x Egg treatment		$1.79_{1,181}$	0.183
Sex x Rearing treatment		$0.013_{1,179}$	0.721

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Supplementary Material

Larcombe et al. Differential effects of α-tocopherol supplementation on blue tit *Cyanistes caeruleus* mothers and offspring

S1 Supplementary food and calculation of a-tocopherol concentration

Blue tits are territorial and exclude conspecifics from the vicinity of the nest site, ensuring that control feeders were not accessible to blue tits at neighbouring supplemented nest boxes or vice versa. The protocol for the targeted manipulation of wild blue tit diets was developed in a previous study at the site, in which lard was instead supplemented with egg (Ramsay & Houston, 1998). Lard is a highly desirable resource (Allen & Harper, 2000), and observations of colour ringed individuals from a 11 control and 11 manipulated nestboxes confirmed that the parent birds did indeed eat the lard (0.5-7 visits/hour, 2hr observation on the last day of manipulation).

We added 250 mg α-tocopherol per kg of lard to supplemented birds in order to provide a biological relevant dose. Overall, the difference in daily α-tocopherol intake between the control and manipulated groups should be greater than variation within the manipulated group. The natural spring diet of blue tits is primarily caterpillars, and daily intake of around 13.5 g caterpillar per day (Crocker et al., 2002) provides adults with 1.35 mg tocopherol (Arnold et al., 2010). Blue tit daily calorie intake, calculated for wintering adults, is around 1 kcal/g body weight; equivalent to 9.8 g lard (Allen & Harper, 2000). It is unlikely that blue tits consumed only lard, as wintering blue tits provided with stable, high-energy artificial feeding site continue to prospect for new feeding opportunities (Herborn et al., 2010), and parents provided with nest-side supplements still provision their chicks with around 70% natural food (Cowie & Hinsley, 1988). Assuming the proportions of artificial food in their own diet are similar, adult consumption of just 3.3 g lard (30% of calorie requirement) and an accompanying one third reduction in caterpillars would increase daily α-tocopherol intake by 0.37 mg from the natural diet, or around 28%. In contrast, control birds replacing around one third of their natural diet with unsupplemented lard would be expected to lose one third of their daily α-tocopherol intake, i.e around 0.45 ng. These changes are within a biologically reasonable range.

To test whether birds were eating Control and vitamin E lard at similar rates, feeder and observational choice data were analysed. When the food was replaced every two days, between mid-nest building to the start of incubation, the areas of pecked lard were scored:

Thereafter, the food was changed every two days and the amount of lard eaten would be scored on a scale from zero to three. Zero represented no visible peck marks, one represented anything from one peck to an area of five centimetre square of pecking or equivalent (i.e. less than this if areas were deep), two represented five to ten centimetre square of pecking or equivalent and a three represented more than ten centimetre square of pecking or equivalent. Since food had been available for different lengths of time at each nest, the median peck score for each feeder was calculated and square root transformed median scores of control and vitamin E feeders were compared using a independent sample t-test ($t = -1.512$, n; control = 60, vitamin E = 60, $df = 118$, $p = 0.868$). There was no significant difference between median scores of control and vitamin E feeders. So both treatment groups used the feeders at a similar rate over the period of the manipulation.

In addition at the beginning of the incubation period, a choice experiment was conducted to establish if birds were able to differentiate between control and vitamin E enriched foods and whether they had a preference for either type of food. During the choice experiment, a randomly selected subset of blue tit pairs was given the choice between control and vitamin E enriched lard simultaneously. Two feeders containing lard (one with control lard and one with vitamin E enriched lard) were placed side by side in the same place as the treatment lard had previously been placed to aid discovery of the feeders. There was no significant difference between total peck scores over the 24 hour period that 'choice' feeders were present (Paired sample t-test of square root transformed peck scores: $t = -0.891$, $n = 22$, $df = 21$, $p = 0.383$).

It was not possible in this study to calculate precisely the quantity of food consumed by each bird. Lard supplements were also consumed by sympatric bird species, preventing direct calculation of intake from food weight. Moreover, α-tocopherol intake cannot be determined by comparison of baseline to post-consumption blood samples for two reasons. First, the rate of absorption of α-tocopherol into the blood stream and then deposition into tissues, hence window for sampling post-lard ingestion, is unknown. And second, natural levels of α-tocopherol are expected to vary daily with dietary intake and caterpillar availability, making comparisons between ‘baseline’ levels prior to manipulation and elevated levels during manipulation unreliable. Moreover, capture for ‘baseline’ measurement is extremely challenging before birds have laid eggs, and repeated capture during incubation increases the probability of nest failure.

S2 Physiological condition measures

To assess physiological condition of mothers and to gain an indication of their 'stress' levels, we measured both blood glucose and heterophil to lymphocyte ratios.

Baseline plasma glucose levels are predicted to reveal something about the nutritional status of an individual but they will probably respond quickly to capture stress, probably increasing. Thus we will needed to measure glucose as soon after capture as possible. Birds in poor condition and/or which have invested heavily in reproduction are predicted to have lower baseline glucose levels (but see Ruiz et al. 2002)

During immunosupression, the number of circulating lymphocytes goes down and the number of heterophils goes up, which makes the host more susceptible to viral infections (Siegel 1985). This change in the immunological function is called lymphocytosis (Siegel 1985). The ratio between heterophils and lymphocytes (H/L ratio) is known to increase in response to various stressors and has long been use to estimate stress in poultry (Gross & Siegel 1983 ; Maxwell 1993), but has also been used to estimate levels of stress in wild bird population ((Horak et al., 1998; Kilgas et al., 2006a; Kilgas et al., 2006b). Kilgas et. al. (2006) found that higher H/L ratio during breeding was negatively correlated to survival probability in great tits. Other studies have found that the H/L ratio is higher in great tits making a more intense reproductive effort (Horak et al. 1998 ; Ots & Horak. 1996). The H/L ratio has also been found to increase with environmental stressors such as heat stress and starvation (Maxwell 1993). Birds living in an urban environment or in captivity also show higher indices of stress including H/L ratio (Ruiz et al. 2002). Therefore the H/L ratio seems to be a reliable measure of chronic stress, indicating that birds are experiencing physiological or psychological stress (Siegel 1985).

At day 5 or 6 post hatching females were caught (see main paper) and we used venipuncture from a wing vein to collect a small drop of blood for a glucose test and to make two air-dried blood smears. Blood was collected within three minutes of capture, since indices of stress can change very quickly in response to handling (Le Maho et al., 1992). Total handling time was minimized and most birds were released within ten minutes. We used a "blood glucose monitoring system" (OneTouch Ultra, Lifescan UK, Bucks, UK) for glucose measurement, designed for on-the-spot blood glucose testing by human diabetics, but used also in studies of avian stress (e.g. Ruiz et al., 2002). Blood smears were stained with a Giemsa stain to allow differentiation and identification of different blood cells. The Heterophil to Lymphocyte (H/L) ratio was determined by examining the air dry blood smears under a light microscope at 1000× oil immersion. A total of at least 200 lymphocytes were counted systematically (to

avoid counting the same cell twice) including lymphocytes, heterophils, monocytes, basophils and eosinophils. The number of heterophils counted was then divided by the number of lymphocyte counted to obtain the H/L ratio. For each bird the better quality of the two blood smears was analysed, and ten slides were examined twice in order to assess repeatability. H/L ratio was highly repeatable within individuals (Pearson correlation; 0.754, n = 11, p = 0.007).

There was no significant difference in measures of physiological condition (heterophil/lymphocyte ratio and glucose concentration) between α-tocopherol and control fed females, when chicks were 5 days old (multivariate GLM, $F_{2,20} = 1.218$ p = 0.317). There was an effect of hatching date on female with birds breeding later in the season having a higher H/L ratio (univariate GLM $F_{1,25} = 4.882$, p = 0.037) and lower glucose concentrations (univariate GLM $F_{1,25} = 4.128$, p = 0.053 Figure S1). There was no effect of total clutch volume (p=0.135), or female mass (p=0.744) on either measure

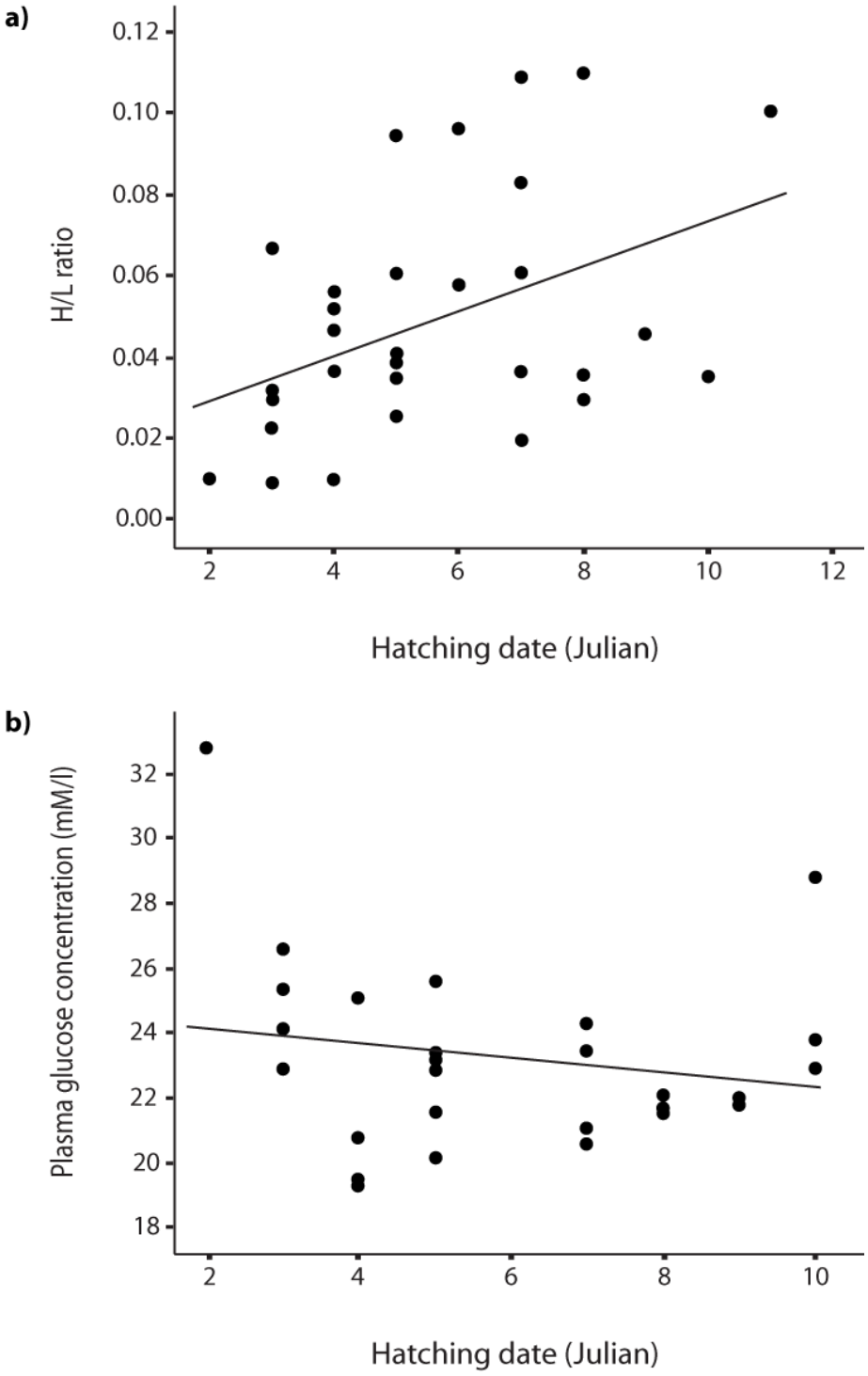


Figure S1 a) Heterophil to lymphocyte ratio increased with brood hatching date in breeding females, and b) glucose concentration decreased with brood hatching date when chicks were 5 days old. Day 1 = 19th May.

There was no difference between treatments in any measure of indicators of stress. We predicted that oxidative stress might limit reproductive effort through links with the physiological stress response. However, there was a significant positive relationship between

blood glucose and hatching date, and a significant negative relationship between heterophil to lymphocyte (H/L) ratio and hatching date. This finding is contrary to some other studies, where stressed birds with a higher H/L ratio, had a higher concentration of blood glucose (Ruiz et al., 2002). High glucose levels may be a sign of acute stress, since glucose is mobilized by corticosterone as part of the stress response. However, blood glucose concentration is known to decrease during fasting or starvation (Savory and Smith, 1987) and the heterophil to lymphocyte ratio is known to increase during starvation (Maxwell, 1993). In this study, both measures may reveal the feeding status of a bird, as opposed to an indicator of acute stress. This explains why the two measures were not positively correlated: as the breeding season progresses and food becomes more scarce, breeding females are under a greater starvation threat, reflected by higher H/L ratio and lower glucose levels.

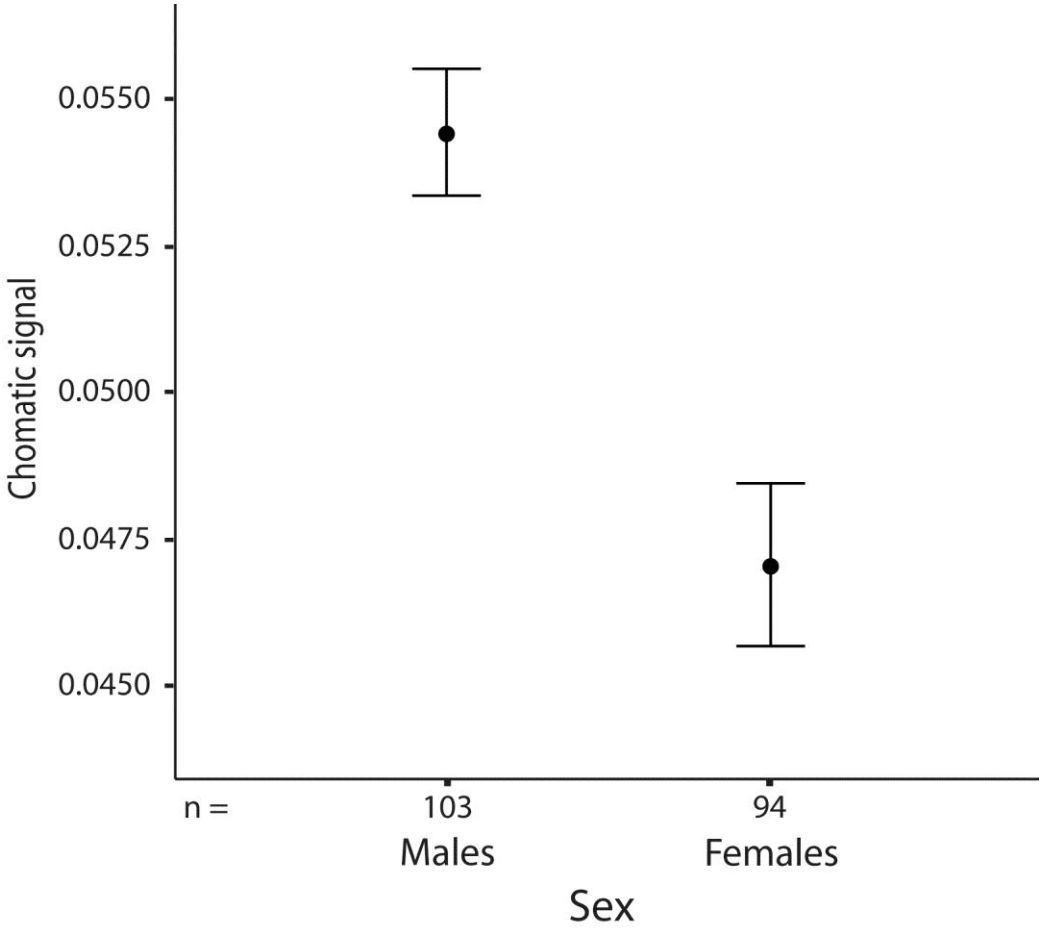
S3 Nestling plumage colouration

In order to assess whether nestlings from parents of either treatment differed in plumage colouration, plumage reflectance was analyzed using the SPEC package (http://www.bio.ic.ac.uk/research/iowens/spec). The SPEC program multiplies cone sensitivities by the reflectance spectrum from the plumage patch (Hadfield & Owens, 2006). This is done for every wavelength to which the cones are sensitive, and these values are then summed for each cone type, to give four quantal cone catches; UVS (Ultraviolet sensitive), SWS (Short wavelength sensitive), MWS (Medium wavelength sensitive) and LWS (Long wavelength sensitive) (Vorobyev & Osorio, 1998). A mean quantal cone catch was calculated from the two readings taken for each plumage area.

In this experiment we used this quantal cone catch from the MWS and LWS cones to analyse carotenoid mediated colour of yellow chest plumage and the UV portion of both chest and crown feathers, since there is considerable interest in how the UV portion of the spectra affects social signalling in blue tits (e.g.(Korsten et al., 2006). From the chest plumage the LWS chromatic signal and the UVS chromatic signal were analysed. From the head feathers, the UVS chromatic signal was analysed. Thus, spectral data from the LWS, MWS, SWS and UVS cones were used for the crown plumage and the UVS, and SWS cones were used for the head plumage. Analyses of chromatic cues permit the distinction of stimuli of different spectral composition regardless of intensity, typically achieved by chromatic opponency of signals from photoreceptors chromatic signals with the following formulae using the quantal cone catches calculated by SPEC (Osorio et al., 1999) : 1) Chest chromatic signals: a) LWS chromatic signal = (LWS - MWS) / (LWS + MWS) b) UVS chromatic signal = (UVS – SWS)/(UVS + SWS; Head feather chromatic signals a) UVS chromatic signal = (UVS – SWS)/(UVS + SWS); b) SWS chromatic signal = (SWS – MWS)/(SWS + MWS).
Males had a greater LWS chromatic signal (relating to yellow chest plumage) than females (GLMM $F_{1, 191} = 19.38$, $p < 0.0001$, Figure S2), however, there were no effects of either treatment on chest LWS chromatic signal. There was no effect of sex (GLMM $F_{1, 173} = 0.12$, $p = 0.729$), rearing treatment (GLMM $F_{1, 17.9} = 0.27$, $p = 0.61$), or egg treatment (GLMM $F_{1, 58.8} = 0.41$, $p = 0.527$) on chest UVS chromatic signal. Interestingly, variation in MWS chromatic signal of chest plumage was significantly affected by ID of rearing parents, but not by ID of egg parents (random factors: egg parent, $Z = 0.32$, $p = 0.374$; rearing parent $Z = 1.85$

p = 0.0321), whereas UVS chromatic signal of chest plumage was affected by ID of egg parents, but not by ID of rearing parent (random factors: egg parent Z = 2.85, p = 0.0022, rearing parent Z =0.23, p = 0.410). Thus some aspects of colouration are likely influenced by genetic effects, whereas others are influenced by the rearing environment.

Neither aspect of crown plumage measured was significantly affected by egg parent treatment (UVS chromatic signal GLMM $F_{1, 30.1} = 0.06$, p = 0.802, SWS chromatic signal GLMM $F_{1, 194} = 0.82$, p = 0.37), rearing parent treatment (UVS chromatic signal GLMM $F_{1, 184} = 2.08$, p = 0.108, SWS chromatic signal GLMM $F_{1, 195} = 1.41$, p = 0.23), or by sex (UVS chromatic signal GLMM $F_{1, 184} = 0.24$, p = 0.625, SWS chromatic signal GLMM $F_{1, 193} = 0.95$, p = 0.33). Crown SWS chromatic signal was not affected by any aspect of treatment or sex (most significant term: treatment of rearing parent GLMM $F_{1, 195} = 1.41$, p = 0.24). Crown UVS chromatic signal was not affected by treatment or sex (most significant term: treatment of rearing parent GLMM $F_{1, 184} = 2.6$, p = 0.11). Neither random factor contributed to either crown UVS (random factors: egg parent Z = 0.79, p = 0.427, rearing parent Z =-0.12, p = 0.902) or SWS (random factors: egg parent Z = -0.08, p = 0.934, rearing parent Z =-0.02, p = 0.986) chromatic signals.



In tit species, several studies have shown an effect of common rearing environment on carotenoid mediated colour, in agreement with our results for the yellow component of chest plumage (Biard et al., 2005; Fitze et al., 2003a). This further supports the idea that carotenoid based plumage colouration is determined sometime during early post hatching development (Fitze et al., 2003b). Conversely, the UV portion of chest plumage was affected by egg effects but not rearing environment. Although carotenoid plumage spectra typically have a peak in the UV, as well as long wavelengths, there is some evidence that the mechanism for the UV colour is not carotenoid pigmentation (Prum, 2006). The results here are in agreement with this suggestion. Interestingly, neither component of crown plumage measured were significantly affected by any aspect of egg quality, rearing environment or feeding treatment.

S4 Recruitment of breeding and juvenile birds

In order to assess parental survival and F1 recruitment, adults were trapped on the nest during the 2007 breeding season. Nest boxes were checked weekly for signs of nest building, then every 3 days for egg-laying once nests were fully built. When no new eggs were laid for two consecutive days, the final clutch size was noted. During incubation females were inspected for existing leg rings by observation either on or off the nest. Females were caught when their chicks were 5 days old. All ringed or unknown females within the study site were caught to assess female recruitment from previous years within our population. Reproductive success was noted for female birds that had been ringed a previous year. Clutch size, hatching success and fledging success were recorded. We also attempted to mist-net survivors during the winter 2007/2008, but with only one 2006 adult female captured, could not distinguish survival from the possibility that the spring population over-wintered elsewhere.

Breeding data from re-trapped mothers, and female F1s in 2007 can be seen in Tables S1 and S2 respectively. The sample size for both F1s and adults is too small for statistical analysis, but there were more α-tocopherol fed adult females (5) reproducing again in 2007, than control birds (3). They also appeared to successfully fledge more chicks in 2007 (means +/- S.E: Control 6.33 +/- 3.17 α-tocopherol 9.6 +/- 1.07) . Though a very small samples size, this might indicate a benefit to the vitamin E supplementation that was only apparent the following year, perhaps due to increased investment in self maintenance following supplementation. There were no apparent differences in either recruitment or breeding success of chicks from either control or α-tocopherol fed parents in 2007. In winter of 2007/2008 mistnetting was used to catch overwintering birds in our population. This data concerns male and female chicks from this study, and can be seen in Table S3. In this case there were more chicks from α-tocopherol supplemented egg parents, than controls. Only one adult from 2006 was re-caught in winter; a control female.

Table S1 2006 feeding treatments of adult females (2006) retrapped in breeding season of 2007. Table includes number of chicks successfully fledged in breeding season of 2007.

Ring number	Treatment	No chicks fledged (2007)
V205208	Control	10
V205007	Control	9
V205012	Control	0
V205013	α-tocopherol	7
V205014	α-tocopherol	9

V205021	α-tocopherol	8
V205015	α-tocopherol	11
V205020	α-tocopherol	13

Table S2 F1 females from 2006 retrapped in 2007. Table shows treatments of both egg parents, and rearing parents, and number of chicks successfully fledged in 2007.

Ring number	Egg Parents' Treatment	Rearing Parents' Treatment	No chicks fledged
V205201	Control	Control	9
V205351	Control	Control	5
V205357	Control	Control	11
V205411	Control	Control	7
V205475	Control	α-tocopherol	11
V205450	α-tocopherol	α-tocopherol	5
V205427	α-tocopherol	α-tocopherol	10
V205326	α-tocopherol	Control	8
V205732	α-tocopherol	α-tocopherol	11
V205383	α-tocopherol	Control	9
V205442	α-tocopherol	α-tocopherol	8

Table S3 Treatments of egg parents and rearing parents of F1 chicks from 2006 recaptured in winter 2007/2008.

Ring number	Egg Parents' Treatment	Rearing Parents' Treatment
V205310	α-tocopherol	α-tocopherol
V205525	α-tocopherol	Control
V205426	α-tocopherol	α-tocopherol
V205476	α-tocopherol	α-tocopherol
V205488	α-tocopherol	α-tocopherol
V205514	α-tocopherol	α-tocopherol
V205352	Control	Control
V205665	Control	Control

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