Functional Ecology
British Ecological Society

Antioxidant supplementation during early development reduces parasite load but does not affect sexual ornament expression in adult ring-necked pheasants Phasianus colchicus

Journal:	*Functional Ecology*
Manuscript ID:	FE-2011-00368.R2
Manuscript Type:	Standard Paper
Date Submitted by the Author:	n/a
Complete List of Authors:	Orledge, Josephine; University of Exeter, Biosciences Blount, Jonathan; University of Exeter, Biosciences Hoodless, Andrew; Game & Wildlife Conservation Trust, Royle, Nick; University of Exeter, Biosciences;
Key-words:	Sexual selection, Oxidative Damage, Antioxidants, Trade-offs, Growth

STANDARD PAPER

Antioxidant supplementation during early development reduces parasite load but does not affect sexual ornament expression in adult ring-necked pheasants *Phasianus colchicus*

Running headline: Early life-history trade-offs in pheasants.

Josephine M. Orledge [1], Jonathan D. Blount[1], Andrew N. Hoodless[2], Nick J. Royle[1,*].

1. Centre for Ecology and Conservation, Biosciences, College of Life and Environmental Sciences, University of Exeter, Cornwall Campus, Penryn, Cornwall, TR10 9EZ, UK.

2. Game and Wildlife Conservation Trust, Fordingbridge, Hampshire, SP6 1EF, UK.

* Author for correspondence: N.J.Royle@exeter.ac.uk

Summary

1. The 'parasite-mediated sexual selection' (PMSS) hypothesis predicts that exaggerated male ornamentation could provide a signal to females of a males ability to resist parasites. Empirical tests of the PMSS have been largely equivocal, however, which may be because most have not considered the role of early life-history effects.
2. Many sexually-selected traits are carotenoid-based. Allocation of dietary-derived carotenoids to sexual ornaments may trade-off with allocation to pro-inflammatory immune response and/or antioxidant functions, mediated by the oxidative status of individuals. Exposure to parasites can increase oxidative stress, so under this scenario sexually-selected traits indicate ability to resist oxidative stress rather than ability to resist parasites per se. Such life-history trade-offs, mediated by oxidative status of individuals, are particularly acute during growth and development.
3. Here we use ring-necked pheasants, *Phasianus colchicus*, a strongly sexually-selected species, to test whether supplementation with dietary antioxidants (vitamin E) can mitigate the effects of early exposure to parasites (the nematode, *Heterakis gallinarum*), via alteration of the oxidative status of individuals, and positively affect the expression of sexual ornaments at adulthood.
4. We found that vitamin E mediated the effect of early exposure to parasites on levels of oxidative damage at 8 weeks of age and reduced the parasite load of individuals at adulthood as predicted. However, the expression of sexual ornaments, immune function, and growth were unaffected by either early vitamin E supplementation or manipulation of parasite load. In contrast to the predictions of the PMSS hypothesis the intensity of sexual ornament expression was not related to either parasite load or oxidative status of individuals (current or long-term). Consequently there was no evidence that the expression of sexual ornaments provided information on the ability of males to resist infection from parasites.

Key words: sexual selection, oxidative damage, antioxidants, trade-offs, growth

INTRODUCTION

Females in many animal species prefer to mate with the most elaborately ornamented males (Andersson & Simmons 2006). In species in which males contribute nothing beyond their sperm (Kirkpatrick & Ryan 1991; Andersson 1994) females are expected to choose mates based on 'indirect benefits' (Borgia 1979; Reynolds & Gross 1990); males differ in their viability and quality so that mate preference confers genetic benefits to the fitness of offspring ('good genes'; Norris 1993; Petrie 1994; Wedell & Tregenza 1999). More specifically, Hamilton and Zuk (1982) suggested that exaggerated male ornamentation could provide a signal to females of their ability to resist parasite infection (the 'parasite-mediated sexual selection' or 'bright male' hypothesis). If the ability to resist parasites is heritable then females could improve the fitness of their offspring by choosing males with the most exaggerated ornaments (Hamilton & Poulin 1997). Experiments with controlled infections show that sexual ornaments are more sensitive to parasite infection than other morphological traits (Zuk, Thornhill & Lignon 1990; Houde & Torio 1992; Møller 1994). Therefore, females could potentially choose males for their genetic quality (disease resistance) based on the expression of their sexually selected traits (Hamilton & Zuk 1982). Tests of Hamilton and Zuk's idea has produced equivocal results however (Hamilton & Poulin 1997; Møller et al. 1999; Getty 2002), and one reason for this may be because the majority of studies only consider parasite infections in adults and do not consider early life-history effects (Borgia et al. 2004).

Sexually selected traits are often highly sensitive to variations in the environmental conditions experienced during growth and development (e.g. David et al. 2000; Ohlsson et al. 2002; McGraw et al. 2005; Royle et al. 2005). Despite this very few studies have assessed how exposure to parasites during life-history stages prior to adulthood affects the expression of sexually selected traits. Borgia *et al.* (2004) studied adult satin bowerbirds to determine whether male display could provide an indication of parasitic infections experienced during juvenile life history stages. They found that the most attractive males were those that had experienced a lower parasite burden as juveniles, whilst no significant relationship was found to exist between current adult parasite burden and male

attractiveness (Borgia et al. 2004). If sexually-selected traits reflect the long-term condition of individuals and/or the ability to cope with environmental insult throughout development this is likely to be more informative of genetic quality than traits that just reflect current condition, which may be more transient in character.

Many sexually-selected traits expressed in birds and fish in particular are carotenoid-based. Carotenoids are dietary derived, highly pigmented antioxidants that have immuno-enhancing properties (Blount et al. 2003; McGraw & Ardia 2003). The intensity of the colouration of carotenoid-mediated traits has been found to be negatively affected by parasite burden in many species (Milinski & Bakker 1990; Zuk et al. 1990; Houde & Torio 1992; Thompson et al. 1997; Brawner, Hill & Sundermann 2000; McGraw & Hill 2000; Baeta et al. 2008; Mougeot et al. 2010). The intensity of parasite infection can affect carotenoid-mediated ornament expression either directly, by reducing the ability of an individual to assimilate carotenoids (Hõrak et al. 2004), or by affecting resource allocation trade-offs between signalling and self-maintenance (Martinez-Padilla et al. 2007).

The allocation of carotenoids to signalling is therefore expected to reduce the amount available for allocation to immune function (Lozano 1994). Moreover, activation of the immune system in response to parasite infection also results in the production of higher amounts of reactive oxygen species (ROS) during the respiratory burst activity of phagocytes (Babior 1984), leading to increased potential for oxidative stress. Oxidative stress results from an imbalance between the production of damaging ROS and antioxidant defences (Sies 1997). Carotenoids are also antioxidants, so the intensity of carotenoid-mediated sexually selected traits may therefore signal the oxidative status of individuals (von Schantz et al. 1999). There is increasing evidence that oxidative stress provides a potentially unifying mechanism that mediates fundamental resource allocation trade-offs underlying the evolution of life-history traits in animals (e.g. Costantini 2008; Monaghan, Metcalfe & Torres 2009; Hall et al. 2010). Under this scenario early exposure to parasite infection can be viewed as a

contributory factor influencing oxidative stress, so that sexually-selected traits do not reflect exposure to parasites *per se*, but the oxidative status of individuals. However, the antioxidant properties of carotenoids are thought to be comparatively poor compared to non-pigmentary antioxidants such as vitamin E (Costantini & Moller 2008) and it has been suggested that the presence of carotenoid based signals may, instead, signal the prevalence of these more efficient, non-pigmentary, antioxidants ('The carotenoid protection theory'; Hartley & Kennedy 2004). This is supported by the observation that oxidation causes the structural alteration of carotenoids, rendering them colourless and therefore not available for signalling (Hartley & Kennedy 2004).

Previous studies testing the carotenoid protection theory have been conducted on adults (e.g. Bertrand, Faivre & Sorci 2006; Pike et al. 2007; Perez, Lores & Velando 2008). However, resource allocation trade-offs are particularly prevalent during early growth and development (e.g. Cucco et al. 2006; Hall et al. 2010) and can lead to long-lasting effects. Early diet can determine the ability to assimilate and metabolise antioxidants in adulthood (Kim et al. 1996; Blount et al. 2003; Koutsos et al. 2003; Orledge et al. 2012) for example, and somatic growth results in the production of higher levels of ROS (Stoks, De Blok & McPeek, 2006). Supplementation of vitamin E during early development resulted in increased circulating vitamin E at adulthood in zebra finches (Blount et al. 2003a) and pheasants (Orledge et al. 2012) suggesting that the quality of the rearing diet may permanently affect the ability of individuals to assimilate circulating antioxidants at adulthood (Blount et al. 2003a). The availability of dietary antioxidants, and the degree of environmental insult (e.g. exposure to parasite infection) may therefore alter the balance of trade-offs during growth and development that affect the expression of phenotypic traits during adulthood, such as sexual ornaments, through affecting the oxidative status of individuals.

We used a sexually dimorphic galliform, the ring-necked pheasant, *Phasianus colchicus* (Fig. 1) as a study species to examine whether supplementation of a non-pigmentary antioxidant (vitamin E) could mitigate the effects of environmental insult (exposure to parasite infection) during early

development on the expression of sexually selected traits at adulthood (one year old), immune function, oxidative damage and growth. Male ring-necked pheasants have bright plumage, conspicuous wattles, long tail feathers, spurs and ear tufts. Females are smaller than males with a duller yellowish buff plumage and a long banded tail. Pheasants exhibit a harem polygyny social mating system and females choose mates based on multiple sexual ornaments (Hill & Robertson 1988). These ornaments include facial wattles (Hillgarth 1990), the colour of which is likely to be carotenoid-mediated (Czeczuga 1979), and the length of spurs on the legs (Göransson et al. 1990). The bright wattle of males is expanded during sexual displays to attract females (Hill & Robertson 1988) and females have been shown to prefer larger males (Göransson et al. 1990), and males with larger wattles (Hillgarth 1990). We used the nematode *Heterakis gallinarum,* a major parasite of wild ring-necked pheasants in the UK (Draycott et al. 2000), to manipulate the health of the birds during development. *H. gallinarum* release single cell eggs into the host faeces that remain in the soil before reaching the infective stage. Infection occurs through ingestion of the eggs from the soil or ingestion of earthworms that can act as transport hosts. The eggs develop into adults in 14 days within the caeca and begin ovipositing 24 to 36 days after infection (Olsen 1974).

If early life-history effects are important in determining the expression of traits in adults then we predict that early exposure to both parasites and antioxidants will have long-term effects. Specifically we predict that early exposure to parasites will lead to increased susceptibility (increased parasite burden) in adulthood, and that access to supplementary dietary antioxidants (vitamin E) during early growth will lead to an increase in circulating levels of antioxidants when mature. Furthermore we predict that if oxidative stress is an important mechanism underlying trade-offs during development then males supplemented with dietary antioxidants will have more resources available to allocate to sexually selected traits than unsupplemented males. In contrast, males infected with parasites will have higher levels of oxidative damage, so will have to allocate more resources to self-maintenance and less will be available for the expression of sexually-selected traits. Individuals supplemented

with vitamin E are therefore expected to have more exaggerated sexual signals than those that receive a control diet or individuals infected with parasites.

MATERIALS AND METHODS

(a) General Methods and experimental design

240 ring-necked day-old pheasants of mixed genetic stock (Holme Farm Hatcheries, Wokingham) were allocated randomly to one of four treatment groups (n=60 in each treatment) at the Game and Wildlife Conservation Trust HQ, Hampshire. The game farm that supplied the pheasants maintains breeding stock in groups of 30 hens with 3 cock pheasants (i.e. replicating the natural harem polygyny mating system). As a result, males and females have multiple potential copulation partners. The pheasants are not intensively farmed or artificially selected for traits such as high egg production or disease resistance either, so there is no evidence that the phenotypes of the pheasants are uncoupled from past natural and sexual selection pressures. Treatment diets over the first 8 weeks were (1) vitamin E supplement with addition of *Heterakis* nematode parasites (P-E) (2) vitamin E supplement without parasites (NP-E) (3) control diet with *Heterakis* parasites (P-C) (4) control diet without parasites (NP-C). An 8 week period of dietary manipulation was chosen to include the early developmental window identified by previous studies on pheasants (Ohlsson & Smith 2001; Ohlsson et al. 2002). Birds supplemented in treatment groups with *Heterakis* nematodes were infected at 21 days of age, the optimal age for successful infection in chickens (Olsen 1974). The diet provided after 8 weeks was identical for all birds. Morphometric measurements were taken initially on day one then subsequently at 8, 21 and 47 weeks of age. To assay plasma concentrations of vitamin E and carotenoids blood samples were taken at 8 and 47 weeks of age and, because vitamin E is fat soluble and known to be an important antioxidant in the lipid-rich cell membrane (Wang & Quinn 1999), oxidative stress was measured by assay of the concentration of a biomarker of lipid peroxidation, malondialdehyde (MDA) at 8 and 47 weeks of age. Phytohaemagglutinin injection was used to measure immune response at 21 weeks of age. Sexual signals including wattle colour, size and shape and spur length were measured at 47 weeks of age. Females may use multiple cues during mate

choice that may reflect different aspects of male quality (Candolin 2003), so although we focused on a carotenoid-mediated trait, wattle colouration, we measured multiple pheasant ornaments. Previous studies have shown that the expression of ornaments is responsive to dietary quality manipulation during development (Ohlsson et al. 2001) and in adulthood (one year old; Smith et al. 2007).

(b) Husbandry

General husbandry followed standard pheasant rearing practice (The Game Conservancy 2006). For the first 8 weeks (commencing in early May) birds were housed in groups of 30 in indoor pens (1.8m x 1.5m) under dim light conditions within a semi-intensive brooder hut system. Additional (non-experimental) birds were reared and introduced to experimental pens following mortality of experimental birds as necessary, in order to maintain standardised rearing densities during the first 8 weeks (N = 8 birds). At 2 weeks of age birds were also given daily access to outdoor pens with wire floors (3m x 1.5m). At 8 weeks of age the birds were sexed and then transferred to two outdoor single-sex pens (30m x 27m) with access to grass for the remainder of the experiment.

(c) Dietary Supplementation

Vitamin E is used as a descriptor a group of compounds that include both tocopherols and tocotrienols. In this study we supplemented treatment groups with α-tocopherol. However, we refer to the supplement using the more general description of vitamin E throughout the paper. Vitamin E was supplemented to the P-E and NP-E treatment groups at a concentration of 100mg/kg of feed. The basal diet of individuals in the P-C and NP-C received no vitamin E supplement (0 mg/kg of feed). Birds were given treatment diets from the day after hatching (day 1) until 8 weeks of age. The concentration of vitamin E supplemented was chosen to match the concentrations used in previous studies on poultry that have shown effects of vitamin E on lipid peroxidation following exposure to a toxin (Hoehler and Marquardt 1996), improved growth and feed utilisation (Guo et al. 2001) and increased plasma vitamin E concentrations (Bartov & Frigg 1992). Supplements were added to a basal diet made to specification with no added vitamin E, low levels of vitamin A (10.0mg/kg) and

selenium (0.20mg/kg) (Target Feeds Ltd., Shropshire). All feed was sprayed daily using a 5 litre spray pump with the following: Vitamin E supplementation (NP-E and P-E) – vitamin E was sprayed in soybean oil onto the feed and stored in refrigerated vacuum pumped containers until it was given to the birds. Soybean oil was selected as a medium for vitamin E supplementation because it contains low levels of α-tocopherol (0.07µg/mg) in comparison to other food oils such as sunflower or olive oils (Carpenter 1979). Equal volumes of soybean oil but without the supplemental vitamin E were sprayed onto the other feeds (NP-E and P-E). Each afternoon the feed was replenished with fresh refrigerated treatment feed. Following standard pheasant rearing practice four basal diets were provided over the 8 week period of supplementation with medium levels of protein (starter crumb 1-2 weeks: 29.8%, starter pellets 3-4 weeks: 25.5%, rearer pellets 5-6 weeks: 21.4%, grower pellets 7-8 weeks: 18.1%). Feed, grit and water were provided *ad libitum*. Protein levels therefore averaged 23.7% over the 8 week experimental period, which is mid-way between the levels used by Ohlsson *et al.* (2001) in a previous experiment that manipulated the amount of protein available during the first 8 weeks of life (low protein diet = 20.5%, high protein diet = 27% protein). The overall protein levels in our experiment were moderate in order to reduce the risk of high protein levels masking among individual variation in quality. After 8 weeks of age all birds were fed a commercial feed with a standard protein content (13%) for adult pheasants (Woodard et al.1977; Sheppard et al. 1998).

(d) *Heterakis infection and counts*

Heterakis gallinarum eggs were embryonated by maintaining female nematodes in 0.5% formalin solution at 21°C for 21 days. Eggs were then released by blending the female nematodes in saline solution. Eggs were counted using a McMaster egg slide (Hawksley Ltd. Z11000) and the solution was diluted with saline solution until a solution containing approximately 100 eggs per ml was produced. Individuals were infected with *Heterakis gallinarum* eggs at 21 days of age. The timing of infection was chosen to match the 'optimal' age of development for infection success (Olsen 1974). A spring survey of wild hen pheasants in England found a median of 84 and range of 9-331 *H. gallinarum* nematode worms per individual bird across 21 sites in England and Wales (Draycott et al.

2000). We also recorded similar numbers of nematodes in a sample of wild pheasants found dead on the road (Orledge et al. *unpublished data*). Individual pheasant chicks were each infected with 100 embryonated *H. gallinarum* eggs administered directly into the throat in 1ml of saline using a pipette (Tompkins et al. 2000; Sage et al. 2002). Tompkins et al. (2000) found that this dosage resulted in a mean infection of 59 (± 14.83 SE) *H. gallinarum* worms. 1ml of saline solution without nematode eggs was administered to individuals in treatment groups without infection. An infective dose of 100 eggs was used, as this was the largest number that could be used to avoid documented density-dependent effects on *H. gallinarum* fecundity (Tompkins & Hudson 1999). The nematode *Heterakis gallinarum* is found in the lumen of the caecum and occasionally in the small intestine. At 47 weeks of age, all individuals were euthanized and dissected and the numbers of *Heterakis gallinarum* were counted. Each caecum was cut open and the contents were scraped from the gut lining into a fine mesh sieve (aperture 100 microns). The worms were then washed into a petri dish and counted (Doster and Goater 1997).

(e) Morphometric measurements

The morphometric measurements of individuals were recorded at 0, 8, 21 and 47 weeks of age. Body mass was measured using a variety of Pesola® spring balances (30g, 60g, 100g, 300g, 600g, 1000g, 2500g). Tarsus length and head to bill length were measured using a sliding calliper (± 0.01mm) and wing length was recorded using a wing rule (± 0.1mm). Spur length was measured at 21 and 47 weeks using dial calliper measurements of the tarsus width just above the spur and by subtracting this from a measurement of the tarsus width and spur length (Ohlsson et al. 2001).

(f) *Measurement of plasma antioxidants and oxidative stress*

Blood samples were taken at 8 weeks (at the end of the supplementation period) and at 47 weeks of age. Whole blood (up to 0.3ml) was collected from the brachial vein under Home Office licence in 5/8" 26 gauge Microlance™ needles (Fisher Scientific UK Ltd.) and BD Plastipak™ 1ml syringes (Fisher Scientific UK Ltd.) flushed with heparin (Sigma-Aldrich Inc.) and microhaematocrit EDTA-

coated capillary tubes (Bilbate Ltd.). Syringe samples were transferred to 1.5ml EDTA-coated micro tubes (Sarstedt) and stored in a dark cool bag. The samples were centrifuged and plasma was removed and stored at -20°C within 1 hour of collection. The samples were then transferred to a -80°C freezer within 5 days before biochemical analysis.

α-Tocopherol was measured within a month using high-performance liquid chromotography (HPLC). Plasma (50µl) was mixed with 5% sodium chloride (50µl) and ethanol (100µl). The mixture was vortexed for 20s. Hexane (600µl) was added to the solution and vortexed for 20s and centrifuged for 4min (13.8 x g). The hexane layer was removed and the absorbance measured at 450nm using a spectrophotometer (Nicolet Evolution 500) to determine total carotenoid concentration using 2500 as an average extinction coefficient for all carotenoids. The hexane (400µl) was dried down and samples redissolved in methanol (150µl), centrifuged for 4 minutes, then injected (50µl) into a Dionex HPLC system (Dionex Corporation, California, USA) fitted with a 3µ C_{18} reverse-phase column (15 cm x 4.6 mm) (Spherisorb S30DS2; Phase separations, Clwyd, UK) and using a mobile phase of methanol:distilled water (97:3) at a flow rate of 1.1ml min^{-1}. Fluorescence detection was carried out at 295 nm (excitation) and 330 nm (emission). Known concentrations of α-tocopherol (Sigma-Aldrich T36634) dissolved in methanol were used for calibration.

To measure plasma concentrations of malondialdehyde (MDA), 20µl butylated hydroxytoluene (BHT) (0.05% w/v in 95% ethanol), 160µl of phosphoric acid (0.44*M*) solution and 20µl of 2-thiobarbituric acid (TBA) (42m*M*) was added to either 20µl of plasma or 1,1,3,3-tetraethoxypropane (TEP) which was used for calibration (see below). The mixture was vortexed for 10s and heated in a dry bath incubator for 1hour at 100ºC. Samples were then cooled on ice for 5 minutes. 80µl of *n*-butanol (HPLC grade) was added and the mixture was vortexed for 20s and centrifuged for 3 minutes at 4°C (13.8 x g) and 20ul of the butanol phase containing MDA-TBA adduct was injected into a Dionex HPLC system fitted with a Hewlett-Packard Hypersil 5µm ODS 100 x 4.6 mm column and a 5µ ODS guard column maintained at 37°C. The mobile phase was 50mM potassium monobasic

phosphate (pH 6.8 adjusted using 5M potassium hydroxide) mixed with methanol (HPLC grade) running isocratically at 60:40 (v/v), at a flow rate of 1ml min^{-1}. Fluorescence detection was performed at 515 nm (excitation) and 553 nm (emission). For calibration a standard curve was prepared using a TEP stock solution (5 m*M* in 40 % ethanol) serially diluted using 40 % ethanol.

(g) Wattle colour measurement and quantification

Wattle reflectance data were collected using a USB2000 UV-Visible spectrophotometer and OOIBase32 Software (Ocean Optics Inc., Dunedin, FL) (Mougeot et al. 2005). The spectrophotometer was fitted with a 90º probe pointer to ensure perpendicular contact with the wattle surface and to exclude ambient light (Mougeot et al. 2005). Reflected radiance was measured across a spectral range of 260-680nm at 0.3nm resolution relative to a WS-1 (Ocean Optics Inc.) white standard. The probe was held against the wattle and the spectra allowed to stabilize before capture (Keyser & Hill 1999). Three spectra were collected for the left wattle and 3 for the right wattle. The brightness of the wattle has been identified as being important in female mate choice (Keyser and Hill, 1999), so we calculated brightness as it is likely to be perceived by female pheasants, using the method detailed in Endler and Mielke (2005). In Galliforms, brightness is likely to be perceived by the double cones which show broader spectral tuning and a greater absolute sensitivity suggesting that they are of greater importance for luminance than for colour vision (Vorobyev et al. 1998; Osorio et al. 1999). Because no data on photoreceptor spectral sensitivity have been collected for ring-necked pheasants we used data for the closely-related species, the blue peafowl (*Pavo cristatus*) (Hart 2002). The pheasants' double cone has a peak sensitivity at 567 nm, and is associated with a carotenoid-coloured oil droplet (Hart 2002). Effective double cone sensitivity functions were modelled using the visual pigment template of Govardovskii et al. (2000) and incorporating the transmittance spectra of the combined ocular media for peafowl (Hart 2002), and estimated oil droplet transmission spectra calculated using the equations of Hart & Vorobyev (2005) and data from Hart (2002). The birds were reared outdoors, so a standard daylight-simulating illumination spectrum (D65) was used in the model (Wyszecki & Stiles 1982).

(h) Wattle Size and Shape parameters

An image of the male wattle at 46 weeks of age was taken with the head held on the same plane as a fixed scale. Image J software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2009) was used to calibrate the scale of the image and a polygon was drawn around the wattle to calculate area. The outlines of the wattles for all individuals were included in a common elliptic fourier analysis (EFA) (Rohlf 1992) using Morpheus *et al.* software (D. E. Slice, *Morpheus et al.: Software for Morphometric Research. Revision 01-31-00* Department of Ecology and Evolution, State University of New York). The EFA decomposed the curved edges of the polygon into a sum of 15 harmonically related ellipses (to produce 60 Fourier coefficients). Normalisation allowed for variation in the size, position and the rotation of images taken of each wattle. The Fourier coefficients were then used as variables in principal component analyses. The number of principal components that described over 95% of the wattle shape variation was used for analyses (South & Arnqvist 2009).

(i) Immune response

Immune response was measured in all birds at 21 weeks of age. Phytohaemagglutinin (PHA) a lectin from the red kidney bean (*Phaseolus vulgaris*) is used as a standard measurement of pro-inflammatory immune response in avian studies (Smits et al. 1999, Vinkler et al. 2010). An area of feathers (approx. 1cm^2) from the patagium of both wings for each bird was plucked and sterilised with ethanol. The wing web diameters were then measured using callipers (0.01mm). In the right patagium 0.2mg of phytohaemagglutinin (PHA) (Sigma-Aldrich Inc.) in 0.1ml of sterilised phosphate buffer solution (PBS) (Sigma-Aldrich Inc.) was injected subcutaneously using 5/8" 26 gauge Microlance^{TM} needles (Fisher Scientific UK Ltd.) and BD Plastipak^{TM}1ml needles (Fisher Scientific UK Ltd.). 0.1ml of sterilised PBS was injected into the left wing patagium. The thickness of the wing patagium of each wing was measured directly before injection using callipers (0.01mm). 24 hours (± 10 minutes) after the injection the thickness of the patagium of the wings was measured.

The original thickness measurement was subtracted from this measurement to identify the pro-inflammatory response to PHA 24 hours after exposure.

(j) Statistical analyses

Normality checks were carried out in SPSS (SPSS Inc., Chicago IL) and data was log-transformed where necessary. Nine individuals died before 47 weeks, approximately equally distributed across the treatment groups. Only measurements taken from individuals that survived to 47 weeks of age were used in analyses (P-E N=59, NP-E N=57, P-C N=57, NP-C N=58). Principal components were produced using the coefficients calculated by an elliptic fourier analysis of wattle shape data. These principal components were used in a multivariate analysis of covariance (MANCOVA) as dependent variables with parasite and vitamin E treatments as fixed effects to determine the effects of treatments on wattle shape. Other response variables were analysed using general linear mixed models (GLMMs) with hatch date (batch) as a random effect. Parasite treatment and vitamin E treatment were included as 2 factors each with 2 levels in a 2 x 2 factorial design in all models. The date on which the HPLC assay was run for each sample was also included as a covariate to control for inter-assay variation, but was dropped from all models during simplification. Growth was analysed using morphometric measurements for males and females at 0, 8, 21 and 47 weeks of age with repeated measures GLMMs. Plasma concentration of either vitamin E or carotenoids were used as the dependent variables in repeated measures GLMMs that included age (for males) as an additional fixed effect to those listed above and bird ID as an additional random effect to determine the effects of the treatments on circulating levels of antioxidants. The effect of the treatments on oxidative damage was examined using a repeated measures GLMM with plasma MDA concentration as the response variable and including sex and age as fixed effects. Similar GLMMs (including sex as a fixed effect, but not repeated measures) were used to examine treatment effects on immunity (PHA measurement as the dependent), parasite burden, and, for males, the expression of secondary sexual traits (spur length, wattle colouration, wattle size and wattle shape). GLMMs were completed in R version 2.9.2 (© R Development Core Team 2009). General linear mixed models were tested

using the *lme* function. All interactions were included in the maximal model. For model simplification we removed the highest order interactions, followed by lower order terms in turn from the maximal model using maximum likelihood tests (Likelihood ratios – LR; Crawley 2007) to identify the minimum adequate model (MAM). For post hoc tests involving treatment groups GLMMs in which the focal treatment groups were paired were compared to the original GLMM (i.e. with unpaired treatments) using ANOVA model comparison.

RESULTS

(a) Parasitic Burden at 47 weeks of age

The number of *Heterakis* worms in the guts of individual pheasants was measured in both males and females at 47 weeks of age (N = 231 individuals). The MAM of a GLMM with parasite burden at adulthood as the dependent variable included significant main effects of sex (LR = 12.87, $p<0.001$), vitamin E treatment (LR = 7.99, $p<0.01$) and parasite treatment (LR = 13.34, $p<0.001$) and a vitamin E treatment * parasite treatment interaction (LR = 6.45, $p=0.03$; see Table 1a for parameter estimates for the MAM). All other interactions were dropped from the model during simplification (all $p>0.20$). Individuals infected with parasites and given a control diet had more parasites at 47 weeks of age than individuals from other treatment groups (Fig. 2). Birds that were infected with parasites but did not receive vitamin E had a higher number of parasites at 47 weeks than those birds that did not receive either vitamin E or parasites in early life. Individuals that received a diet with supplementary vitamin E during development had a lower parasite burden at 47 weeks of age, whereas individuals that were infected with parasites during early life had a higher parasite burden at 47 weeks of age than those individuals that did not receive the parasite treatment (Fig. 2). Males had a significantly higher mean parasitic burden than females (Table 1a).

(b) Concentrations of plasma antioxidants

The concentration of α-tocopherol (vitamin E) decreased from a mean across groups of 87.66 μg/ml at 8 weeks to 2.59 μg/ml by 47 weeks of age in male pheasants (N = 115 individuals and 218

observations) The MAM of a repeated measures GLMM with bird ID and hatch date as random effects and plasma vitamin E concentration as the response variable included main effects of vitamin E supplementation group (LR = 75.00, p<0.001) and age (LR = 204.91, p<0.001), and a significant interaction between age and vitamin E supplementation (LR = 115.19, p<0.001; see Table 1b for parameter estimates). The greatest decrease in plasma vitamin E concentration occurred in those birds that received vitamin E in their diet up to 8 weeks of age (Table 1b, Fig. 3a, b).

In analyses separated by age (N = 115), males in groups that were supplemented with vitamin E had higher concentrations of plasma vitamin E at 8 weeks of age than males given a control diet (Vitamin E treatment, LR = 98.36, p<0.001). Plasma concentrations of vitamin E in males that received a diet supplemented with vitamin E in early life remained higher at 47 weeks than birds given a diet without the vitamin E supplement (Vitamin E treatment, LR = 45.63, p<0.001). Infection with parasites did not affect the concentration of vitamin E in the plasma at 8 (parasite treatment, LR = 2.42, p=0.11; vitamin E * parasite, LR = 2.28, p=0.14) or 47 weeks of age (parasite treatment, LR = 0.88, p=0.35; vitamin E * parasite, LR = 0.10, p=0.76), and males did not differ from females in the concentrations of vitamin E circulating in the plasma at 8 weeks of age (Sex, LR = 0.85, p=0.47; N = 231 individuals;). There were no effects of vitamin E supplementation (LR = 0.93, p=0.69), parasite treatment (LR = 1.37, p=0.33), age (LR = 0.42, p=0.85) or any significant interactions between these variables on the concentrations of carotenoids circulating in plasma (all interactions were p>0.06; The MAM included just the model intercept; Fig. 3c, d).

(c) Oxidative Stress

The concentration of MDA in plasma did not differ between males and females (LR = 0.11, p=0.74), or parasite treatment (LR = 1.36, p=0.26) but decreased with age (from an overall mean of 6.61 µg/ml at 8 weeks to a mean of 1.61 µg/ml at 47 weeks of age; LR = 252.12, p<0.001; Fig. 3e, f). The MAM included significant interactions between vitamin E treatment and age (LR = 9.47, p=0.002), parasite treatment and age (LR = 4.18, p=0.041) and vitamin E treatment and parasite treatment (LR

= 5.70, p=0.017) respectively (N = 231 individuals and 462 observations; Table 1c). GLMMs separated by age for males showed that birds given a control diet and infected with parasites had a higher concentration of plasma MDA at 8 weeks of age (Parasite treatment * vitamin E treatment: LR = 3.92, p=0.03; vitamin E treatment, LR = 9.39, p<0.01; parasite treatment, LR = 2.85, p=0.09; Fig. 3e, f). However, by 47 weeks there were no differences in plasma MDA concentrations between individuals given the parasite treatment or the vitamin E treatment (GLMM for birds at 47 weeks: vitamin E treatment* parasite treatment: LR = 2.42, p=0.12; vitamin E treatment: LR= 1.72, p=0.17; parasite treatment: LR = 1.38, p=0.24; Fig. 3e, f).

(d) Morphometric measurements

There were no initial differences in the size of chicks allocated to different vitamin E or parasite infection treatments (GLMM, N = 231 individuals: treatment group, LR = 6.22, p=0.10; sex, LR = 0.44, p=0.51; treatment * sex, LR = 2.83, p=0.42). Repeated measures GLMMs with mass, tarsus length, wing length or head-bill length as response variables (N = 231 individuals and 693 observations) and sex, age and treatment group as explanatory variables showed that males were larger and faster growing than females (mass, LR = 91.87, p<0.001; head-bill length, LR = 87.19, p<0.001; tarsus, LR = 124.15, p<0.001, wing length, LR = 12.18, p=0.04), but that there were no significant differences in growth among treatments, either for vitamin E supplementation (mass, LR = 0.03, p=0.98; head-bill length, LR = 0.27, p=0.89; tarsus, LR = 0.28, p=0.84; wing length, LR = 0.81, p=0.67) or in relation to parasite treatment (mass, LR = 1.47, p=0.55; head-bill length, LR = 2.45, p=0.43; tarsus, LR = 0.25, p=0.87; wing length, LR = 2.01, p=0.11). There were also no significant interaction terms in any of the respective MAMs (all interactions p>0.29; parameter estimates for the MAMs are given in Table 2)).

(e) Immune function

The MAM of a model including immune response at adulthood as the dependent variable and vitamin E treatment, parasite treatment and sex with hatch date as a random effect included only the

intercept (N = 231 individuals). Immune response did not vary in relation to either sex (LR = 0.54, p = 0.46), parasite treatment (LR = 0.83, p=0.36), or vitamin E treatment (LR = 0.20, p=0.65). All interactions were also dropped from the model during simplification (all p>0.38).

(f) Secondary Sexual Signals

The expression of sexual signals in males (N = 115 individuals) was not affected by parasite load (parasite treatment: wattle size LR = 2.10, p=0.15, spur length: LR = 2.62, p=0.11, wattle brightness: LR = 0.59, p=0.44) or the supplementation of vitamin E (vitamin E treatment: wattle size LR = 2.23, p=0.14, spur length: LR = 0.29, p=0.59, wattle brightness: LR = 0.18, p=0.67). A MANCOVA of the 5 principal components that collectively described 95% of the shape variation calculated by EFA analysis indicated that there was also no difference in the shape of the wattles of males in relation to parasite treatment (F = 0.34, df = 1,110, p=0.54) or vitamin E treatment (F = 1.25, df = 1,110, p=0.23). There were no significant interaction terms in any of these models (all p>0.09).

DISCUSSION

The results show that, contrary to expectations, the expression of sexually-selected traits in adulthood was unaffected by the experimental manipulation of parasite load or antioxidant (vitamin E) availability during the first 8 weeks of development. However, adult males had greater numbers of parasites than females in their guts at 47 weeks of age regardless of which treatment they had received during development. In addition the experimental treatments did not have any effect on the growth or immune response of individual ring-necked pheasants of either sex, but early exposure to parasites and vitamin E did, as predicted, have some long-term effects. Individuals exposed to *Heterakis* nematode worms at 21 days of age had higher numbers of the parasite at adulthood (47 weeks) than individuals that were not infected with *Heterakis*, unless they also received supplementary vitamin E during early growth. Early exposure to parasites without supplementary vitamin E was also associated with elevated levels of oxidative damage at 8 weeks of age. In

contrast, the reduced oxidative stress (lower levels of damage during early growth and higher circulating levels of vitamin E throughout development) and lower numbers of intestinal parasites at adulthood (47 weeks) of individuals that received supplementary vitamin E during the first 8 weeks of growth may have positive downstream effects on fitness prospects, even if sexually-selected traits were unaffected.

Sexual traits can show higher condition dependence in response to environmental stress during early development than morphological traits (e.g. Hunt & Simmons, 1997, David et al. 2000). The negative effects of nutritional stress during early development on sexual signals have mostly been documented for vocal sexual signals (song e.g. Buchanan et al 2003; Spencer et al. 2003) but little is known about the connection between development and evolution of sexual ornaments in response to an early environmental insult such as parasite infection. Borgia et al. (2004) proposed that if females have evolved to gain the greatest "good genes" benefits from mate selection that they should choose male display traits that include information from life history stages when parasites are most harmful. The results of the Borgia et al. (2004) study with satin bowerbirds indicated that immunocompetence handicap studies should consider the effects of exposure to infection in non-reproductive, not just reproductive, age classes. In contrast with the results of previous experiments (Borgia et al. 2004; Spencer et al. 2005) the expression of sexually selected traits in ring-necked pheasants in the current study were largely unaffected by exposure to parasites (*H. gallinarum*) during development.

Furthermore, we also found that the intensity of male sexual signals did not correspond with current *H. gallinarum* burden. The results of the current study therefore do not support the 'parasite-mediated sexual selection' theory (Hamilton & Zuk, 1982) which proposes that females choose bright males because elaborate displays are effective indicators of heritable male-parasite resistance traits. None of the multiple ornaments measured, whether carotenoid-mediated (wattle colour) or not (spur length, wattle size or body size) were related to parasite load. Previous studies have provided evidence that carotenoid-mediated sexual traits can be affected by parasitic infection. Male house

finches infected with *Mycoplasma gallicepticum*, show reduced carotenoid plumage colour without direct disruption of carotenoid absorption or transportation (Hill et al. 2004). Experimental reduction of infection levels has been shown to reduce carotenoid based signalling in red grouse combs (nematode; Martinez-Padilla et al. 2007) and in great tits (hemoparasite; Horak et al. 2001). Møller *et al.* (1999) suggested that inconsistent results in tests of the 'parasite-mediated sexual signal' theory may result from the use of relatively harmless parasites in studies. Previous studies on pheasants have provided some support for parasite-mediated effects on sexual display. Hillgarth (1990), for example, found a correlation between female mate-choice, coccidian numbers and male display rate. Our experiment used *H. gallinarum,* a common nematode in wild pheasants which may be less pathogenic than some other parasites. We found no negative effects of *H. gallinarum* infection on body mass or growth, consistent with other studies (Tompkins et al. 1999; Draycott et al. 2000; Tompkins et al. 2001; Woodburn et al. 2002). However, Tompkins et al. (2001) found that pheasants infected with *H. gallinarum* following infection with 100 embryonated eggs, the same dosage used in this study, produced a lower mass of caecal droppings, and suggested that reduced caecal activity may result in reduced nutrient absorption and therefore reduce the fecundity and survival of pheasants in the wild if food is limiting (see also Holmes, 1995; Coop & Holmes, 1996). In the current study birds infected with parasites that were not also provided with supplementary antioxidants had higher levels of oxidative damage at 8 weeks of age and higher parasite loads at adulthood, which indicates that there may be significant costs of early exposure to *H. gallinarum*.

Activation of the immune system in response to parasite infection results in the production of higher amounts of reactive oxygen species during the respiratory burst activity of phagocytes (Babior 1984). Individuals may also experience higher levels of oxidative damage if parasitism impairs the uptake of antioxidants from the diet. As a result it was predicted that individuals infected with *H. gallinarum* would experience a higher degree of oxidative damage. Supplementation with vitamin E however, mitigated the oxidative effects of early exposure to parasites, as P-E birds had significantly lower levels of oxidative damage than infected birds given a control diet, and had similar levels of MDA to

uninfected individuals at 8 weeks of age. In addition, our results complement the results of previous studies showing that vitamin E can reduce nematode infection. Vitamin E deficiency has been shown to impair resistance to secondary nematode infection 30 days after inoculation in adult mice (Smith et al. 2005). Reduced vitamin E concentrations may affect the ability of a host to respond to nematode infection of the gastro-intestinal tract due to increases in oxidative stress and alterations to both signal transduction and transcription factor activation (Smith et al. 2005). Supplementation with vitamin E during the first 8 weeks in our experiment also resulted in increased levels of circulating vitamin E (i.e. elevated antioxidant defences) at adulthood. However, there were no differences in oxidative stress at 47 weeks of age despite significantly higher numbers of parasites in the P-C group. As a result there was also no evidence that sexually-selected traits reflected the long-term oxidative status of individuals.

Despite monitoring individuals for a year post-hatch treatment effects on sexual signal expression were not detected, in contrast to a previous study on pheasants that manipulated protein content of early diet and found treatment effects on the expression of sexually-selected traits on one-year old adults (Ohlsson et al. 2002). However, it is possible that measurement of the sexual ornaments of males at one-year of age failed to identify the longer term effects of supplementation. Hillgarth (1990) found no female preferences for male morphological traits in captive birds during a study on one year old ring-necked pheasants. Spur length is reportedly the most important predictor of harem size in ring-necked pheasants (Göransson et al. 1990), but spur length at one year of age has been found to have less influence on female mate choice than the spur length of older males (Grahn & von Schantz 1994). In addition, the effects of higher circulating vitamin E at 47 weeks found in birds supplemented with vitamin E during development on the oxidative status of individuals beyond the first year of life are unknown.

Previous supplementation experiments during post-natal development involving vitamin E only (in barn swallows; de Ayala et al. 2006) and a cocktail of antioxidants including vitamin E (in red-

winged blackbirds; Hall et al. 2010) have shown that additional antioxidant resources are preferentially allocated to growth. Related work on pheasants showed that supplementation of a combination of carotenoids and vitamin E, but not vitamin E by itself, resulted in preferential allocation of resources to achieving a large body size rather than to sexually-selected traits (Orledge et al. 2012). This is likely to be because in ring-necked pheasants attaining a larger body size has beneficial downstream effects. Smith et al. (2007) found that pheasants in better body condition, measured as residual mass, showed increased wattle colour when carotenoid supplemented as first year adult males. By maintaining a better body condition it is likely that birds will be able to capitalise on environmental fluctuations in carotenoid availability to allocate resources to sexual signalling as adults (Smith et al. 2007). Göransson et al. (1990) and Grahn et al. (1993) also found that increased body mass is correlated with dominance in pheasant male-male interactions. However, in the current study extra antioxidant resources were preferentially allocated to self-maintenance (reducing parasite load and oxidative damage) instead of growth or reproduction (i.e. sexually-selected traits). Consequently it may be that selection favours allocation of resources to self-maintenance in parasitized birds related to increased survival prospects during the first year of life. Individuals ingest a cocktail of natural antioxidants and a number of studies have identified synergistic interactions of dietary antioxidants when supplemented in combination (Pike et al. 2007; Catoni et al. 2008; Perez et al. 2008; Orledge et al. 2012). Thus it may be that selection favours the allocation of resources to self-maintenance in parasitized birds, which is related to increasing survival prospects during the first year of life, or that unless vitamin E is supplemented in conjunction with carotenoids it is effectively unavailable for preferential allocation towards growth (Orledge et al. 2012).

Males had significantly larger numbers of adult *H. gallinarum* at adulthood than females. Previous studies have also shown that males are more likely to be infected with parasites and have a higher load than females (Zuk & McKean 1996). Folstad and Karter (1992) have argued that immunosuppressive effects of high testosterone levels that contribute to bright displays may cause

males to have more rather than fewer parasites. Despite evidence that vitamin E has immuno-enhancing capacities we found no evidence for improved immune response to PHA injection at 21 weeks of age in individuals that had been supplemented with vitamin E during development. In addition, we found no effect of parasite load on the degree of immune response. In this study, we measured the pro-inflammatory immune response following PHA injection at 21 weeks of age, which is likely to incorporate broad elements of both innate and acquired immunity, so we were unable to measure more specific immune responses. In this case it may have been that humoral immunity was affected by the treatments, and/or there were treatment effects at 47 weeks, but these were not measured. It is also possible that the nematode *H. gallinarum* was not pathogenic enough to affect the pro-inflammatory immune response, although the reduced numbers of nematodes in the guts of birds supplemented with vitamin E indicates that the costs of parasite infection at the given dose was sufficient to lead to treatment differences in parasite loads at 47 weeks.

In conclusion, we found that supplementation of additional vitamin E during development reduced the parasite load of adults and the oxidative stress associated with maintaining a higher parasite load. However, we did not find that the availability of extra antioxidant resources during development resulted in increased allocation to sexual signals if infected with nematode parasites, or that the degree of ornamentation in pheasants reflected either the parasite load of *H. gallinarum* or the oxidative status of males. It is possible that the parasite used in our study did not produce a sufficiently strong pathological response to lead to detectable differences in the allocation of resources to sexually-selected traits. However, given that *H. gallinarum* is a common intestinal parasite of pheasants and was administered in doses within the natural range found in wild birds, if the dose was not sufficient to stimulate a strong enough response that is visibly expressed in a sexual signal of quality it raises questions about how generally informative such a signal can be to females if it is only expressed when males have experienced very high parasite loads. In such circumstances signals may effectively become redundant. It is also possible that the effects of parasite manipulation and supplementation of vitamin E in relation to the quality of the general nutritional environment

were too weak to detect treatment effects on sexually-selected traits in males that were not fully developed (i.e. 1st year as opposed to 2nd year birds). However, the long-term effects of early exposure to parasites and vitamin E on parasite load and circulating levels of vitamin E at adulthood indicate that there are likely to be downstream fitness effects of the treatments that are not evident at 47 weeks, when the expression of sexually-selected traits is largely uninformative of the environment experienced during the first 8 weeks of life in pheasants.

ACKNOWLEDGEMENTS

We would like to thank Tom Pike for help with the colour analyses and Maureen Woodburn for advice about *Heterakis gallinarum* infection. Thanks also to Chris Davis (MRCVS) and Matt Ford at the Game and Wildlife Conservation Trust (GWCT) for help with the husbandry and welfare of the pheasants, to Matthew Cooke, Sam Cruickshank, Mark Hillsley, John Simper and Amy Williams for assistance in the field and to three anonymous referees for their constructive comments. This research was funded by a NERC studentship (NE/F007450/1) for JMO, awarded to NJR and JDB in CASE partnership with ANH at the GWCT. JDB was funded by a Royal Society Research Fellowship.

REFERENCES

Andersson, M., & Simmons, L. (2006) Sexual selection and mate choice. *Trends in Ecology and Evolution*, **21**, 296-302.

Andersson, M. (1994) *Sexual Selection*, Princeton Univ. Press.

de Ayala, R.M., Martinelli, R., & Saino, N. (2006) Vitamin E supplementation enhances growth and condition of nestling barn swallow (*Hirundo rustica*). *Behavioral Ecology and Sociobiology*, **60**, 619-630.

Babior, B.M. (1984) The respiratory burst of phagocytes. *Journal of Clinical Investigation*, **73**, 599-601.

Baeta, R., Faivre, B., Motreuil, S., Gaillard, M. & Moreau, J. (2008) Carotenoid trade-off between parasitic resistance and sexual display: an experimental study in the blackbird (*Turdus merula*). *Proceedings of the Royal Society B-Biological Sciences,* **275**, 427-434.

Bartov, I., & Frigg, M. (1992) Effect of high concentrations of dietary vitamin-E during various age

periods on performance, plasma vitamin-E and meat stability of broiler chicks at 7 weeks of age. *British Poultry Science*, **33**, 393-402.

Bertrand, S., Faivre, B., & Sorci, G. (2006) Do carotenoid-based sexual traits signal the availability of non-pigmentary antioxidants? *Journal of Experimental Biology*, **209**, 4414-4419.

Blount, J.D., Metcalfe, N.B., Birkhead, T.R., & Surai, P.F. (2003) Carotenoid modulation of immune function and sexual attractiveness in zebra finches. *Science*, **300**, 125-127.

Borgia, G. (1979) Sexual selection and the evolution of mating systems. *Sexual selection and reproductive competition* (eds. M. Blum and A. Blum), pp.67-107. New York Academic Press.

Borgia, G., Egeth, M., Uy, J.A., & Patricelli, G.L. (2004) Juvenile infection and male display: testing the bright male hypothesis across individual life histories. *Behavioral Ecology*, **15**, 722-728.

Brawner, W.R., Hill, G.E., & Sundermann, C.A. (2000) Effects of coccidial and mycoplasmal infections on carotenoid- based plumage pigmentation in male House Finches. *Auk*, **117**, 952-963.

Buchanan, K.L., Spencer, K.A., Goldsmith, A.R., & Catchpole, C.K. (2003) Song as an honest signal of past developmental stress in the European starling (*Sturnus vulgaris*). *Proceedings of the Royal Society of London B*, **270**, 1149-1156.

Candolin, U. (2003) The use of multiple cues in mate choice. *Biological Reviews*, **78**, 575-595.

Carpenter, A.P. (1979) Determination of tocopherols in vegetable-oils. *Journal of the American Oil Chemists Society*, **56**, 668-671.

Catoni, C., Peters, A., & Schaefer, H. M. (2008) Life history trade-offs are influenced by the diversity, availability and interactions of dietary antioxidants. *Animal Behaviour*, **76**, 1107-1119.

Coop, R.L., & Holmes, P.H. (1996). Nutrition and parasite interaction. *International Journal for Parasitology,* **26**, 951-962.

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

Costantini, D., & Moller, A. (2008) Carotenoids are minor antioxidants for birds. *Functional Ecology*, **22**, 367-370.

Crawley M.J. (2007) The R Book, Wiley, London.

Cucco, M., Guasco, B., Malacarne, G., & Ottonelli, R. (2006) Effects of beta-carotene supplementation on chick growth, immune status and behaviour in the grey partridge, *Perdix perdix*. *Behavioural Processes*, **73**, 325-332.

Czeczuga, B. (1979) Carotenoids in the skin of certain species of birds. *Comparative Biochemistry and Physiology Part B: Comparative Biochemistry*, **62**, 107-109.

David, P., Bjorksten T., Fowler K., & Pomiankowski, A. (2000) Condition-dependent signalling of genetic variation in stalk-eyes flies. *Nature,* **406**, 186-188.

Doster G.L., & Goater C.P. (1997) Collection and quantification of avian helminths and protozoa. *Host-parasite evolution- general principles and avian models* (eds. D.H.Clayton & J. Moore), pp 396-418, Oxford University Press, Oxford.

Draycott, R., Parish, D.M., Woodburn, M.I., & Carroll, J.P. (2000) Spring survey of the parasite *Heterakis gallinarum* in wild-living pheasants in Britain. *Veterinary Record*, **147**, 245-246.

Endler, J.A., & Mielke, P.W. (2005) Comparing entire colour patterns as birds see them. *Biological Journal of the Linnean Society,* **86**, 405-431.

Folstad, I., & Karter, A.J. (1992) Parasites, bright males, and the immunocompetence handicap. *The American Naturalist*, **139,** 603–622.

Getty, T. (2002) Signalling health versus parasites. *American Naturalist*, 159, 363-371.

Göransson, G., von Schantz, T., Fröberg, I, Helgee, A., & Wittzell, H. (1990) Male characteristics, viability and harem size in the pheasant, *Phasianus colchicus*. *Animal Behaviour*, **40**, 89-104.

Govardovskii, V.I., Fyhrquist, N., Reuter, T., Kuzmin, D.G., and Donner, K. (2000) In search of the visual pigment template. *Visual Neuroscience,* **17**:509-528.

Grahn, M., Göransson G., & von Schantz, T. (1993) Spacing behaviour of male pheasants, Phasianus colchicus, in relation to dominance and mate acquisition. *Animal Behaviour,* **45**, 93-103.

Grahn, M., & von Schantz T. (1994). Fashion and age in pheasants: age differences in mate choice. *Proceedings of the Royal Society of London- Series B*, **255**, 237-241.

Guo, Y., Tang, Q., Yuan, J., & Jiang, Z. (2001) Effects of supplementation with vitamin E on the performance and the tissue peroxidation of broiler chicks and the stability of thigh meat against oxidative deterioration. *Animal Feed Science and Technology*, **89**, 165-173.

Hall, M.E., Blount, J.D., Forbes, S., & Royle, N.J. (2010) Does oxidative stress mediate the trade-off between growth and self-maintenance in structured families? *Functional Ecology,* **24**, 365-373.

Hamilton, W., & Zuk, M. (1982) Heritable true fitness and bright birds- A role for parasites. *Science*, **218**, 384-387.

Hamilton, W., & Poulin, R. (1997) The Hamilton and Zuk hypothesis revisited: A meta-analytical approach. *Behaviour*, **134**, 299-320.

Hart, N. (2002) Vision in the peafowl (Aves : Pavo cristatus). *Journal of Experimental Biology*, **205**, 3925-3935.

Hart, N., & Vorobyev, M. (2005) Modelling oil droplet absorption spectra and spectral sensitivities of bird cone photoreceptors. *Journal of Comparative Physiology A- Neuroethology, Sensory, Neural and Behavioral Physiology,* **191**, 381-392.

Hartley, R.C., & Kennedy, M.W. (2004) Are carotenoids a red herring in sexual display? *Trends in Ecology & Evolution*, **19**, 353-354.

Hill, D. & Robertson, P. (1988) *The Pheasant: Ecology, Management and Conservation*, BSP, Kent.

Hill, G.E., Farmer, K.L., & Beck, M.L. (2004) The effect of mycoplasmosis on carotenoid plumage coloration in male house finches. *The Journal of Experimental Biology*, **207**, 2095-2099.

Hillgarth, N. (1990) Parasites and female choice in the ring-necked pheasant. *American Zoologist*, **30**, 227-233.

Holmes, J.C. (1995) Population regulation: a dynamic complex of interactions. *Wildlife Research,* **22**, 11-19.

Hõrak, P., Ots I., Vellau, H., Spottiswoode, C., & Møller, A.P. (2001) Carotenoid-based plumage coloration reflects hemoparasite infection and local survival in breeding great tits. *Oecologia*, **126**, 166-173.

Hõrak, P., Saks, L., Karu, U., Ots, I., Surai, P.F., & McGraw, K. (2004) How coccidian parasites affect health and appearance of greenfinches. *Journal of Animal Ecology*, **73**, 935-947.

Houde, A., & Torio, A. (1992) Effect of parasitic infection on male color pattern and female choice in guppies. *Behavioral Ecology*, **3**, 346-351.

Hudson, P.J., Dobson, A.P., Newborn, D. (1992) Do parasites make prey vulnerable to predation? Red grouse and parasites. *Journal of Animal Ecology*, **61**, 681-692.

Hunt, J., & Simmons, L.W. (1997) Patterns of fluctuating asymmetry in beetle horns: an experimental examination of the honest signalling hypothesis. *Behavioral Ecology and Sociobiology,* **42**, 447–451.

Isomursu, M., Rätti, O., Helle, P., & Hollmén, T. (2008) Parasitised grouse are more vulnerable to predation as revealed by a dog assisted hunting study. *Annales Zoologici Fennici*, **45**, 496-502.

Keyser, A.J., & Hill, G.E. (1999) Condition-dependent variation in the blue-ultraviolet coloration of a structurally based plumage ornament. *Proceedings of the Royal Society of London-Series B Biological Sciences*, **266**, 771-777.

Kim, H., Arai, H., Arita, M., Ogihara, T., Tamai, H., Inoue, K., & Mino, M. (1996) Age-related changes of alpha-tocopherol transfer protein expression in rat liver. Journal *of Nutritional Science and Vitaminology,* **42**, 11-18.

Kirkpatrick, M., & Ryan, M.J. (1991) The evolution of mating preferences and the paradox of the lek. *Nature* **350**, 33-38.

Lozano, G.A. (1994) Carotenoids, Parasites, and Sexual Selection. *Oikos*, **70**, 309-311.

Martinez-Padilla, J., Mougeot, F., Perez-Rodriguez, L., & Bortolotti, G.R. (2007) Nematode parasites reduce carotenoid-based signalling in male red grouse. Biology Letters, **3**, 161-164.

McGraw, K.J., & Hill, G.E. (2000) Differential effects of endoparasitism on the expression of carotenoid- and melanin-based ornamental coloration. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **267**, 1525-1531.

McGraw, K., & Ardia, D. (2003) Carotenoids, immunocompetence, and the information content of sexual colors: An experimental test. American Naturalist*,* **162**, 704-712.

McGraw, K., Adkins-Regan, E., & Parker, R.S. (2005) Maternally derived carotenoid pigments affect offspring survival, sex ratio, and sexual attractiveness in a colourful songbird. *Naturwissenschaften*, **92**, 375-380.

Millán, J., Gortázar, C., Tizzani, P., & Buenestado, F.J. (2002) Do helminths increase the vulnerability of released pheasants to fox predation? *Journal of Helminthology*, **76**, 225-229.

Milinski, M., & Bakker, T.C.M. (1990) Female Sticklebacks Use Male Coloration in Mate Choice And Hence Avoid Parasitized Males. *Nature*, **344**, 330-333.

Møller, A. (1994) *Sexual selection in the barn swallow*. Oxford University Press, Oxford.

Møller, A.P., Christe, P., & Lux, E. (1999) Parasitism, host immune function, and sexual selection. *The Quarterly Review of Biology*, **74**, 3-20.

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

Mougeot, F., Redpath, S., & Leckie, F. (2005) Ultra-violet reflectance of male and female red grouse, *Lagopus lagopus scoticus*: sexual ornaments reflect nematode parasite intensity. *Journal of Avian Biology*, **36**, 203-209.

Mougeot, F., Martinez-Padilla, J., Blount, J.D., Perez-Rodriguez, L., Webster, L.M.I., & Piertney,, S.B. (2010) Oxidative stress and the effect of parasites on a carotenoid-based ornament. *Journal of Experimental Biology,* **213**, 400-407

Norris, K. (1993) Heritable variation in a plumage indicator of viability in male great tits *Parus major*. *Nature*, **362**, 537-539.

Ohlsson, T., & Smith H.G. (2001) Early nutrition causes persistent effects on pheasant morphology. *Physiological and Biochemical Zoology,* **74**, 212-218.

Ohlsson, T., Smith H.G., Råberg L., & Hasselquist, D. (2002) Pheasant sexual ornaments reflect nutritional conditions during early growth. *Proceedings of the Royal Society of London Series B Biological Sciences,* **269**, 21-27.

Olsen, O.W. (1974) *Animal parasites: their life cycles and ecology*, University Park Press.

Orledge, J.M., Blount, J.D., Hoodless, A. N., Pike, T.W. & Royle, N.J. (2012) Synergistic effects of supplementation of dietary antioxidants during growth on adult phenotype in ring-necked pheasants, *Phasianus colchicus. Functional Ecology,* **26***,* 254-264.

Osorio, D., Vorobyev, M. and Jones, C.D. (1999) Colour vision of domestic chicks. *Journal of Experimental Biology*, **202**, 2951-2959.

Perez, C., Lores, M., & Velando, A. (2008) Availability of nonpigmentary antioxidant affects red coloration in gulls. *Behavioural Ecology*, **19**, 967-973.

Petrie, M. (1994) Improved growth and survival of offspring of peacocks with more elaborate trains. *Nature*, **371**, 598-599.

Pike, T.W., Blount, J.D., Lindström & Metcalfe, N.B. (2007) Availability of non-carotenoid antioxidants affects the expression of a carotenoid-based sexual ornament. *Biology Letters*, **3**, 353-

356.

Reynolds, J., & Gross, M. (1990) Costs and benefits of female mate choice-Is there a lek paradox? *American Naturalist*, **136**, 230-243.

Rohlf, F.J. (1992) The analysis of shape variation using ordinations of fitted functions. *Ordination in the study of morphology, evolution and systematics of insects: Applications and quantitative genetic rationals* (eds.J.T., Sorenson & R., Foottit), pp. 95-112, Elsevier, Amsterdam.

Royle, N.J., Lindström, J., & Metcalfe, N. (2005) A poor start in life negatively affects dominance status in adulthood independent of body size in green swordtails *Xiphophorus helleri*. *Proceedings of the Royal Society of London-B*, **272**, 1917-1922.

Sage, R.B., Woodburn, M.I., Davis, C., & Aebischer, N.J. (2002) The effect of an experimental infection of the nematode Heterakis gallinarum on hand-reared grey partridges *Perdix perdix*. *Parasitology*, **124**, 529-535.

von Schantz, T., Bensch, S., Grahn, M, Hasselquist, D., & Wittzell, H. (1999) Good genes, oxidative stress and condition-dependent sexual signals. *Proceedings of the Royal Society of London Series B Biological Sciences*, **266**, 1-12.

Sheppard, C., Dierenfeld, E. & Burnett, M. (1998) Recommendations for diets of captive pheasants, based on information from diets of wild birds. *WPA News* **56**, 27–33.

Sies, H. (1997) Oxidative stress: Oxidants and antioxidants. *Experimental Physiology*, **82**, 291-295.

Smith, A., Madden, K.B., Au Yeung, K.J., Zhao A., Elfrey J., Finkelman F., Levander O., Shea-Donohue T., & Urban, J.F. (2005) Deficiencies in Selenium and/or Vitamin E Lower the Resistance of Mice to *Heligmosomoides polygyrus* Infections. *Journal of Nutrition*, **135**, 830-836.

Smith, H.G., Råberg, L., Ohlsson, T., Grandbom, M., & Hasselquist, D. (2007) Carotenoid and protein supplementation have differential effects on pheasant ornamentation and immunity. *Journal of Evolutionary Biology*, **20,** 310-319.

Smits, J., Bortolotti, G., & Tella, J. (1999) Simplifying the phytohaemagglutinin skin-testing technique in studies of avian immunocompetence. *Functional Ecology*, **13**, 567-572.

South, S. & Arnqvist, G. (2009) Morphological variation of an ornament expressed in both sexes of the mosquito *Sabethes cyaneus*. *Evolutionary Ecology Research*, **11**, 1-21.

Spencer, K.A., Buchanan, K.L., Goldsmith, A.R. & Catchpole, C.K. (2003) Song as an honest signal of developmental stress in the zebra finch (*Taenopygia guttata*). *Hormones and Behavior*, **44**, 132-139.

Spencer, K.A., Buchanan, K.L., Leitner, S., Goldsmith, A.R., & Catchpole, C.K. (2005) Parasites affect song complexity and neural development in a songbird. *Proceedings of the Royal Society of London*, **272**, 2037-2043.

Stoks, R., De Block, M., & McPeek, M.A. (2006) Physiological costs of compensatory growth in a damselfly. *Ecology*, **87**, 1566-1574.

Surai, A.P. (2002) *Natural antioxidants in avian nutrition and reproduction*, Nottingham University Press, UK.

Game Conservancy Trust (2006) *Gamebird Rearing*. Game Conservancy Ltd., Cheltenham.

Thompson, C.W., Hillgarth, N., Leu, M., & McClure, H.E. (1997) High parasite load in housefinches (*Carpodacus mexicanus*) is correlated with reduced expression of a sexually selected trait. *American Naturalist*, **149**, 270-294.

Tompkins, D.M., and Hudson, P.J. (1999) Regulation of nematode fecundity in the ring-necked Pheasant (*Phasianus colchicus*): not just density dependence. *Parasitology,* **118**, 417-423.

Tompkins, D.M., Greenman, J.V., & Hudson, P.J. (2001) Differential impact of a shared nematode parasite on two gamebird hosts: implications for apparent competition. *Parasitology*, **22**, 187-193.

Vinkler, M., Bainova, H., and T. Albrecht. 2010. Functional analysis of the skin-swelling response to phytohaemagglutinin. Functional Ecology 24:1081-1086.

Vorobyev, M., Osorio, D., Bennett, A.T.D., Marshall, N.J. & Cuthill, I.C. (1998) Tetrachromacy, oil droplets and bird plumage colours. *Journal of Comparative Physiology A*, **183**, 621-633.

Wang, X., & Quinn, P.J. (1999) The effect of α-tocopherol on the thermotropic phase behaviour of dipalmitoylphosphatidylethanolamine. A synchrotron X-ray diffraction study. *European Journal of Biochemistry*, **264**, 1–8.

Wedell, N., & Tregenza, T. (1999) Successful fathers sire successful sons. *Evolution*, **53**, 620-625.

Woodard, A.E., Vohra, P. & Snyder, R.L. (1977) Effect of protein levels in the diet on the growth of pheasants. *Poultry Science* **56**, 1492–1500.

Woodburn, M., Sage, R.B., & Carroll, J.P. (2002) The efficacy of a technique to control parasitic worm burden in pheasants *(Phasianus colchicus*) in the wild. *Zeitschrift fur Jagdwissenschaft,* **48,** 364–372.

Wyszecki, G., & Stiles W.S. (1982) Color Science: Concepts and methods, quantitative data and Formulae. Wiley, New York.

Zuk, M., Thornhill, R., & Lignon, J.D. (1990) Parasites And Mate Choice In Red Jungle Fowl. *American Zoologist*, **30**, 235-244.

Zuk, M., & McKean, K.A. (1996) Sex differences in parasite infections: patterns and processes. *International Journal of Parasitology*, **26**, 1009-102

Table 1. Parameter estimates of explanatory terms in Minimum Adequate Models for parasite load and plasma concentrations of vitamin E and the lipid peroxidation product MDA , respectively. See main text for further model details.

Explanatory term	Parameter estimate	SE of estimate	DF	t-value	p-value
a) Parasite burden of males and females at 47 weeks of age					
Intercept	24.57	3.82	225	6.44	<0.0001
Vitamin E treatment	-5.95	4.59	225	-1.30	0.196
Parasite treatment	10.71	4.60	225	2.33	0.021
Sex	-11.45	3.18	225	-3.60	<0.001
Vitamin E * Parasite	-3.38	6.38	225	-0.53	0.031
b) Plasma vitamin E concentration (µg/ml) of males					
Intercept	48.50	5.21	113	9.32	<0.0001
Vitamin E treatment	107.47	6.20	112	17.32	<0.0001
Age	-1.00	0.13	113	-7.53	<0.0001
Vitamin E * Age	-2.26	0.18	113	-12.26	<0.0001
c) Plasma MDA concentration (µg/ml) of males and females					
Intercept	8.13	0.47	454	17.29	<0.0001
Vitamin E treatment	-0.84	0.58	454	-1.45	0.148
Parasite treatment	2.18	0.58	454	3.75	0.0002
Age	-0.15	0.01	454	-11.16	<0.0001
Vitamin E * Parasite	-2.04	0.58	454	-3.54	0.0004
Vitamin E * Age	0.05	0.01	454	3.07	0.002
Parasite * Age	-0.03	0.01	454	-2.03	0.043

Table 2. Parameter estimates of explanatory terms in Minimum Adequate Models for growth of morphological response variables. See main text for further model details.

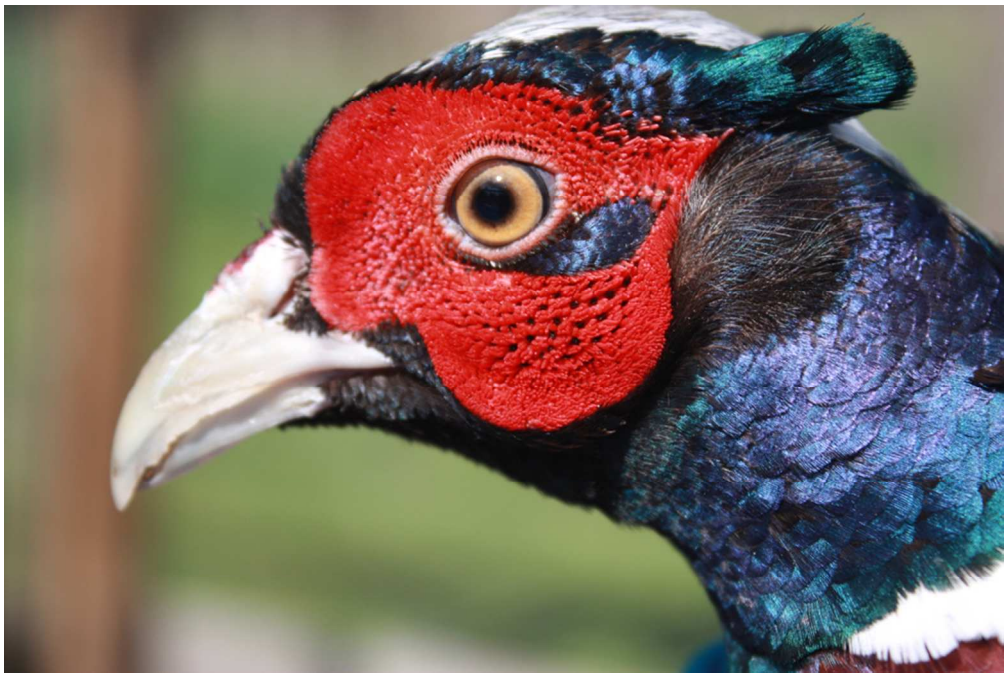
Explanatory term	Parameter estimate	SE of estimate	DF	t-value	p-value
a) Mass (g)					
Intercept	624.54	16.76	461	37.27	<0.0001
Sex	-161.59	14.72	228	-10.98	<0.0001
Age	16.12	0.45	461	35.53	<0.0001
b) Head-bill length (mm)					
Intercept	62.77	0.27	461	228.51	<0.0001
Sex	-3.09	0.29	228	-10.71	<0.0001
Age	0.22	0.01	461	33.30	<0.0001
c) Tarsus length (mm)					
Intercept	76.85	0.33	461	235.43	<0.0001
Sex	-7.09	0.42	228	-16.99	<0.0001
Age	0.15	0.01	461	28.00	<0.0001
d) Wing length (mm)					
Intercept	18.88	0.13	461	148.79	<0.0001
Sex	-1.23	0.12	228	-10.22	<0.0001
Age	0.12	0.01	461	32.17	<0.0001

Figure legends

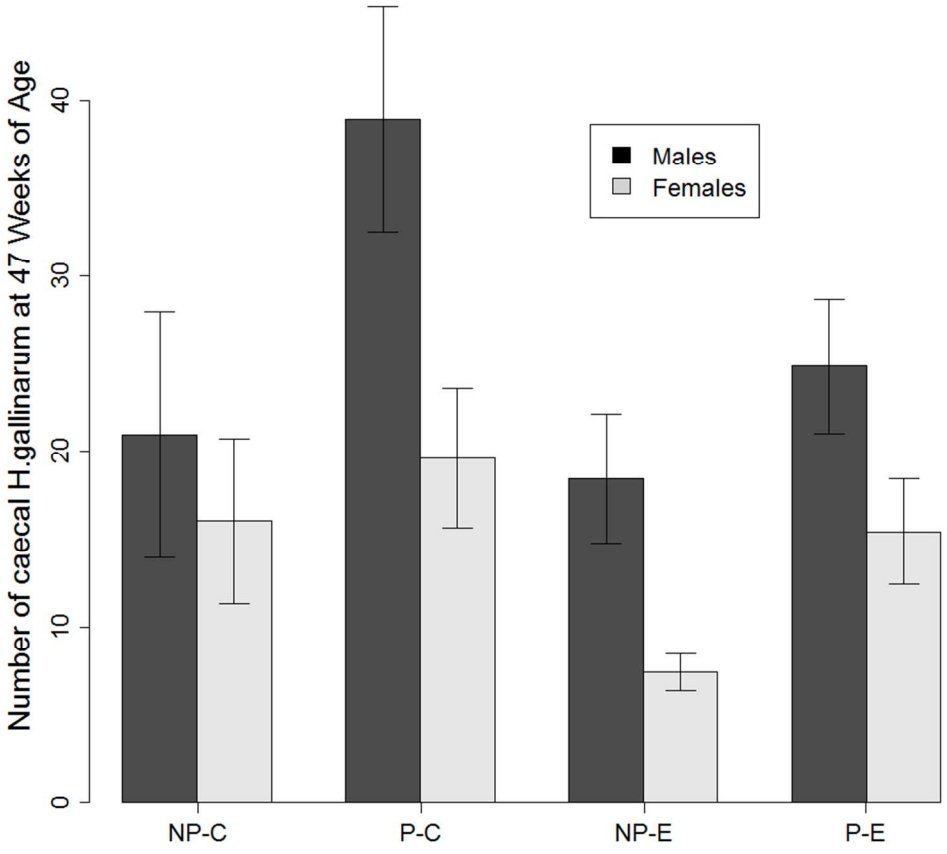
Fig. 1: A male ring necked pheasant [*Phasianus colchicus*] showing sexually selected ornament, the facial wattle. Photo credit N.J. Royle.

Fig. 2: Levels of parasitic burden (*H.gallinarum*) at 47 weeks of age in relation to sex and treatment group. Means are shown with 95% confidence intervals. Sample sizes are provided for each mean.

Fig. 3: Plasma α-tocopherol (a and b) carotenoid (c and d) and MDA (e and f) concentrations (μg/ml) in relation to treatment and age at (a, c and e) 8 and (b, d and f) 47 weeks of age. Means are shown with 95% confidence intervals. Note that scales differ considerably between 8 and 47 weeks of age.



270x180mm (96 x 96 DPI)



345x300mm (72 x 72 DPI)

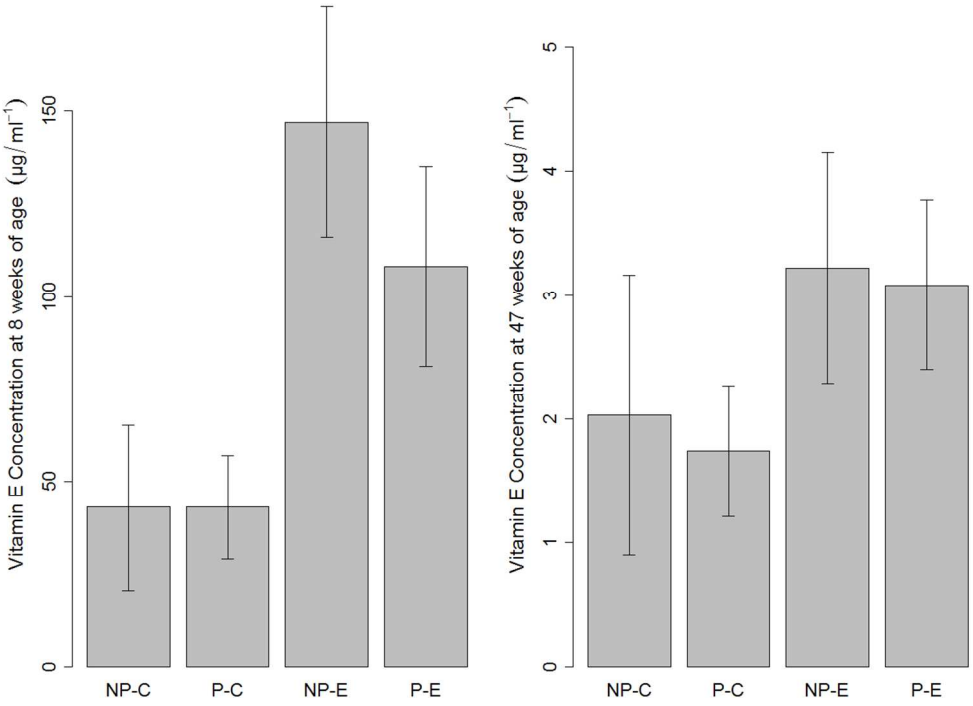


Figure 3 a & b
451x319mm (72 x 72 DPI)

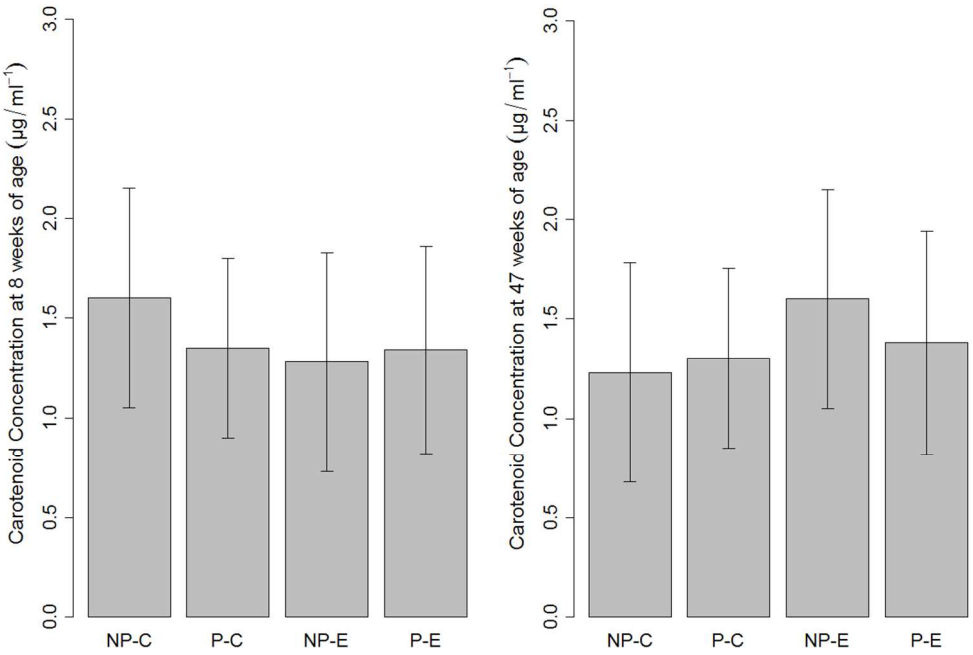
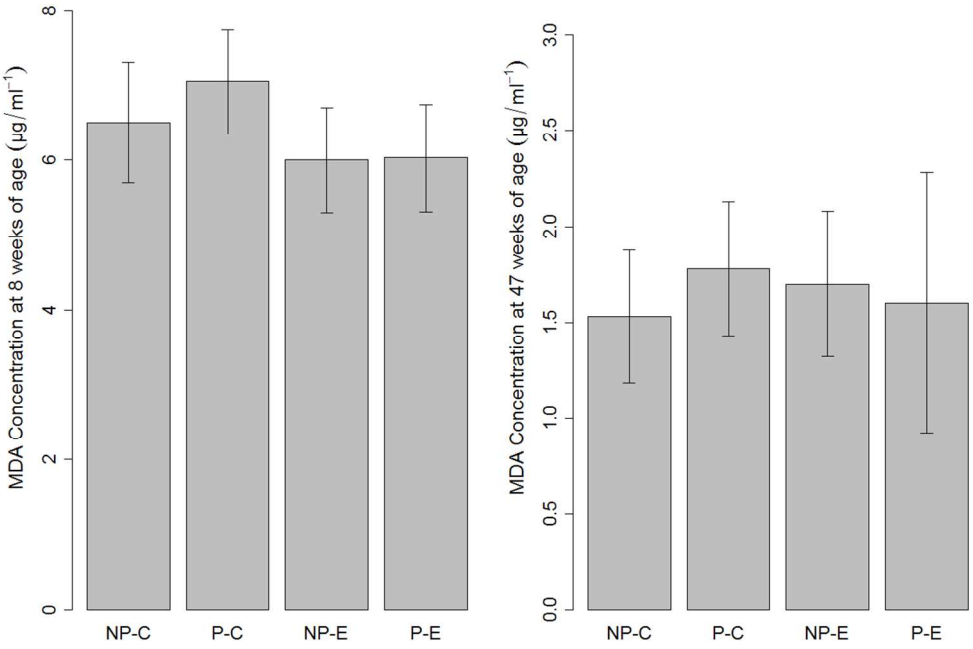


Figure 3c & d
445x300mm (72 x 72 DPI)



451x319mm (72 x 72 DPI)