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

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Running head: Vitamin E effects on blue tit reproduction

Abstract

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 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.

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 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. At points in life where the endogenous antioxidant system may be assumed to 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 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, thus reproduction as used 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; Blount et al., 2015; Christe et al., 2011; Larcombe et al., 2010b; Metcalfe & Monaghan, 2013; Monaghan et al., 2009; Speakman & Garratt, 2014). 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., 2015; Arnold et al., 2007; Salin et al., 2015; Speakman et al., 2015), reproductive investment has been linked to a decrease in antioxidant defences (Alonso-Alverez et al. 2004; Losdat et al. 2011). 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 (Monaghan & Spencer, 2014; Sahin & Gumuslu, 2007). 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 lipophillic 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 (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. 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 (Costantini, 2008; Machlin, 1991; Sies & Murphy, 1991).

α-tocopherol is suggested to be 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), effects generally attributed to its antioxidant properties specifically (Traber & Atkinson, 2007) . α-tocopherol can play a role in mediating gene-expression (Azzi et al., 2004; Azzi & Stocker, 2000) and in immune processes (Leshchinsky & Klasing, 2001; Wintergerst et al., 2007), but it 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). 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 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). 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 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 α -tocopherol supplement delivery was based on methods established at the site (Ramsay & Houston, 1998) and designed to provide a biologically relevant dose of 0.37mg 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. This assumption was tested in a later experiment in spring, where we found a small decrease in detectable vitamin E in lard after 48 hours (maximum decease of 25% from starting concentration at point that new feeders were provided: see supplementary material).

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 -40 ºC 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 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. 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. 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 data 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 (approximately 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 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 (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 (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, yolk mass (5^{th} egg), antioxidant concentrations, total egg volume and fledging success. Treatment was entered as a fixed factor model, 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. 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) 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 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, 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 5^{th} 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 5^{th} 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 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 two hour 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

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, 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 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, F1, 79.7 = 0.35, p = 0.55, rearing treatment, F1, 19.4 = 0.19, p = 0.67). There were no sex differences in MDA (GLMM F1, 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 (if not significantly) with increased MDA (GLMM F1, 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, 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.

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 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. Further, 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, av. egg volume, 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 though 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 - that vitamin E supplementation did not have any demonstrable benefits to mothers, and that there was strong evidence of an impact on body mass in early nestling development - would have been upheld regardless.

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 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 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, though 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 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 insofar as providing enhanced vitamin E to birds, as they 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, 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, 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 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. 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 pay an increased cost in terms of lipid peroxidation. There is often assumed to be a cost to "catch-up" growth, potentially paid later in life (Metcalfe & Monaghan, 2001; Metcalfe & Monaghan, 2003). This catch up growth may be considered a cost rather than benefit of the treatment, though 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 re-capture rates were too low to be conclusive (see ESM).

We calculated growth rate from the change in mass between days 3-13. 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 to nestlings increased tarsus size but not body size (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 (Raberg et al., 2005). Without further information on adult survival and fitness, we cannot conclude whether greater skeletal size vs 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. Though 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 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). 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 (Biard et al., 2005; Hõrak et al., 2002; Török et al., 2007). Alternatively, other yolk constituents that impact size and development e.g. 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. Firstly, is it possible that previously identified benefits of vitamin E or carotenoids attributed to antioxidant function, were related to other functions of these molecules (Hartley & Kennedy, 2004). This is also the case for α-tocopherol, which, whilst a strong in vivo free radical scavenger, 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, though 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 are present in caterpillars in this population (Arnold et al., 2010b). The high fledging and hatching success suggest 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 limiting to 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. We attempted 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 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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738 **Figure legends**

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

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744 **Figure 2** Mean (± 1 S.E.) differences between nestlings from eggs laid by females that had
745 received either α-tocopherol, or control diet in: **a)** Mass of nestlings age 3 days; **b)** Mass gain
746 per day between days 3-13; **c)** Body condition of nestlings aged 14 days (residuals of ln
747 (mass) on 3*ln (tarsus) **d)** MDA concentration.

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750 **Figure 3** Differences (Mean ± 1 S.E.) between nestlings a). Mass aged 14 days of male and
751 female nestlings, reared by parents from different treatment groups b) Tarsus length nestlings,
752 laid by either α-tocopherol, or control treated parents, and reared by α-tocopherol, or control
753 treated parents

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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

References

Alonso-Alvarez C, Bertrand S, Devevey G, Prost J, Faivre B, Sorci G. 2004. Increased susceptibility to oxidative stress as a proximate cost of reproduction. *Ecology letters* **7:** 363-368.

Alonso-Alvarez C, Bertrand S, Devevey G, Prost J, Faivre B, Sorci G. 2004. Increased susceptibility to oxidative stress as a proximate cost of reproduction. *Ecology letters* **7:** 363-368.

Arnold KE, Herborn KA, Adam A, Alexander L. 2015. Individual variation in the oxidative costs of personality traits. *Functional Ecology* **29:** 522-530.

Arnold KE, Larcombe SD, Ducaroir L, Alexander L. 2010a. Antioxidant status, flight performance and sexual signalling in wild-type parrots. *Behavioral ecology and sociobiology* **64:** 1857-1866.

Arnold KE, Ramsay SL, Donaldson C, Adam A. 2007. Parental prey selection affects risk-taking behaviour and spatial learning in avian offspring. *Proceedings of the Royal Society B-Biological Sciences* **274:** 2563-2569.

Arnold KE, Ramsay SL, Henderson L, Larcombe SD. 2010b. Seasonal variation in diet quality: antioxidants, invertebrates and blue tits Cyanistes caeruleus. *Biological Journal of the Linnean Society* **99:** 708-717.

Azzi A, Gysin R, KempnÁ P, Munteanu A, Negis Y, Villacorta L, Visarius T, ZINGG J. 2004. Vitamin E mediates cell signaling and regulation of gene expression. *Annals of the New York Academy of Sciences* **1031:** 86-95.

Azzi A, Stocker A. 2000. Vitamin E: non-antioxidant roles. *Progress in Lipid Research* **39:** 231-255.

Berthouly A, Helfenstein F, Tanner M, Richner H. 2008. Sex-related effects of maternal egg investment on offspring in relation to carotenoid availability in the great tit. *Journal of Animal Ecology* **77:** 74-82.

Biard C, Surai PF, Moller AP. 2005. Effects of carotenoid availability during laying on reproduction in the blue tit. *Oecologia* **144:** 32-44.

Bize P, Devevey G, Monaghan P, Doligez B, Christe P. 2008. Fecundity and survival in relation to resistance to oxidative stress in a free-living bird. *Ecology* **89:** 2584-2593.

Blount JD, Metcalfe NB, Arnold KE, Surai PF, Devevey GL, Monaghan P. 2003. Neonatal nutrition, adult antioxidant defences and sexual attractiveness in the zebra finch. *Proceedings of the Royal Society of London Series B-Biological Sciences* **270:** 1691-1696.

Blount JD, Surai PF, Nager RG, Houston DC, Moller AP, Trewby ML, Kennedy MW. 2002. Carotenoids and egg quality in the lesser black-backed gull Larus fuscus: a supplemental feeding study of maternal effects. *Proceedings of the Royal Society of London Series B-Biological Sciences* **269:** 29-36.

Blount JD, Vitikainen EI, Stott I, Cant MA. 2015. Oxidative shielding and the cost of reproduction. *Biological Reviews*.

Christe P, Glaizot O, Strepparava N, Devevey G, Fumagalli L. 2011. Twofold cost of reproduction: an increase in parental effort leads to higher malarial parasitaemia and to a decrease in resistance to oxidative stress. *Proceedings of the Royal Society of London B: Biological Sciences***:** rspb20111546.

Costantini D. 2008. Oxidative stress in ecology and evolution: lessons from avian studies. *Ecology letters* **11:** 1238-1251.

Costantini D, Casasole G, AbdElgawad H, Asard H, Eens M. 2015. Experimental evidence that oxidative stress influences reproductive decisions. *Functional Ecology*.

Costantini D, Møller A. 2008. Carotenoids are minor antioxidants for birds. *Functional Ecology* **22:** 367-370.

de Ayala RM, Martinelli R, Saino N. 2006. Vitamin E supplementation enhances growth and condition of nestling barn swallows (Hirundo rustica). *Behavioral ecology and sociobiology* **60:** 619-630.

Deeming DC, Pike TW. 2013. Embryonic growth and antioxidant provision in avian eggs. *Biology letters* **9:** 20130757.

Dowling DK, Simmons LW. 2009. Reactive oxygen species as universal constraints in life-history evolution. *Proceedings of the Royal Society of London B: Biological Sciences***:** rspb. 2008.1791.

Finkel T, Holbrook NJ. 2000. Oxidants, oxidative stress and the biology of ageing. *Nature* **408:** 239-247.

Gasparini J, Boulinier T, Gill V, Gil D, Hatch S, Roulin A. 2007. Food availability affects the maternal transfer of androgens and antibodies into eggs of a colonial seabird. *Journal of evolutionary biology* **20:** 874-880.

Giraudeau M, Sweazea K, Butler MW, McGraw KJ. 2013. Effects of carotenoid and vitamin E supplementation on oxidative stress and plumage coloration in house finches (Haemorhous mexicanus). *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **166:** 406-413.

Giraudeau M, Ziegler A-K, Tschirren B. 2016. Long-term effect of yolk carotenoid levels on testis size in a precocial bird. *Biology letters* **12:** 20160008.

Gorman HE, Nager RG. 2004. Prenatal developmental conditions have long-term effects on offspring fecundity. *Proceedings of the Royal Society of London Series B-Biological Sciences* **271:** 1923-1928.

Griffiths R, Double MC, Orr K, Dawson RJG. 1998. A DNA test to sex most birds. *Molecular Ecology* **7:** 1071-1075.

Hartley RC, Kennedy MW. 2004. Are carotenoids a red herring in sexual display? *Trends in ecology & evolution* **19:** 353-354.

Helfenstein F, Berthouly A, Tanner M, Karadas F, Richner H. 2008. Nestling begging intensity and parental effort in relation to prelaying carotenoid availability. *Behavioral Ecology* **19:** 108-115.

Hodum PJ, Sydeman WJ, Visser GH, Weathers WW. 1998. Energy expenditure and food requirement of Cassin's Auklets provisioning nestlings. *Condor***:** 546-550.

Hõrak P, Surai PF, Moller A. 2002. Fat-soluble antioxidants in the eggs of great tits Parus major in relation to breeding habitat and laying sequence. *Avian Science* **2:** 123-130.

Hoyt DF. 1979. Practical methods of estimating volume and fresh weight of bird eggs. *The Auk***:** 73-77.

Karadas F, Pappas AC, Surai PF, Speake BK. 2005. Embryonic development within carotenoid-enriched eggs influences the post-hatch carotenoid status of the chicken. *Comparative Biochemistry and Physiology B-Biochemistry & Molecular Biology* **141:** 244-251.

Kim S-Y, Monaghan P. 2006. Effects of early incubation constancy on embryonic development: an experimental study in the herring gull Larus argentatus. *Journal of Thermal Biology* **31:** 416-421.

Larcombe S, Coffey J, Bann D, Alexander L, Arnold K. 2010a. Impacts of dietary antioxidants and flight training on post-exercise oxidative damage in adult parrots. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology* **155:** 49-53.

Larcombe S, Tregaskes C, Coffey J, Stevenson A, Alexander L, Arnold K. 2008. The effects of short-term antioxidant supplementation on oxidative stress and flight performance in adult budgerigars Melopsittacus undulatus. *Journal of Experimental Biology* **211:** 2859-2864.

Larcombe S, Tregaskes C, Coffey J, Stevenson A, Alexander L, Arnold K. 2015. Oxidative stress, activity behaviour and body mass in captive parrots. *Conservation Physiology* **3:** cov045.

Larcombe SD, Mullen W, Alexander L, Arnold KE. 2010b. Dietary antioxidants, lipid peroxidation and plumage colouration in nestling blue tits Cyanistes caeruleus. *Naturwissenschaften* **97:** 903-913.

Leclaire S, Bourret V, Blanchard P, de Franceschi C, Merkling T, Hatch SA, Danchin É. 2015. Carotenoids increase immunity and sex specifically affect color and redox homeostasis in a monochromatic seabird. *Behavioral ecology and sociobiology* **69:** 1097-1111.

Leshchinsky T, Klasing K. 2001. Relationship between the level of dietary vitamin E and the immune response of broiler chickens. *Poultry Science* **80:** 1590-1599.

Losdat S, Helfenstein F, Blount JD, Marri V, Maronde L, Richner H. 2012. Nestling erythrocyte resistance to oxidative stress predicts fledging success but not local recruitment in a wild bird. *Biology letters***:** rsbl20120888.

Machlin LJ. 1991. VITAMIN E. *Machlin, L. J. (Ed.). Food Science and Technology, 40. Handbook of Vitamins, Second Edition. Xi+595p. Marcel Dekker, Inc.: New York, New York, USA; Basel, Switzerland. Illus***:** 99-144.

Maronde L, Richner H. 2014. Effects of increased begging and vitamin E supplements on oxidative stress and fledging probability. *Behavioral Ecology***:** aru215.

Marri V, Richner H. 2014. Yolk carotenoids increase fledging success in great tit nestlings. *Oecologia* **176:** 371-377.

Marri V, Richner H. 2015. Immune response, oxidative stress and dietary antioxidants in great tit nestlings. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology* **179:** 192-196.

Matrková J, Remeš V. 2014. Vitamin E improves growth of Collared Flycatcher Ficedula albicollis young: A supplementation experiment. *Journal of Avian Biology* **45:** 475-483.

Metcalfe NB, Alonso-Alvarez C. 2010. Oxidative stress as a life-history constraint: the role of reactive oxygen species in shaping phenotypes from conception to death. *Functional Ecology* **24:** 984-996.

Metcalfe NB, Monaghan P. 2001. Compensation for a bad start: grow now, pay later? *Trends in ecology & evolution* **16:** 254-260.

Metcalfe NB, Monaghan P. 2003. Growth versus lifespan: perspectives from evolutionary ecology. *Experimental gerontology* **38:** 935-940.

Metcalfe NB, Monaghan P. 2013. Does reproduction cause oxidative stress? An open question. *Trends in ecology & evolution* **28:** 347-350.

Monaghan P, Metcalfe NB, Torres R. 2009. Oxidative stress as a mediator of life history trade-offs: mechanisms, measurements and interpretation. *Ecology letters* **12:** 75-92.

Monaghan P, Spencer KA. 2014. Stress and life history. *Current Biology* **24:** R408-R412.

Nager RG, Monaghan P, Houston DC. 2000. Within-clutch trade-offs between the number and quality of eggs: experimental manipulations in gulls. *Ecology* **81:** 1339-1350.

Navara KJ, Hill GE, Mendonca MT. 2006. Yolk testosterone stimulates growth and immunity in house finch chicks. *Physiological and Biochemical Zoology* **79:** 550-555.

Orledge JM, Blount JD, Hoodless AN, Royle NJ. 2012. Antioxidant supplementation during early development reduces parasite load but does not affect sexual ornament expression in adult ring-necked pheasants. *Functional Ecology* **26:** 688-700.

Parolini M, Romano M, Caprioli M, Rubolini D, Saino N. 2015. Vitamin E deficiency in last-laid eggs limits growth of yellow-legged gull chicks. *Functional Ecology* **29:** 1070-1077.

Raberg L, Stjernman M, Nilsson JA. 2005. Sex and environmental sensitivity in blue tit nestlings. *Oecologia* **145:** 496-503.

Ramsay S, Houston D. 1998. The effect of dietary amino acid composition on egg production in blue tits. *Proceedings of the Royal Society of London B: Biological Sciences* **265:** 1401-1405.

Remes V, Krist M, Bertacche V, Stradi R. 2007. Maternal carotenoid supplementation does not affect breeding performance in the Great Tit (Parus major). *Functional Ecology* **21:** 776-783.

Romero LM, Soma KK, Oreilly KM, Suydam R, Wingfield JC. 1997. Territorial behavior, hormonal changes, and body condition in an arctic-breeding song bird, the redpoll (Carduelis flammea). *Behaviour* **134:** 727-747.

Sahin E, Gumuslu S. 2007. Stress-dependent induction of protein oxidation, lipid peroxidation and anti-oxidants in peripheral tissues of rats: Comparison of three stress models (immobilization, cold and immobilization-cold). *Clinical and Experimental Pharmacology and Physiology* **34:** 425-431.

Saino N, Ferrari R, Romano M, Martinelli R, Moller AP. 2003. Experimental manipulation of egg carotenoids affects immunity of barn swallow nestlings. *Proceedings of the Royal Society of London Series B-Biological Sciences* **270:** 2485-2489.

Salin K, Auer SK, Rudolf AM, Anderson GJ, Cairns AG, Mullen W, Hartley RC, Selman C, Metcalfe NB. 2015. Individuals with higher metabolic rates have lower levels of reactive oxygen species in vivo. *Biology letters* **11:** 20150538.

Satiroglu-Tufan NL, Tufan AC. 2004. Amelioration of ethanol-induced growth retardation by all-trans-retinoic acid and α-tocopherol in shell-less culture of the chick embryo. *Reproductive Toxicology* **18:** 407-412.

Senar JC, Figuerola J, Pascual J. 2002. Brighter yellow blue tits make better parents. *Proceedings of the Royal Society of London Series B-Biological Sciences* **269:** 257-261.

Sies H. 1991. Oxidative stress: from basic research to clinical application. *The American journal of medicine* **91:** S31-S38.

Sies H, Murphy ME. 1991. Role of tocopherols in the protection of biological systems against oxidative damage. *Journal of Photochemistry and Photobiology B: Biology* **8:** 211.

Siitari H, Alatalo R, Pihlaja M, Hämäläinen J, Blount JD, Groothuis TG, Hytönen VP, Surai P, Soulsbury CD. 2015. Food supplementation reveals constraints and adaptability of egg quality in the magpie Pica pica. *Avian Biology Research* **8:** 244-253.

Speakman JR, Blount JD, Bronikowski AM, Buffenstein R, Isaksson C, Kirkwood TB, Monaghan P, Ozanne SE, Beaulieu M, Briga M. 2015. Oxidative stress and life histories: unresolved issues and current needs. *Ecology and Evolution* **5:** 5745-5757.

Speakman JR, Garratt M. 2014. Oxidative stress as a cost of reproduction: Beyond the simplistic trade-off model. *Bioessays* **36:** 93-106.

Surai AP. 2002. *Natural Antioxidants in Avian Nutrition and Reproduction*. Nottingham University Press: Nottingham.

Surai AP, Surai PF, Steinberg W, Wakeman WG, Speake BK, Sparks NHC. 2003. Effect of canthaxanthin content of the maternal diet on the antioxidant system of the developing chick. *British poultry science* **44:** 612-619.

Surai PF, Ionov IA, Kuchmistova EF, Noble RC, Speake BK. 1998. The relationship between the levels of alpha-tocopherol and carotenoids in the maternal feed, yolk and neonatal tissues: Comparison between the chicken, turkey, duck and goose. *Journal of the Science of Food and Agriculture* **76:** 593-598.

Surai PF, Noble RC, Speake BK. 1996. Tissue-specific differences in antioxidant distribution and susceptibility to lipid peroxidation during development of the chick embryo. *Biochimica et Biophysica Acta-Lipids and Lipid Metabolism* **1304:** 1-10.

Surai PF, Sparks NHC, Acamovic T, McDevitt RM. 2002. Antioxidant systems in the developing chicken: vitamins E and C in the liver of broiler chicks. *British poultry science* **43:** S64-S65.

Tappel AL. 1962. VITAMIN-E AS THE BIOLOGICAL LIPID ANTIOXIDANT. *Vitamins and Hormones-Advances in Research and Applications* **20:** 493-510.

Török J, Hargitai R, Hegyi G, Matus Z, Michl G, Péczely P, Rosivall B, Tóth G. 2007. Carotenoids in the egg yolks of collared flycatchers (Ficedula albicollis) in relation to parental quality, environmental factors and laying order. *Behavioral ecology and sociobiology* **61:** 541-550.

Traber MG, Atkinson J. 2007. Vitamin E, antioxidant and nothing more. *Free Radical Biology and Medicine* **43:** 4-15.

Verboven N, Monaghan P, Evans DM, Schwabl H, Evans N, Whitelaw C, Nager RG. 2003. Maternal condition, yolk androgens and offspring performance: a supplemental feeding experiment in the lesser black-backed gull (Larus fuscus). *Proceedings of the Royal Society of London Series B-Biological Sciences* **270:** 2223-2232.

Weimerskirch H, Ancel A, Caloin M, Zahariev A, Spagiari J, Kersten M, Chastel O. 2003. Foraging efficiency and adjustment of energy expenditure in a pelagic seabird provisioning its chick. *Journal of Animal Ecology* **72:** 500-508.

Wintergerst ES, Maggini S, Hornig DH. 2007. Contribution of selected vitamins and trace elements to immune function. *Annals of Nutrition and Metabolism* **51:** 301-323.

Young IS, Trimble ER. 1991. Measurement of Malondialdehyde in Plasma by High-Performance Liquid-Chromatography with Fluorometric Detection. *Annals of Clinical Biochemistry* **28:** 504-508.

Zingg JM. 2007. Vitamin E: An overview of major research directions. *Molecular Aspects of Medicine* **28:** 400-422.