Consequences of Trade-Offs During Growth and Development in Pheasants
(*Phasianus colchicus*)

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signed:

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Abstract

Oxidative stress may provide a proximate link mediating the trade-offs between the allocation of resources to growth and/or reproduction and investment in self-maintenance. Dietary antioxidants, such as carotenoids and vitamin E, provide potentially important roles in regulating these trade-offs. Recent work suggests that carotenoids may have important synergistic effects in combination with non-pigmentary antioxidants (e.g. vitamin E) on the expression of sexually-selected traits at adulthood. However, these studies involved the supplementation of antioxidants to adults and did not take into account early life-history effects. In this thesis, I test the independent and combined roles of supplementation of carotenoids and vitamin E during early growth in regulating the expression of traits at adulthood, in ring-necked pheasants, *Phasianus colchicus*. Individuals supplemented with a combination of carotenoids and vitamin E were larger at adulthood than individuals receiving other treatment diets (including vitamin E or carotenoids alone), but there were no differences in ornament expression, immune function or levels of oxidative damage. In addition, there were no effects of early antioxidant supplementation on primary sexual traits and I found no relationship between primary and secondary sexual traits to support the phenotype-linked fertility hypothesis. This suggests that the allocation of limited antioxidant resources is prioritised towards traits that increase competitive ability rather than sexual attractiveness or primary reproductive traits in this strongly sexually-selected species.

I also measure male ornament expression to test the 'parasite-mediated sexual signalling' hypothesis that predicts that ornamentation could provide a signal to females of a male's ability to resist parasites. Allocation of dietary-derived carotenoids to sexual ornaments may trade-off with allocation to immune and/or antioxidant functions mediated by the oxidative status of individuals. In this thesis I test whether supplementation with dietary antioxidants (vitamin E) can mitigate the effects of early exposure to parasites (the nematode, *Heterakis gallinarum*), via the alteration of the oxidative status of individuals, and positively affect the expression of sexual ornaments at adulthood. I find 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 predictions, the intensity of sexual ornament expression was not related to either the parasite load or oxidative status of individuals. Finally, I present the data from a novel experiment showing that feather odour changes in response to antioxidant status, but not intestinal parasite levels, at adulthood and in light of these results describe a potential role for feather odour in mate choice.

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Author's Declaration

During the research contributing to this thesis I was supported by a studentship from the Natural Environment Research Council in case partnership with the Game and Wildlife Conservation Trust (GWCT). The birds used during the experiments were maintained from 10 weeks of age onwards by the Rearing Field Technician, Matt Ford, at the GWCT.

All of the chapters in this thesis were written by J. Orledge with comments from and completed under the guidance of Dr. Nick Royle.

All of the experiments and data collection detailed in this thesis were completed by J. Orledge. In each chapter the spectrophotometer data was processed by Dr. Tom Pike before final data analysis was completed by J. Orledge.

Chapter 1 General introduction

The initial draft of this chapter was written by J. Orledge and had substantial revision from Dr. Nick Royle. A version of this chapter has been included as a chapter in the following book, accepted for publication:

Royle, N.J., Orledge, J.M., and Blount, J.D. *Early life-history effects, oxidative stress and the evolution and expression of animal signals*. *In* Animal Signaling: Functional and Evolutionary Perspectives (Eds. Irschick, D., Podos, J., and Briffa, M.)

Chapter 2 General methods

The methods described within this chapter were the result of planning and guidance from Dr. Nick Royle, Dr. Jon Blount, Dr. Andrew Hoodless and Dr. Tom Pike. The laboratory methods described to measure vitamin E concentrations within the testes were adapted from methods developed by Dr. Sam Weber.

Chapter 3 Synergistic effects of supplementation of dietary antioxidants during growth on adult phenotype in ring-necked pheasants

I conducted the experiment, the data collection and data analysis for this experiment with the guidance of Drs Nick Royle, Jon Blount and Andrew Hoodless. Dr. Tom Pike provided comments on this chapter. Nia Denman, James Connell, Marc Edwards and John Simper provided field assistance during blood sampling.

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Synergistic effects of supplementation of dietary antioxidants during growth on adult phenotype in ring-necked pheasants, *Phasianus colchicus*.

Josephine M. Orledge, Jonathan D. Blount, Andrew N. Hoodless, Thomas W. Pike, Nick J. Royle.

Chapter 4 Effects of neonatal nutrition on ornamentation and sperm quality in ring-necked pheasants

I conducted the experiment, the data collection and data analysis for this experiment with guidance from Drs Jon Blount, Nick Royle and Andrew Hoodless. Dr. Stefan Lüpold measured sperm motility and prepared semen and testes samples for storage. Dr. Stefan Lüpold provided comments on a draft of this chapter.

Chapter 5 Antioxidant supplementation during early development reduces parasite load but does not affect sexual ornament expression in adult ring-necked pheasants

I conducted the experiment, the data collection and data analysis for this experiment with the guidance of Drs Nick Royle, Jon Blount and Andrew Hoodless. Matthew Cooke, Sam Cruickshank, Amy Williams, Mark Hillsley, Annie Browne and Lauren Hill provided field assistance during blood sampling. Dr. Maureen Woodburn provided advice regarding the use of *H. gallinarum*.

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Josephine M. Orledge, Jonathan D. Blount, Andrew N. Hoodless, Nick J. Royle.

Chapter 6 Feather odour changes in response to antioxidant status, but not intestinal parasite levels, in ring-necked pheasants

I conducted the experiment, the data collection and data analysis for this experiment with the guidance of Drs Nick Royle, Jon Blount and Andrew Hoodless. The initial concept was developed by Dr. Tom Pike and J. Orledge. Dr. Tom Pike provided guidance on the statistical analysis of GCMS data and with Dr. Nick Royle provided comments and advice during the writing of the chapter. Chris Mitchell provided training in the use of the GCMS.

A shortened version of this chapter is in preparation for submission as a correspondence article to *Current Biology*:

Odour as a cue to antioxidant status in a bird

Josephine M. Orledge, Nick J. Royle, Jonathan D. Blount, Andrew N. Hoodless and Thomas W. Pike.

Chapter 7 General Discussion

The conclusions presented in this chapter are my own interpretation of the previous data chapters under the guidance of Dr. Nick Royle.

Chapter 1 General Introduction

A version of this chapter has been accepted for publication:
Royle, N.J., Orledge, J.M., and Blount, J.D. Early life-history effects, oxidative stress and the evolution and expression of animal signals. *In* Animal Signaling: Functional and Evolutionary Perspectives (Eds. Irschick, D., Podos, J., and Briffa, M.) (*In press*)

1.1. Early life history effects and resource allocation trade-offs

1.1.1. Early life history effects

The period from conception to sexual maturity is referred to as the early life-history of an organism. Early life-history effects are the long-term consequences of the conditions experienced over the developmental period. Generally, the earlier the disruption to an individual's development the more pronounced the downstream effects (Lindström 1999). Fluctuations in the environmental conditions that an individual experiences during development, and maternal (and paternal) effects, the effects of parents on the phenotype of offspring that are unrelated to the offspring's genotype (Bernardo 1996), have longer-term effects on growth and the allocation of resources to competing functions (e.g. the development of the immune system). One of the most fundamental traits affected by perturbations in resource availability during an individual's early development is the rate of growth.

1.1.2. Costs of growth

Many advantages have been identified associated with growing rapidly, including faster growth through the 'mortality window' (Campana 1996; Brunton and Booth 2003; Takasuka et al. 2003; Arendt and Reznick 2005); earlier access to a greater range of prey (Shoji and Tanaka 2006); increased competitive ability (Moran 2007) and earlier and more successful reproductive output (Metcalfe and Monaghan 2003). Despite the expectation that rapid growth should be advantageous and lead to greater fitness (Metcalfe and Monaghan 2003), the prevalence of sub-maximal growth rates suggests that there are significant costs to rapid growth. The costs of rapid growth are varied, and include for example, reduced investment in protein maintenance (in rats; Samuels and Baracos 1995), deferred sexual maturation (salmonids; Morgan and Metcalfe 2001), weight loss during metamorphosis in butterflies (Fischer et al. 2004), reduced lifespan (zebra finches; Birkhead et al. 1999; mice; Ozanne and Hales 2004), lower competitive ability (swordtails; Royle et al. 2005), impaired locomotor performance (salmonids; Farrell et al. 1997; larval anurans; Arendt 2003; swordtails; Royle et al. 2006a,b) and an increased risk of predation (damselflies; Stoks et al.

2005). The timescale over which these costs are paid varies, from the immediate (e.g. reduced rate of bone ossification in bluegill sunfish; Arendt and Wilson 2000) to longer term costs (e.g. increased risk of heart disease in humans; Singhal and Lucas 2004; Singhal et al. 2004). As a result, on experiencing marked fluctuations in resource availability during early development there will be an optimal balance between immediate investment in increased growth rate and the costs of this growth.

1.1.3. Trade-offs during growth and development

Resource allocation trade-offs are believed to be particularly acute for strongly sexually-selected species, where significant investment in costly secondary sexual traits can only be achieved at a cost to other traits. The preferential allocation of resources to sexual attractiveness and growth, despite major costs, has been demonstrated in zebra finches, *Taeniopygia guttata.* Individuals that received a sub-optimal nestling diet were comparatively stunted at fledging, but on receiving an improved diet underwent a period of compensatory growth and 'caught' up with individuals given an optimal nestling diet. Later measurements indicated those individuals that had experienced rapid growth, had done so at the expense of a reduced adult lifespan (Birkhead et al. 1999).

The main selective forces for the allocation of resources to rapid growth and investment in secondary sexual traits are likely to include competition for limited resources during development (e.g. Royle et al. 1999), reduced fitness associated with small size (increased mortality; Metcalfe and Monaghan 2001) and poorly developed sexual signals (reduced mating success; Blount et al. 2003). Numerous studies have identified a variety of functional outcomes of resource allocation during growth and development, however, very little is known about the mechanism(s) underlying such trade-offs. Recent evidence suggests that oxidative stress may provide a proximate link between investment in growth/and or reproduction (primary and secondary sexual traits) and self-maintenance in animals (Blount 2004; Catoni et al. 2008; Costantini 2008; Dowling and Simmons 2009; Monaghan et al. 2009).

1.2. Oxidative stress as a mediator of resource allocation trade-offs

1.2.1. Reactive oxygen species

Oxygen is a necessary requisite for survival. However, in the form of reactive oxygen species (ROS), it has the capacity to act as a potent stressor associated with ageing and a multitude of degenerative diseases (Genestra 2007). Generated by aerobic cells as a routine product of regular metabolic activity and immune function (Castro and Freeman 2001; Surai 2002), it is estimated that 1-3% of the oxygen absorbed by a cell is converted to ROS (Seifried et al. 2007). Mitochondria fulfil the energy requirements of the living cell as a result of the synthesis of the high-energy biochemical compound, adenosine triphosphate (ATP). The mitochondrial respiratory chain that generates ATP is separated into discrete multienzyme complexes affixed to the inner mitochondrial membrane. Mitochondria use approximately 90% of the oxygen consumed and constitute the principal manufacturers of ROS (Dalle-Donne et al. 2006).

Characterisation of the 'respiratory burst,' a result of the incitement of phagocytes during immune activation has identified this event as a source of ROS. Peroxisomal fatty acid metabolism, microsomal enzymes and flavoprotein oxidases also contribute to levels of ROS (Pryor et al. 2006). ROS are predominantly highly reactive oxygen-derived free radicals, the most prevalent of which are superoxide ions (O_2^-), hydrogen peroxide (H_2O_2) and hydroxide radicals (OH^-) (Pryor et al. 2006; Cash et al. 2007). Nitric oxide and its derivatives react with free radicals to form reactive nitrogen species (RNS) (Patel et al. 1999). Oxidative stress is the net outcome of a physiological imbalance between the production of and the removal, by antioxidants, of ROS and RNS resulting in increased cellular levels (Kregel and Zhang 2007).

1.2.2. Oxidative stress

ROS/RNS induced damage to essential intracellular macromolecules can culminate in profound cellular abnormalities. High exposure to reactive species results in lipid peroxidation (altering cellular membrane permeability and ultimately resulting in leakage), DNA damage (leading potentially to uncontrolled cell proliferation or accelerated cell death) and protein oxidation (Kregel and Zhang 2007; Seifried et al.

2007). The brief coupling of mitochondrial DNA (mtDNA) to the inner mitochondrial membrane results in its intense exposure to ROS. Moreover, the lower efficiency of mitochondrial DNA repair apparatus results in the increased susceptibility of mtDNA to oxidative stress and the higher persistence of detrimental changes (Chow et al. 1999; Berg et al. 2004). Cell signalling pathways disrupted by oxidative damage to transcriptional factors and regulatory macromolecules can result in the activation or inactivation of essential proliferation and differentiation pathways. In addition, detrimental adjustments to cellular signalling can result in cellular senescence, an irreversible cellular state in which a cell remains metabolically active but is incapable of cellular division, and even apoptosis (Berg et al. 2004). However, the effects of ROS are not always negative. ROS also have a beneficial role as an essential component of pathways involved in the regulation of gene expression, neurotransmitter actions, control of blood flow and inflammatory signalling reactions (Castro and Freeman 2001; Genestra 2007; Seifried et al. 2007).

1.2.3. Antioxidant defences

The progressive shift towards an oxygen rich atmosphere has resulted in the evolution of a multitude of antioxidants to reduce levels of oxidative damage (Castro and Freeman 2001). Antioxidants significantly delay, inhibit or prevent free radical induced tissue damage by impeding the formation of radicals, scavenging them, or by promoting their decomposition (Young and Woodside 2001). Antioxidants are a collection of compounds distinguished by their proficiency to be oxidised more readily than vital cellular components and to upregulate repair systems (Genestra 2007). Situated in subcellular compartments, organelles and extracellular spaces the antioxidant system is both ubiquitous and diverse comprising natural fat soluble antioxidants such as vitamins A and E, carotenoids and ubiquinones, antioxidant enzymes, water soluble antioxidants such as uric acid and ascorbic acid, and the thiol redox system.

Antioxidant protection may be assigned to three functional levels. The first functional level has the role of abating free radical formation by removing the precursors of free radicals or by inactivating catalysts (Surai 2002). This level consists primarily of three antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT) and a selenoprotein, glutathione peroxidise (GPx) (Surai 2002; Salganak 2000). Superoxide

dismutase removes the free radical superoxide by converting it to peroxide which in turn may be converted by CAT or GPx to water (Surai 2002; Rodrigo et al. 2006). The antioxidant catalase, a remarkably efficient enzyme that cannot become saturated by H_2O_2, obstructs its release from the cell by converting it into molecular oxygen and water. Glutathione peroxidise also converts H_2O_2 to water and oxygen but is better suited to respond to low-level oxidative stress and defence against lipid and other organic hydroperoxides (Mates 2000). Metal binding proteins such as iron binding transferrin and copper binding ceruloplasmin, adhere to, and therefore isolate, metal ions, disabling them from acting as catalysts of reactions which would otherwise produce hydroxyl radicals (Surai 2002).

The second level of antioxidant defence consists of chain breaking antioxidants which include vitamin E, vitamin A, uric acid and ubiquinones. Lipid soluble chain breaking antioxidants disturb free radical lipid peroxidation, a chain reaction that culminates in an alteration of the fluidity and permeability of biological membranes. Chain breaking antioxidants prevent the propagation step of lipid peroxidation by scavenging the peroxyl radical intermediates required for a chain reaction (Surai 2002).

The third level of antioxidant defence incorporates the recognition of, and the repair or destruction of, molecules damaged by oxidative stress. The proteasome complex ameliorates oxidative damage by minimising the aggregation of proteins and degrading damaged proteins by proteolysis, a chemical reaction that breaks peptide bonds. DNA repair enzymes such as the DNA glycosylases, endonucleases, polymerases and ligases maintain the stability of the genome and lipases remove oxidised membrane lipids (Surai 2002). A delicate and critical balance exists in the body between antioxidant defence, repair systems and free radical generation.

1.2.4. Dietary derived antioxidants

Animals have evolved an effective systemic/enzymatic antioxidant system, however, there are some important antioxidants, most notably vitamins C and E and carotenoids, that must be derived solely from the diet. Carotenoids, for example, are derived from a diet of higher plants and algae, where they confer protection from photo-damage and contribute to photosynthetic machinery. The eight compounds, α-, β-, δ- and γ-tocopherol and α-, β-, δ- and γ -tocotrienols for which vitamin E is a

generic descriptor are, in contrast, derived from oil seeds and the leaves of higher plants (Sen 2007). Selenium, an essential component of several antioxidant proteins called selenoproteins can be derived from cereals, grains and vegetables. Optimal diet composition therefore improves antioxidant protection; however, it is also determined by bioavailability, the rate and extent at which a compound appears in the blood. Nine principal determinants of bioavailability after carotenoid consumption have been identified, including the type and amount of carotenoids consumed, the matrix in which the carotenoid is incorporated, the nutrient status of the host and genetic factors (van het Hof et al. 2000).

1.2.5. Interactions among dietary antioxidants

Increasingly research into antioxidant capabilities has indicated that it is no longer sufficient to consider the effect of a single antioxidant but necessary to investigate the interactions of multiple antioxidants (Catoni et al. 2008). For example, one molecule of α-tocopherol, an extremely effective antioxidant, is capable of reacting 100-1000 times faster than vulnerable membrane phospholipids with peroxyl radicals (Zhang and Omaye 2000). However, in the presence of α-ubiquinone, and the resulting competition for membrane binding sites, the antioxidant capabilities of α-tocopherol are reduced (Gille et al. 2008). Similarly, increasing ß-carotene levels lowered the availability of α-tocopherol in the plasma of rats (Blakely et al. 1990). The absorption pathways of carotenoids and vitamin E are similar, involving their incorporation into lipoprotein particles and release into the circulation via lymphatic pathways. Vitamin E absorption is described as a sensitive process and it is likely that an impairment in any of these stages will decrease vitamin assimilation from the diet (Surai 2002). It is possible that ß-carotene may compete for the same binding sites on lipoproteins required for absorption by α-tocopherol (Zhang and Omaye 2000). For example, when protein damage was measured it was lower with a combination of ascorbic acid, ß-carotene and α-tocopherol than with only a single antioxidant (Gille et al. 2002). However, synergistic interactions between carotenoids and vitamin E have also been measured (Palozza and Krinsky 1992). Synergistic interactions may result from the ability of carotenoids to recycle vitamin E by the transfer of an electron to the α-tocopherol radical formed during oxidation (Surai 2002).

1.2.6. Antioxidants, oxidative stress and rapid growth

The increased energetic requirements and higher oxygen intake during rapid growth are believed to result in the production of higher levels of ROS (Stoks et al. 2006). Rapid growth in response to antioxidant supplementation during early development has been identified in a number of different species (e.g. Cucco et al. 2006; de Ayala et al. 2006; O'Brien and Dawson 2008). As predicted, several of these supplementation studies measured an increase in oxidative damage following a period of rapid growth (Alonso-Alvarez et al. 2007; Nussey et al. 2009) or an increase in the production of antioxidant enzymes (De Blok and Stoks 2008). Previous studies have either measured oxidative stress in relation to growth (Stoks et al. 2006; Alonso-Alvarez et al. 2007; Nussey et al. 2009) or the effect of antioxidant supplementation on growth (Cucco et al. 2006; de Ayala et al. 2006; O'Brien and Dawson 2008).

Hall *et al*. (2010) measured the differential effects of antioxidant supplementation on red-winged blackbird chicks, *Agelaius phoeniceus*, hatching asynchronously in highly structured families. Nestling blackbirds have extremely rapid rates of growth and exhibit high intrafamilial conflict over parental investment. The high levels of conflict measured within the species is believed to result from low within nest relatedness due to high rates of extra-pair paternity (Royle et al. 1999). Sibling scramble competition for resources therefore selects for rapid growth as larger, more competitive, individuals can secure disproportionate shares of parental resources, which enables them to grow faster and makes them even more competitive (Mock and Parker 1997). Marginal offspring suffer considerably higher rates of mortality than core brood chicks. In order to be able to compete with higher quality nest-mates, that are better provisioned, marginal offspring must allocate relatively more resources to growth compared to self-maintenance. However, marginal offspring that do survive to fledging age grow just as rapidly as their older, core siblings, but have greater oxidative damage. Supplementation of nestlings with dietary antioxidants confirmed the outcome of this trade-off in favour of growth, as individuals that received more antioxidants throughout development allocated these extra resources to increasing growth rate, rather than reducing oxidative damage, irrespective of whether they were core or marginal offspring (Hall et al. 2010). This study showed that availability of dietary antioxidants can have differing effects on compensatory resource allocation

strategies depending upon the position of individuals within structured families, which affected the developmental trajectories of the chicks. Such trade-offs during early life are likely to have long-term effects on the phenotype of individuals at adulthood (Metcalfe and Monaghan 2001). The consequences of these trade-offs are expected to be particularly acute for species with exaggerated expression of sexual signals when adult (Royle et al. 2005).

1.3. Signals expressed at adulthood

1.3.1. Nutrition during growth and the expression of sexual signals as an adult

There is evidence that ornamental traits show a greater susceptibility to environmental stress than morphological traits. In Malaysian stalk eyed flies, *Cyrtodiopsis dalmanni*, a species in which females choose mates based upon eye span, the interaction between genetic and environmental influences on the sexual signal was investigated. Variation in the non sexual traits, wing length and female eye span, measured in flies exposed to reduced food quality, was directly proportional to body size. However, male eye span, a sexually-selected trait, was not proportional to body size and showed heightened condition-dependence. In some males development was unaffected by environmental stress demonstrating a strong genetic influence and potentially signalling the possession of 'good genes' to females (David et al. 2000).

1.3.2. Exposure to parasites during growth and the expression of sexual signals

The sensitivity of sexually-selected traits to the environment experienced during growth and development includes exposure to parasitic infection. Borgia *et al.* (2004) studied adult satin bowerbirds, *Ptilonorhynchus violaceus,* to determine whether male display could provide an indication of the parasite infections experienced during the juvenile life history stages. The study found that more attractive males had experienced a lower parasite burden as juveniles, but no significant relationship was found to exist between current adult parasite burden and male attractiveness (Borgia et al. 2004). Such findings suggest that females using sexually selected traits could acquire information on an individual's health during development.

1.3.3. Carotenoid-based sexual signals and immunity

Many sexual signals involve colour. Carotenoids are responsible for the red to yellow colouration of many birds, fish, reptiles, amphibians, insects and plants. Numerous mate choice studies have demonstrated the preference of females for males possessing signals with greater carotenoid pigmentation (Blount 2004). The capacity of carotenoids to act as sexual signals is believed to result from their relative rarity in nature combined with their roles in critical physiological functions, such as antioxidant defence and immunoenhancing properties (Saks et al. 2003).

In 1982, Hamilton and Zuk hypothesised a negative relationship between parasite encumbrance and the brightness of plumage. The parasite-mediated sexual selection hypothesis proposed that a higher intensity of carotenoid-plumage provided an indication of increased genetic resistance to parasites (Hamilton and Zuk 1982). Parasites of the gastrointestinal tract, such as *coccidia*, can disrupt epithelial cell permeability and in turn influence the absorption of carotenoids (Baeta et al. 2008). In addition, ectoparasites may directly reduce male colouration (Blount 2004). Experiments to induce immune activation using parasite infection (Brawner et al. 2000; Hoodless et al. 2002; Hõrak et al. 2004), analysis of infection clearance rates (Lindström and Lundström 2000) and heterophil counts (Dufva and Allander 1995) have provided evidence to link immune function and carotenoid-mediated plumage colouration. The basis for the hypothesis that organisms encounter a trade-off between deploying carotenoids to immune function and colourful display is the premise that activation of the immune system diminishes the level of carotenoid provisions available for signalling (Lozano 1994) which has been identified in a number of studies (e.g. Faivre et al. 2003; McGraw and Ardia 2003; Alonso-Alvarez et al. 2004). This could be because investment in immune function incurs an increase in the production of ROS, which is predicted to reduce expression of sexual signals (von Schantz et al. 1999). A study on surviving male house finches, *Carpodacus mexicanus*, following an epidemic of mycoplasmal conjunctivitis found survivors had significantly redder plumage than male non-survivors suggesting that plumage colour provides an honest signal of male condition (Nolan et al. 1998).

It has been suggested that both carotenoid-based sexual signals and immune function are ultimately influenced by nutritional status (Hill 2000; McGraw 2005). If immune

function and carotenoid-dependent display are reliant upon nutrient uptake, correlations may occur in the absence of a direct relationship between the two (Navara and Hill 2003). A carotenoid supplementation experiment on blackbirds, *Turdus merula*, for example showed that circulating carotenoid levels stabilise despite further supplementation (Baeta et al. 2008). Whether such a 'carrying capacity' occurs as a result of a limitation on an individual's absorption or transportation capabilities was unresolved (Baeta et al. 2008). Currently, interpretation of carotenoid supplementation studies is hindered by a lack of knowledge of the natural intake rates of antioxidants in the wild (Monaghan et al. 2009).

1.3.4. Carotenoid-based signals as indicators of oxidative stress

Due to the role of carotenoids as antioxidants, the intensity of carotenoid-mediated male sexual ornaments could signal the oxidative status of individuals because carotenoids are bleached on oxidation (von Schantz et al. 1999). Recently the antioxidant, free-radical scavenging properties of carotenoids have been questioned (Costantini and Møller 2008; Isaksson et al. 2008). However, animals ingest a cocktail of antioxidants which may interact either antagonistically (e.g. competition during absorption) or synergistically (e.g. carotenoid recycling by vitamin E; Surai 2002, Catoni et al. 2008). Hartley and Kennedy (2004) suggested that the presence of carotenoid based signals may signal the prevalence of more efficient non-pigmentary antioxidants ('The carotenoid protection theory'). This is because oxidation, a consequence of free radical scavenging, results in the structural alteration of carotenoids leaving them colourless and unavailable for use in sexual signalling. Non-pigmentary antioxidants may therefore protect carotenoids from oxidation, retaining them for use in sexual signalling (Hartley and Kennedy 2004). Several studies have since provided evidence in support of the 'carotenoid protection theory' (zebra finches, *Taenopygia guttata*; Bertrand et al. 2006; sticklebacks, *Gasterosteus aculeatus,* Pike et al. 2007; yellow-legged gulls, *Larus michahellis,* Pérez et al. 2008). Carotenoid-mediated signals could therefore provide an indication of the collective antioxidant defences, and indirectly the oxidative status, of an individual (Hartley and Kennedy 2004). Research has focused on the synergistic effects of antioxidants at adulthood and therefore the synergistic and antagonistic effects of antioxidants during growth and development on the expression of sexually-selected traits are untested.

1.3.5. Testosterone, carotenoid-mediated sexual signals and the oxidative handicap hypothesis

Testosterone may play an important role in antioxidant interactions because it can also affect the expression of male sexually-selected traits (Blas et al. 2006; Buchanan et al. 2003), and act as an immune suppressant (Peters 2000, 2007). An increase in the expression of testosterone dependent ornamentation, as a result, may compromise male immune system function (Folstad and Karter 1992). Recent research has suggested that the role of carotenoid pigments and testosterone are unlikely to be mutually exclusive, rather that they represent two components of a complex integrated physiological mechanism connecting ornamentation and immunity (Peters 2007). The basis for this research has been the investigation of the lipoprotein molecules that mediate the delivery of carotenoid molecules binding to them and conveying carotenoids from the intestine to the integument. There is a strong correlation between carotenoid-mediated beak colouration and the accumulation of lipoproteins found within the blood of zebra finches (Peters 2007), and evidence suggests that testosterone aids the formation of pivotal carotenoid transporting lipoproteins by upregulating the production of cholesterol, a constituent of lipoproteins (McGraw and Parker 2006).

The existence of a link between testosterone (immunosuppressant) and carotenoid (immunoenhancing) pathways would account for the ability of testosterone rich males to withstand the elevated levels of oxidative stress associated with high testosterone concentration (Peters 2007). The 'oxidative handicap hypothesis' suggests that an increase in testosterone leads to elevated metabolic rates, which increase ROS production and oxidative stress. The experimental elevation of testosterone levels in male red-legged partridges, *Alectoris rufa*, for example, resulted in higher levels of circulating carotenoids. However, increased testosterone levels also resulted in a reduction in oxidative status at the cost of carotenoid-mediated sexual signalling (Alonso-Alvarez et al. 2008), indicating that testosterone, in this case, promoted the allocation of resources to self-maintenance over reproduction (in contrast to the study of Hall et al. 2010). In male red grouse, *Lagopus lagopus scoticus,* increased testosterone levels result in enhanced ornamentation and circulating antioxidants levels, but also increased oxidative damage (Mougeot et al. 2009). These studies

support the hypothesis that sexually-selected traits can signal the oxidative status of a male (Alonso-Alvarez et al. 2008; Mougeot et al. 2009).

1.3.6. Early life history effects and the expression of carotenoid-mediated sexual signals

Considerable research has centred on the expression of carotenoid-mediated sexual signals following the manipulation of carotenoid availability at adulthood. However, the downstream effects of antioxidant availability during development have received relatively little attention despite evidence that early availability of antioxidants affects physiological function at adulthood (e.g. Blount et al. 2003) and that resource availability during development can have more influence on sexual signal expression than at adulthood (e.g. Borgia et al. 2004; McGraw 2005). Carotenoid supplementation during development has been found to affect the ability of individuals to assimilate carotenoids at adulthood regardless of the carotenoid content of the diet at adulthood (e.g. Koutsos et al. 2003; Biard et al. 2005, 2007; McGraw 2005; Isaksson et al. 2008). Similarly, variation in the protein content of developmental diet can affect physiological function and male sexually selected traits at adulthood (Ohlsson et al. 2002; Blount et al. 2003). The environment experienced during early life is therefore expected to affect the expression of carotenoid-mediated sexual signals later in life.

1.3.7. Early life history, oxidative stress and avian song

The effects of fluctuations in resources during development are not only seen in the expression of morphometric and carotenoid-based signals, but have also been observed in studies of the complexity of avian song. In many avian species females choose mates based on the quality or quantity of their song (Searcy and Nowicki 2005). Experimental manipulation of the environment experienced over early life through imposing nutritional stress or injection with the stress hormone corticosterone can lead to a reduction of the High Vocal Centre (HVC), a song control nucleus known to be a key determinant of song complexity (Buchanan et al. 2004). The 'developmental-stress hypothesis,' an extension of the 'nutritional-stress hypothesis' proposes that learned features of song development can serve as reliable indicators of male quality signalling a male's experience of, and developmental response, to stress

during early life (Buchanan et al. 2003, 2004; Nowicki et al. 2002; Zann and Cash 2008). The HVC has a higher susceptibility to stress experienced during development than other areas of the brain (Buchanan et al. 2004), and it has been suggested that the physiological mechanism underlying this trade-off in the allocation resources to HVC development, may be related to depleted energy levels and the response of corticosteroid receptors on increased levels of corticosterone (Buchanan et al. 2004; MacDonald et al. 2006). The vertebrate brain has a disproportionately high requirement for oxygen, contains a high volume of vulnerable polyunsaturated fatty acids and has many mitochondria releasing free radicals (Halliwell and Gutteridge 2007). Therefore, the HVC may be particularly susceptible to oxidative damage by free radicals (von Schantz et al. 1999). This high susceptibility of the HVC to oxidative stress suggests that it is not only traits involved in visual signalling that are affected by oxidative stress.

1.3.8. Avian olfaction

Most research to date addressing the question of how animals can assess the health of potential mates has focused on visual cues such as carotenoid-based ornaments, and vocal signals which may, through physiological trade-offs, provide an honest signal of the bearer's antioxidant or immune status during mate choice (e.g. Faivre et al. 2003; Munoz et al. 2010). It is not always clear, however, which visual trait could be used when assessing current antioxidant or immune status; for example, the females of many species lack the ornamentation that could provide cues to antioxidant status, yet their antioxidant levels are key determinants of their reproductive success (e.g. Amundsen and Forsgren 2001). One unexplored means by which this information could be acquired is via physiologically-mediated olfactory cues. In contrast with mammalian olfaction, avian olfaction is poorly understood, with birds widely regarded as primarily using visual and auditory signals. However, increasing anatomical, neuroanatomical and physiological evidence has demonstrated that avian olfactory structures are in fact complex (Steiger et al. 2008) and that the functional role of avian olfaction may have been underestimated (Balthazart and Taziaux 2009). Research has shown that in some avian species individuals may be able to utilise odours in social situations, such as to recognise conspecifics (Hagelin et al. 2003; Whittaker et al. 2010), related individuals (Whittaker et al. 2010) or social partners (Bonadonna and Nevitt 2004; Jouventin et al. 2007; Mardon et al. 2010).

Preen oil, a waxy lipid secretion from the uropygial gland, is thought to contribute largely to the odour of birds' feathers (Reneerkens et al. 2002). Preen oil has a role in delaying feather wear, maintaining feathers flexibility, waterproofing and has antimicrobial properties (Balthazart and Taziaux 2009). In addition, females of some species experience a compositional shift in uropygial secretions during courtship and incubation indicating an additional unknown function for preen oil (Piersma et al. 1999). During incubation females of some species have been found to reduce the relative amount of highly volatile compounds in preen oil, which may be an adaptation to make them less susceptible to depredation by olfactory predators (Zhang et al. 2010). However, the potential role of olfaction during courtship is poorly understood. While odours are undoubtedly complex (Soini et al. 2007) and may differ significantly between individuals (Bonadonna et al. 2007), it is unclear whether feather surface odour acts as a cue during courtship and, if so, what information could be transmitted. For example, dietary intake may affect volatile composition on feathers (e.g. through antioxidant effects on the degradation of preen oils) which opens up the possibility that the odour profiles of individuals could provide a cue of the antioxidant status or parasite burden of an individual during mate choice.

1.4. Sexually-selected traits and fertility

If male ornamentation provides an indication of 'direct benefits' for a female such as avoiding infectious diseases or the quantity of resources, including food, territories or parental care that a male can offer, the benefits of female mate choice are clear (Houle and Kondrashov 2001). Sheldon (1994) proposed that females seek extra-pair copulations as insurance against the functional infertility of their mates (Gibson and Jewell 1982; Wetton and Parkin 1991), and that functional fertility correlates with male phenotype. Studies have since identified a positive relationship between the degree of male sexual ornamentation and aspects of sperm quality, quantity or testes size (Merilä and Sheldon 1999; Pilastro et al. 2002; Peters et al. 2004; Malo et al. 2005; Pitcher et al. 2007; Helfenstein et al. 2010).

There is growing evidence that oxidative damage provides a potentially unifying mechanistic link between male ornament expression and sperm quality (Blount et al. 2001; Velando et al. 2008). Sperm is believed to be particularly susceptible to

oxidative damage caused by ROS and ROS are believed to contribute to 30-80% of cases of human male infertility (Tremellen 2008). At low concentrations ROS assist with normal key processes in spermatozoa including capacitation, acrosome reaction, fertilisation and motility (Agarwal 2004; Desai et al. 2010). However, high levels of ROS formed by morphologically and/or functionally abnormal or immature spermatozoa, excessive leukocyte infiltration into the semen (Agarwal 2004) and ROS leakage from the mitochondria within the midpiece of the spermatozoa (Desai et al. 2010) can be highly damaging. Sperm are particularly susceptible to oxidative damage because of the high content of polyunsaturated fatty acids in the cell membrane (Desai et al. 2010), the condensed DNA in sperm and lower concentration of DNA transcription proteins reducing the ability of sperm to repair DNA damage (Tremellen 2008).

A study by Helfenstein et al. (2010) tested the role of oxidative damage as a mechanistic link between sexual signals and sperm quality by artificially increasing the workload and oxidative stress experienced by male great tits, *Parus major*, using brood size manipulation. Males with a greater workload and duller breast plumage had higher lipid peroxidation levels and lower sperm motility than more colourful males. Supplementation with carotenoids enabled paler males to reduce lipid peroxidation damage in the ejaculate reaching the levels of unsupplemented brighter males (Helfenstein et al. 2010). Studies that have manipulated the nutritional stress experienced during development have also provided evidence of downstream effects of early environmental conditions on sperm (Gage and Cook 1994; Wedell 1996). For example nutritional stress in the form of reduced protein during development in the meal moth, *Plodia interpunctella,* resulted in the production of lower numbers of sperm at adulthood (Gage and Cook 1994) and larval host plant affected the protein content of spermataphores at adulthood in male comma butterfly, *Polygonia c-album* (Wedell 1996).

1.5. Competition-dependent signals

Individuals are affected by the social environment that they experience. Life history trade-offs result from competition for a limited and shared resource by multiple traits (Stearns 1989). Allocation to one trait results in a reduction in the resources available

for investment in the other (Zera and Harshman 2001). Greater acquisition of a resource therefore reduces trade-offs in resource allocation between multiple traits. However, when a resource is scarce within the environment the competitiveness of an individual may determine their level of acquisition. Resource availability during growth and development can affect the expression of sexual signals during development and at adulthood (e.g. Ohlsson et al. 2002). As a result any signal that is sensitive to early life history effects and is affected by the competitiveness of an individual during resource acquisition is a 'competition-dependent' signal (Wolf et al. 2008).

The social environment that an individual experiences can have both genetic and environmental effects (Moore et al. 1997; Wolf et al. 1998). Indirect genetic effects (IGEs) are genes expressed by one individual that have an effect on the phenotype of another (Wolf et al. 1998). These effects can often result in complex and non-intuitive evolutionary responses to selection (Moore et al. 1997; Wolf 2003). Models of condition-dependence (Harris et al. 2008; Wolf et al. 2008) have shown that competitive interactions between conspecifics can affect an individual's ability to acquire limited resources and affect their overall condition affecting their expression of condition-dependent signals. Condition-dependent signals can provide a good indication of relative competitive ability and male quality within a group (Wolf et al. 2008). The potential for social competition to influence traits arises during the development and/or the expression of the trait involved, because individuals often develop in groups with other conspecifics (Wolf et al. 2008). The impact of the social environment experienced will depend on a range of factors such as the number of competitors, the relatedness of the individuals, the mechanism of resource partitioning and the degree to which competitive dominance relations among genotypes are transitive (Wolf et al. 2008). The oxidative status of an individual can be affected by competition directly due to increased metabolic rate, or indirectly by increasing resource allocation to growth at a cost to maintaining good health (e.g. Alonso-Alvarez et al. 2007; Hall et al. 2010) proximately mediating the expression of competition-dependent signals.

1.6. Outline and aims of thesis

I have used ring-necked pheasants, *Phasianus colchicus,* as a model species to examine the role of antioxidants in modulating resource allocation trade-offs during growth and development in this thesis. The ring-necked pheasant is a highly sexually dimorphic species, in which males have multiple sexual ornaments (Hill and Robertson 1988). Wattle size is also positively affected by the nutrition experienced during early development (Ohlsson et al. 2002). Moreover, parasite infestations are common in pheasants (Hill and Robertson 1988) and the level of parasite infection is known to affect carotenoid-based pigmentation in other species (Hill et al. 1999). As a result pheasants are an ideal species to use to assess the effects of trade-offs during early life on the expression of sexually-selected traits in adulthood. In particular this thesis aims to examine whether the availability of dietary antioxidants during early life: (1) is important in mediating the trade-offs between the allocation of resources to growth and/or reproduction or self-maintenance through effects on levels of oxidative stress, (2) has synergistic effects on the degree of trait expression in adulthood (particularly primary and secondary sexual traits), (3) modifies the effect of early exposure to parasites on the expression of sexually-selected traits, and (4) affects the long-term odour profiles of individuals and therefore the potential to signal quality and/or status.

Chapter 2 provides the general methods used in the following experiments. **Chapter 3** describes an experiment investigating the independent and combined (synergistic) effects of supplementation of carotenoids and vitamin E during early growth on the expression of traits in adulthood. **Chapter 4** extends this to investigate the idea that one of the unifying mechanistic links between male sexual ornament expression and functional fertility involves oxidative damage (Blount et al. 2001; Velando et al. 2008) by quantifying the allocation of resources to primary sexual characteristics (gamete production) in relation to allocation to secondary sexual characteristics (e.g. wattle size and colouration) following supplementation of carotenoids and vitamin E during early growth.

The 'parasite-mediated sexual signalling' (PMSS) hypothesis predicts that exaggerated male ornamentation could provide a signal to females of a male's ability to resist parasites. **Chapter 5** describes an experiment to test whether supplementation

with antioxidants (vitamin E) can mitigate the effects of early exposure to parasites, via alteration of the oxidative status of individuals, on the expression of sexual ornaments at adulthood. In **Chapter 6** the effects of the supplementation of vitamin E levels and the level of parasite infection during early life on the volatile profile of feathers and caecal matter of females were quantified in order to investigate the potential role of odour as a cue indicating long-term antioxidant and/or parasite status of individuals. Finally, **chapter 7** consists of a general discussion of the work described in this thesis.

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Chapter 2 General Methods

2.1. Project introduction

The project was carried out on the rearing field at the Headquarters of the Game and Wildlife Conservation Trust (GWCT) based in Fordingbridge, Hampshire. The experiments required a Home Office Project Licence (30/2527) and a Personal Licence (30/8186). Ring-necked pheasants, *Phasianus colchicus*, were used as a model species. The birds used in experiments were purchased from Holme Park Hatcheries, Wokingham. This game farm processes eggs from over 2000 laying hens per year. This reduces the likelihood of multiple siblings amongst experimental birds. In both years (2008 and 2009) experiments began during the first two weeks of May at the peak of the laying season during which hatchability is at its highest (Anderson Brown and Robbins 2002).

2.1.1. The ring-necked pheasant

The ring-necked pheasant is a highly sexually dimorphic galliform with a native range in Asia. Pheasants are believed to have been introduced to the UK by the Normans in the 14th Century. They are now widespread and over 7 million pheasants are hand-bred and released each year for sporting purposes (Johnsgard 1998). Female ring-necked pheasants are smaller than males with a duller yellowish buff plumage, mottled chestnut pattern and long banded tail. Males have bright plumage, conspicuous wattles, long tail feathers, spurs and ear tufts. The ring-necked pheasant is an omnivorous and opportunistic species in the wild and tends to consume energy-rich foods, such as cultivated grains, the growing shoots of crops, wild seeds and invertebrates (Hill and Robertson 1988).

During the winter wild ring-necked pheasants commonly form temporary flocks in which individuals move and feed together. Male pheasants are territorial (Hoodless et al. 1999, 2001; Papeschi et al. 2003) and dispersal and territorial establishment in the approach to the breeding season is associated with greater male display towards females and antagonistic male-male interactions to establish a dominance hierarchy.

Pheasants exhibit a harem polygyny social mating system and females choose mates based on multiple sexual ornaments (Hill and Robertson 1988). These ornaments include facial wattles (Hillgarth 1990), the colour of which is thought to be carotenoid-mediated (Czeczuga 1979) and tarsal spurs (Göransson et al. 1990). The bright wattle of males is expanded during sexual displays to attract females (Hill and Robertson 1988) and females have been shown to prefer males with larger wattles (Hillgarth 1990). Body mass has also been found to be an important determinant of success in mating (Göransson et al. 1990). A number of studies have identified the role of multiple cues by females during mate choice that may reflect different aspects of male quality (Candolin 2003). Harems are formed during a period of male displays and the breeding season occurs between mid-March and early June. Females separate from the males and the harem to nest. Pheasants are ground nesting birds and females construct simple nests in shrubby vegetation, laying on average 12 eggs (Hill and Robertson 1988) which are incubated for an average of 23 days. Chicks are precocial at hatching and remain with the hen for the following 6 to 7 weeks before dispersal. Foxes (*Vulpes vulpes*) are the main natural predator of ring-necked pheasants and nest predators include corvids, foxes, badgers, rats, stoats and hedgehogs (Hill and Robertson 1988).

2.1.2. Rearing techniques and husbandry

Experimental birds were reared using a standard semi-intensive system (Game Conservancy Trust 2004) for the first eight weeks in a timber brooder hut (6 x 5 m) with an apex roof (central height of 2.4m) and concrete floor. The brooder hut had a mains supply of electricity and a water supply. Access to the hut was through a single door with a tarpaulin inner cover. Along each 6m length of the hut were 4 x 0.5 m square pane static square glass windows. Two 350mm extractor fans were positioned centrally at each end of the hut. The brooder hut contained 3 strip lamps (Arcadia 2.4% UVB-12% UVA 58W 150mm) and 4 red bulbs (60 watt). The hut was divided into 8 purpose built MDF pens (1.8m x 1.5m) each with a sliding pop-hole (200 mm^2) allowing access to an outdoor run. A central aisle provided access to each pen.

All pens were disinfected before and between experiments. Each pen had a wooden frame and plastic netting lid to which an electric brooder lamp with dull emitter heat

lamp bulb (up to 175 watt), spun aluminium shade (210mm diameter) and protective guard was attached (Solway Feeders Ltd. stock code 1108). The outdoor pens (2008: 3 x 1.5 m, 2009: 6 x 1.5m) were made from standard panels, a raised wire floor and a standard gate panel. Birds were given access to each outdoor pen after 14 days through a pop-hole and a wire ramp connected the pop-hole edge to the wire flooring of the outdoor pen (**Figure 2.1.**). Treatment diets were manipulated up to 8 weeks of age before all birds received the same diet and same access to grass. Wire floors were therefore used to ensure that chicks only had access to treatment diets up to 8 weeks of age.

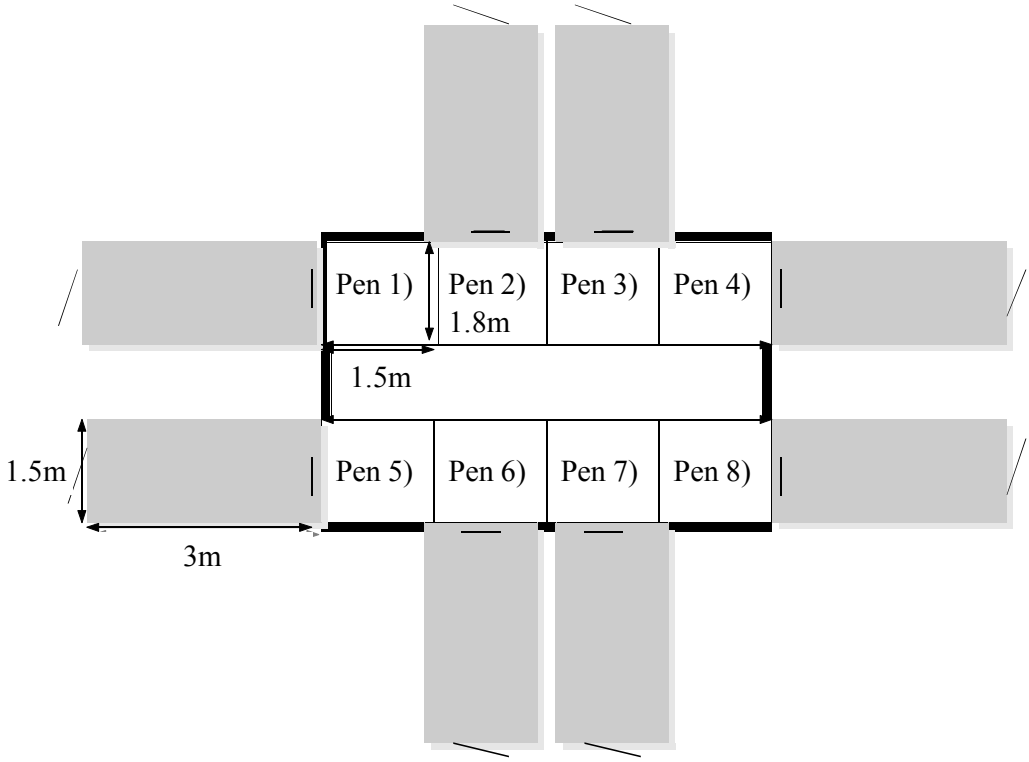

Figure 2.1: Diagram of the brooder hut design. The areas in grey represent the outdoor pen areas and black represents the outer wall of the brooder hut.

Internal pens were heated several days before the arrival of chicks. On arrival birds were kept within clipped plywood nursery circles and water was provided by nipple and font drinkers (Solway Feeders Ltd., Stock code 6101). The floor of each indoor pen was covered in a layer of cardboard bedding squares (Walmsley Premier bedding) that were replenished over the 8 week period. The temperature within the centre of the nursery circle under the heat lamp was checked regularly and maintained at 37°C.

The birds were delivered on two separate days in 4 boxes containing approximately 35 birds per box. Birds were placed in the pens using a randomised design. 4 birds were taken from each box and placed under each of the four heat lamps in a nursery circle until the box was empty. Chicks from the following box were then placed in the same order of circles with a new first pen. The birds were numbered using 5mm flatband leg rings (A.C. Hughes Ltd.) on day one. These were replaced with 8mm and 12.7mm numbered rings as appropriate.

For each experiment an additional 40 chicks were ordered and reared in small outdoor brooder huts with adjacent night shelters and outdoor arcs. These birds were used to maintain experimental densities in the event of any mortality in experimental groups or to observe the establishment of nematodes in the caecae following infection with the parasite, *Heterakis gallinarum* in 2009.

At 6 days of age the nursery circles were removed and birds were given access to the whole of the internal pen. Plywood triangles were positioned in the corners of the internal pens to remove the risk of mortalities following 'huddling' behaviour. Light levels were kept low and chicks were observed regularly to check for signs of cannibalism. Birds were given access to outdoor pens through pop-holes at 2 weeks of age. The exact days on which birds were given access to outdoor pens at this early age was dependent on weather conditions. At 21 days of age the chicks were bitted with 'B' size bits. These were replaced at 6 weeks of age with size 'C' bits. Bits were removed at 10 weeks of age.

Items (i.e. perches, brash etc.) were added to each outdoor pen for the purpose of enrichment and the provision of shelter. At eight weeks the birds were sexed and transferred in high density polyethylene transport crates to two outdoor single-sex pens (30 x 27 m) with netting roof and net props. Each pen was divided into two sides by a standard pen section wall and included four $3m^2$ capture pens (**Figure 2.2.**). Both pens included 4 quill nipple drinkers (Solway Feeders Ltd.). Feed was provided in 25 litre feeders with rain covers (Solway Feeders Ltd.). The birds remained in these single-sex pens until completion of the experiment. The GWCT Rearing Field Technician maintained the birds following release into the outdoor pens.

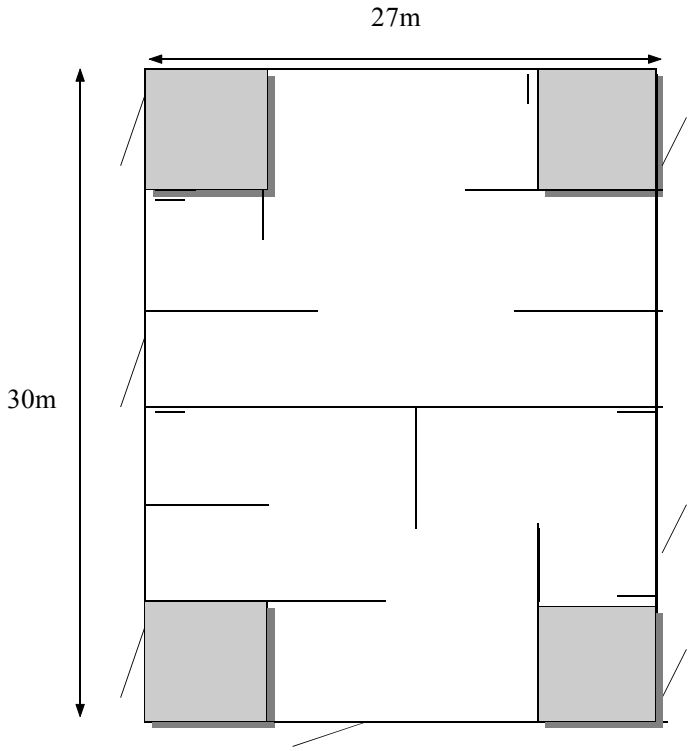

Figure 2.2.: Diagram of the outdoor single-sex pens. The areas in grey show the position of the 'capture pens' (1.5m x 1.5m)

2.1.3. Supplementary feeding

Each year the antioxidant availability in feed was manipulated for the first 8 weeks post-hatch. It was necessary to use a more basic feed than those commercially available in order to successfully manipulate antioxidant availability. Commercial pheasant feed contains a high level of protein, added vitamin E and selenium. Feed was made to specification by Target Feeds Ltd., Shropshire. The basal diet included approximately 49% wheat. Wheat is a naturally high source of vitamin E (4.8mg/lb Cort et al. 1983). The function of the major form of the antioxidant enzyme glutathione peroxidase (GSH-Px), is dependent on the presence of selenium. GSH-Px is a key component of the first level of antioxidant defence that acts to prevent free radical formation by removal of precursors or inactivation of free radical catalysts (Surai 2002). Therefore, experimental feed included a reduced level of selenium to ensure that antioxidant supplementation was not masked by the activity of GSH-Px. Protein levels in the feed were chosen to avoid the effects of a protein rich diet or protein poor diet that could mask the effects of antioxidant supplementation. For example, pheasants fed moderate protein levels (27%) in the first three weeks after hatching had significantly larger and brighter wattles than those that received a low

protein diet (Ohlsson et al. 2002). Vitamin E levels (α,ß,ɣ,δ-tocopherols and tocotrienols) and carotenoids were kept low to ensure that the concentrations available to the pheasants could be measured and supplemented accurately.

The birds were provided with starter crumb (0-2 weeks of age), starter pellets (2mm: 2-4 weeks of age), grower pellets (3mm: 4-6 weeks of age) and rearer pellets (3mm: 6-8 weeks of age) over the first 8 weeks, a standard practice in pheasant game keeping (GCT 2004) (**Table 2.1.**).

Table 2.1.: Feed specification supplied by Target Feeds Ltd.

	Starter Crumb (%)	Starter Pellet (%)	Grower Pellet (%)	Rearer Pellet (%)
Barley	0.00	10.00	20.00	20.00
Wheat	49.15	49.10	49.05	49.05
Prairie Meal	7.50	5.00	2.50	0.00
Soya Extract	20.00	17.50	15.00	15.00
Full Fat Soybeans Extruded	10.00	7.50	5.00	2.50
Provimi 66 White Fish meal	10.00	7.50	5.00	2.50
Lysine Hydrochloride	0.10	0.10	0.10	0.10
DL Methionine	0.05	0.05	0.05	0.05
Soya Oil	1.50	1.50	1.50	1.50
Monocalcium Phosphate	0.20	0.20	0.20	0.20
Salt	0.10	0.15	0.20	0.24
Sodium Bicarbonate	0.10	0.10	0.10	0.10
Choline Chloride	0.05	0.05	0.05	0.05
Oil Ether extract	5.24	4.02	4.20	3.67
Protein	29.80	25.53	21.39	18.13
Fibre	2.85	3.07	3.20	3.34
Ash	6.30	5.74	5.18	4.70
Metabolisable Energy	12.84	12.08	12.52	12.34
Tlysine	1.62	1.38	1.13	0.96
Avlysine	1.49	1.27	1.05	0.89
Methionine	0.60	0.51	0.41	0.32
Methionine + Cystine	1.06	0.90	0.75	0.62
Threonine	1.08	0.92	0.75	0.62
Tryptohan	0.30	0.24	0.23	0.21
Calcium	1.23	1.07	0.90	0.36
Phosphate	0.91	0.71	0.65	0.13
Available phosphorus	0.67	0.54	0.43	0.30
Salt	0.38	0.37	0.37	0.35
Sodium	0.18	0.18	0.18	0.18
Essential fatty acids	2.81	2.52	2.26	1.93
Protein	29.80	25.60	21.39	18.13

Table 2.2.: Nutrients added to the feed by Target Feeds Ltd.

Nutrient	Starter Crumb (0-2 weeks) mg/kg	Starter Pellet (2-4 weeks) mg/kg	Grower Pellet (4-6 weeks) mg/kg	Rearer Pellet (6-8 weeks) mg/kg
Vit A	10.00	10.00	10.00	10.00
Vit D3	3.00	3.00	3.00	3.00
Vit E	0.00	0.00	0.00	0.00
Selenium	0.20	0.20	0.20	0.20

Feed was prepared daily with one of four treatments:

i. Carotenoids: 100mg/kg ORO-GLO® brand 11 Liquid Pigmenter (Kemin Industries, Inc. - Ingredients include water, marigold extract, Potassium Hydroxide, Soybean Oil and Ethoxyquin – 0.05%). The ORO-GLO® contained 11.0 grams of xanthophyll activity per kilo (lutein and zeaxanthin, 20:1 w/w).

ii. Vitamin E: 100 mg/kg (Sigma-Aldrich T36634) (+)-α-Tocopherol from vegetable oil.

iii. Carotenoids and Vitamin E: 50mg/kg of ORO-GLO® and (+)-α-Tocopherol from vegetable oil 50mg/kg.

iv. Control

Feed was weighed daily and then spread onto a heavy duty sheeting until the feed was one pellet/crumb deep. (+)-α-Tocopherol from vegetable oil was used because it is highly bioavailable. The ORO-GLO® has been used in previous ecological experiments (Blount et al. 2004; Blount and Matheson 2006).

Table 2.3.: Daily feed volume suggestions (Target Feeds Ltd.):

Diet	Feed required for 240 birds (kg)
Starter Crumb 0-2 weeks	64
Starter Pellet 2-4 weeks	130
Growers Pellet 4-6 weeks	180
Rearer pellet	220

Following preliminary tests the feed was sprayed using a 5 litre pump spray gun (B&Q) with the specified treatment. α-Tocopherol was mixed with soybean oil (10.1mg/100g; Carpenter 1979) and ORO-GLO® was mixed with water and sprayed onto the feed. Soybean was chosen as a vehicle for α-tocopherol because it has low natural levels of tocopherols and tocotrienols (10.1mg/100g; Carpenter 1979). Feed for the carotenoid and the control treatment group was sprayed with the same volume of soybean oil as that of the treatment groups containing vitamin E. Feed for the control and vitamin E treatment group was sprayed with the same volume of water as that of the treatments groups containing carotenoids. Feed was sprayed daily and provided to the birds twice daily. Feed was stored in airtight containers and refrigerated until required for the afternoon. Air was removed from the containers using a vacuum pump and stoppers.

2.1.4. Medication

All birds were medicated with an anticoccidiat, Avatec (lasalocid sodium) added to the feed at a dose of approximately 125 ppm and 200 micrograms of Doramectin per kilogram body weight for 'gape worm,' as is standard practice (GCT 2004), prescribed and administered by the named Veterinary Surgeon at the GWCT.

2.2. Morphometric measurements

Measurements were taken of the head to bill length and tarsus length using callipers accurate to 0.05 mm (RS Components). Wing length was measured using a wing rule accurate to 0.1 mm. Mass was measured using a Pesola® spring scale (30g± 0.3%, 60g± 0.3%, 100g± 0.3%, 300g± 0.3%, 600g± 0.3%, 1000g± 0.3%, 2500g± 0.3% depending on age) (Alana Ecology). Spur length was measured by subtracting the diameter of the tarsus directly above the spur from the combined length of the tarsus and spur (Ohlsson et al. 2002). Other notes were taken when each bird was measured regarding any distinguishing marks or abnormalities in the birds such as twisted bills were recorded, and the severity of feather pecking was recorded (**Table 2.4.**).

Table 2.4.: Feather condition record

Score	Description
1	Badly denuded feathers (50% + cover missing)
2	Large bald patches (>1.5cm)
3	Small bald patches (<1.5cm)
4	Evidence of some feather pecking but no bald areas
5	No evidence of feather pecking

2.3. Blood sampling

Blood samples were taken at 8 weeks and 47 weeks of age using 26 gauge 5/8"BD Microlance™ needles and BD Plastipak™ 1ml syringes (Fisher Scientific). Syringes were flushed with heparin sodium salt from bovine intestinal mucosa (0.2ml per 100ml) (Sigma-Aldrich H0777). A small area of feathers was removed from the underside of the wing, the area was sterilised and 0.35ml of blood was taken from the brachial vein. The blood was transferred to a 1.3 ml micro tube coated with EDTA (Sarstedt) and kept in a coolbag with freezer blocks. A microhaematocrit EDTA-coated capillary tube (Bilbate Ltd. CAP-MH-75H-EDTA) was filled for each bird and sealed with cristaseal (Hawksley Ltd.). The samples were centrifuged (Hawksley Ltd. Haematospin 1400) at (8,000 rpm) for 5 minutes. Plasma was then removed using a finnpipette and stored in a 0.5ml eppendorf at -27°C. The samples were then transferred to a -80°C freezer within 5 days of blood sampling ready for biochemical analysis. Haematocrit readings were taken using a heamatocrit measurement reader (Hawskley Ltd.) for each microhaematocrit tube. The microhaematocrit tubes were then cut using a diamond tipped file and the plasma was blown into, and stored in, an additional 0.5ml eppendorf. The times at which the blood sample was taken and the time at which the plasma was placed in the freezer were recorded.

2.4. Plasma concentrations of α-tocopherol and carotenoids

α-Tocopherol was measured within a month of the storage of samples in the -80°C using high-performance liquid chromatography (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 3µm 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.

Total vitamin E levels (µg/ml) were calculated by dividing the peak area of a standard by the peak area of the sample. The calculated vitamin E levels were then multiplied by the volume of methanol in which the sample was redissolved and multiplied by 5/4 to account for the amount of hexane following extraction that was then removed to be dried down. The volume calculated was then adjusted to allow for the amount of plasma originally used (50µl) to provide the accurate concentration of tocopherol per ml of plasma in each individual.

2.5. Lipid peroxidation assays

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^{o}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.

2.6. Phytohaemagglutinin

To measure the immunocompetence of the pheasants, the birds were injected with 1mg of phytohaemaggluttinin (PHA, Sigma Aldrich) dissolved in 0.1ml of phosphate buffered saline (PBS) subcutaneously into the left wing-patagium. PHA injection results in local skin swelling that reflects the pro-inflammatory potential of the immune system (Tella et al. 2008; Vinkler et al. 2010). As a control, we injected 1ml of PBS into the right wing-web. The thickness measurement of the left-wing patagium was subtracted from the measurement taken from the right wing-web to identify the pro-inflammatory potential to PHA 24 hours after exposure (Vinkler et al. 2010).

2.7. Wattle colour

Wattle colour was quantified at 47 weeks of age. Wattle reflectance data were collected using a USB2000 UV-Visible spectrophotometer and OOIBase32 Software (Ocean Optics Inc., Dunedin, FL). 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 stabilise before capture (Keyser and Hill 1999). Three spectra were collected for the left wattle and 3 for the right wattle on each male. The brightness of the wattle has also been identified as being important in female mate choice (Hillgarth and Wingfield 1997). We calculated brightness as it would be perceived by a conspecific female, using the method detailed in Endler and Mielke (2005). In galliforms, brightness is likely to be perceived by the double cones (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 pheasant 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 and 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 and Stiles 1982).

2.8. Wattle size

Photographs were taken of each bird positioned on a plane with a ruler scale at 46 weeks of age (**Figure 2.3.**). Polygons were drawn around the wattle outline and a text file with the coordinates was compiled using ImageJ software (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA). The data was then imported into the *Morpheus et al*. (D.E. Slice *Morpheus et al.: Software for Morphometric Research. Revision 01-31-00* Department of Ecology and Evolution, State University of New York) software program for elliptic Fourier analysis (EFA) (Rohlf 1992).

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 60 fourier coefficients were then used as variables in principal component analyses.

Figure 2.3.: Photographs were taken of each bird with the wattle in the same plane as the scale before analysis using ImageJ software**.**

2.9. Preparation of testes and ejaculate samples

In 2009 94 of the 122 males reared were killed using a schedule 1 method over a period of 5 days in order to collect sperm samples. The remainder of the males were

retained by the Game and Wildlife Conservation Trust for breeding. The birds were dissected immediately after death to reduce cumulative damage to samples before storage. Immediately post-mortem semen samples were collected using a pipette from the distal end of the *vas deferens* following dissection. Ten microlitres of semen were immediately diluted in 200µl of Dulbecco's modified Eagle medium (Invitrogen Ltd) and 15µl of the diluted sample was placed under a phase-contrast microscope on a heated stage (40°C) and video recorded at 200× magnification (Lüpold et al. 2009). The remainder of the undiluted semen samples were collected for lipid peroxidation analysis. Testes were removed and the length (*L*), width (*W*) and height (*H*) of each testis was measured using a sliding calliper (±0.01mm). These measurements were used to calculate the volume of each testis as the volume of a regular ellipsoid ($1/6\pi \times L \times W \times H$). The mass of each testis was also quantified (±0.01g).

Sections of testes were taken for laboratory analysis of vitamin E concentration. All such samples were immediately snap-frozen in liquid nitrogen and stored at –80°C. 150mg of testes sample was measured and placed in a 30ml glass vial and capped on ice. 5ml of ethanolic pyrogallol 5% (Sigma Aldrich) a powerful reducing agent was added to each sample to preserve α-tocopherol. Each sample was homogenised for 30 seconds (Ultra-Turrax IKA 1800 Basic). 0.4ml of 50% aqueous KOH was added to the sample solution to break ester bonds and releasing fatty acid salt and glycerin and the vial was capped under nitrogen. Preliminary laboratory tests were carried out to establish the optimum level of KOH with pheasant testes. The blade of the homogeniser was washed with milli-Q water and dried between samples. The samples were heated in a water bath (Clifton NE-4-D) for 70ºC for 30 minutes and chilled in ice for a further 5 minutes. 5 ml of hexane (Sigma-Aldrich- HPLC grade) was added to each sample to extract the α-tocopherol and the sample was washed for 1 minute. 10ml of Milli-Q water was added to the sample and the sample was washed for 1 minute. The samples were centrifuged (Thermofisher Haraeus Fresco 17) for 4 minutes at 1000 rpm. 3ml of the non-aqueous layer was removed and placed in a rotary speedvac (Savant 1SS110) for 15 minutes. The sample was redissolved in 0.5ml methanol and 10µl was injected into the HPLC (Dionex).

2.10. *Heterakis gallinarum*

The caecal nematode *Heterakis gallinarum,* is found in wild and reared ring-necked pheasants. The reproductive potential of *H. gallinarum*, defined in terms of the production of nematode eggs ingested, is greater in the ring-necked pheasant compared to other gamebirds (Hofstad 1984). Eggs are released from the caecum in the faeces in the single-cell stage and reach the infective stage in the soil and in approximately 2 weeks in average environmental conditions. Eggs are very resistant to extreme temperatures and desiccation and can remain infective after 230 weeks within the soil and can survive freezing conditions for up to 167 days (Olsen 1974). The larval stage is contained within the egg. Pheasants can acquire the infective egg by eating invertebrates that have themselves ingested the parasite eggs (Hofstad 1984) or through direct ingestion from the soil. The invertebrates are not hosts to any part of the egg/larval development. Following ingestion it takes approximately 6.5 hours for the eggs to arrive at the caeca without further development. The larvae hatch and are closely associated with the mucosa for the first 12 days. The larvae moult at approximately 4 to 6 days after ingestion, again between 9 to 10 days post-ingestion, are fully grown at 14 days and begin ovipositing between 24-36 days post ingestion (Olsen 1974).

2.10.1. *Heterakis gallinarum* collection and the inoculum

Live adult *Heterakis* were collected during the dissection of pheasant road kill and the collection of caeca from chickens during food processing at Dean's foods Ltd. (Gainsborough, UK). The contents of the caecum were sieved using warm water and *H. gallinarum* were removed and maintained in vials of 5% formalin solution until they could be transferred in the laboratory to petri dishes. *Heterakis* were then sexed under a light microscope. Males (7-13mm in length) have two spike-like copulatory spicules that are absent in females (10-15mm) (**Figure 2.4.**). Males were discarded and the females counted and transferred to fresh solution in a new petri-dish.

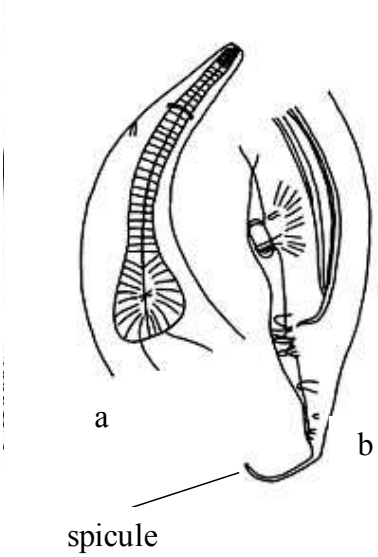

Figure 2.4.: A diagram of the adult nematode *H. gallinarum* a) female b) male showing the spicule of the male (adapted from Pinto et al. 2006)

The female *Heterakis* were then kept in a temperature controlled cabinet maintained at 21-23ºC for 21 days and formalin solution was regularly replenished. At 21 days the *Heterakis* were transferred to a large container in solution and the adults were blended using a hand held blender. A McMaster cell counting chamber and cover slide (Hawksley Ltd.) was used to estimate the number of infective *Heterakis* eggs per ml of solution. It is possible to distinguish an infective egg by the presence of larvae within the egg using a light microscope at 20X magnification. The solution was further diluted until the 100 eggs per ml of solution was the average of McMaster cell count. The birds were infected aged 21 days. Development of lumen dwelling larvae in chicks less than 10 days old is inhibited but is optimal in birds 21 days old and this is believed to be the most susceptible age (Olsen 1974). The presence of bacterial flora in the intestine is essential for the development of the larvae. The role of the bacteria is unknown. Worms are fully grown within 14 days after deposit in the lumen. The females do not begin to oviposit until 24 to 36 days after infection (Olsen 1974). The birds were given 1ml of solution using a finnpipette (Fisher Scientific).

2.10.2. *Heterakis gallinarum* counts

The number of nematodes in the caecum of pheasants were counted at 47 weeks of age at the same age as morphometric measurements and measurements of sexual signals were taken. *H. gallinarum* is found in the lumen of the caecum and

occasionally in the small intestine. The gut was laid out on a dissection tray. 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 (Olsen 1974).

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Chapter 3

Synergistic effects of supplementation of dietary antioxidants during growth on adult phenotype in ring-necked pheasants *Phasianus colchicus*

3.1. Introduction

Life history trade-offs result from competition for a limited and shared resource by multiple traits (Stearns 1989), such that allocation to one trait results in a reduction in the resources available for investment in another (Zera and Harshman 2001). Resource allocation trade-offs may be particularly acute for strongly sexually-selected species, where significant investment in sexual characteristics can only be achieved at a cost to other traits (Royle et al. 2005). Little is known about the mechanistic costs underlying such trade-offs, although there is growing evidence for the role of oxidative stress as a potentially unifying mechanism (Costantini 2008; Monaghan et al. 2009; Hall et al. 2010). Oxidative stress results from an imbalance between the production of damaging reactive oxygen species (ROS) and antioxidant defences, in favour of the former (Sies 1997). An important level of antioxidant defence involves fat-soluble antioxidants such as vitamin E and carotenoid pigments which cannot be synthesised *de novo* by vertebrates and are therefore limited by dietary intake. Vitamin E is a colourless and highly effective antioxidant (Surai 2002) which may also have roles in signal transduction and gene expression (Brigelius-Flohé 2009). Carotenoids, on the other hand are highly pigmented antioxidants that are expressed in many sexually-selected traits and also have immunoenhancing properties (McGraw and Ardia 2003).

A large proportion of the research on the role of oxidative stress in life history trade-offs has focused exclusively on carotenoid supplementation (Catoni et al. 2008), often producing conflicting results. In particular, recent analyses have questioned the effectiveness of carotenoids as antioxidants *in vivo*, concluding that they have only minor antioxidant capabilities (Costantini and Møller 2008; Cohen and McGraw 2009). However, animals ingest a cocktail of antioxidants which may interact either antagonistically (e.g. competition during absorption) or synergistically (e.g. carotenoid recycling by vitamin E; Surai 2002; Catoni et al. 2008), and several supplementation studies have now demonstrated experimentally such synergistic effects of antioxidants on the expression of sexually-selected traits (e.g. Pike et al. 2007; Pérez et al. 2008). However, these studies have all been conducted at adulthood so do not take account of early life-history effects on the expression of traits in adulthood.

Somatic growth results in the production of high levels of ROS (Stoks et al. 2006) and the period of rapid growth and development early in life constitutes a period of heightened vulnerability to oxidative stress (Surai 2002). Individuals often experience variation in resource availability during early development and selection favours rapid growth when competition for resources is acute, so trade-offs between allocation of resources to growth and self-maintenance are expected (Hall et al. 2010). Such early life history effects of heightened levels of oxidative stress during development are therefore likely to play an important role in the expression of sexually selected traits at adulthood (Blount et al. 2003a; Walling et al. 2007). Sexually-selected traits are known to have an increased susceptibility to environmental stress during development (Hunt and Simmons 1997; David et al. 2000) and nutritional quality can influence circulating antioxidant levels at adulthood (Blount et al. 2003a). If antioxidant availability during development has long-term effects on the ability of adults to assimilate antioxidants it is therefore also likely to affect the expression of sexually selected traits at adulthood.

Trade-offs between growth and self-maintenance during development are expected to be particularly strong in sexually dimorphic species of birds such as the ring-necked pheasant, *Phasianus colchicus*. In this species males have bright plumage, conspicuous wattles, long tail feathers, spurs and ear tufts. Females are smaller, with a duller yellowish buff plumage with a mottled chestnut pattern and long banded tail. Pheasants have a harem polygyny mating system and females choose mates based on multiple sexual ornaments (Hill and Robertson 1988). These ornaments include facial wattles (Hillgarth 1990), the colour of which is likely to be carotenoid-mediated (Czeczuga 1979), long spurs on the legs (Göransson et al. 1990) and long tails (Mateos and Carranza 1995). The bright wattle of males varies in shape and is expanded during sexual displays to attract females (Hill and Robertson 1988); females have been shown to prefer larger wattles (Hillgarth 1990). Body mass has also been found to be an important determinant of success in mating and is correlated with spur length (Göransson et al. 1990). Previous work has shown that a low intake of dietary protein during the early growth and development of males resulted in reduced wattle size and brightness at adulthood (Ohlsson et al. 2002), demonstrating that the expression of at least one sexual ornament in pheasants is sensitive to environmental conditions experienced post-hatching in this species.

In the current study we manipulated the dietary antioxidant availability of pheasants during early life and quantified effects on growth and self maintenance (oxidative damage and immunity) in relation to the expression of multiple sexual ornaments of males at adulthood. More specifically, we supplemented pheasants with either carotenoids, vitamin E, or a combination of both vitamin E and carotenoids, or provided a control diet, during the first 8 weeks of life. This allowed us to examine the synergistic and independent effects of carotenoids and vitamin E in regulating trade-offs during growth and development that affect the expression of traits important in reproductive success as adults. If oxidative stress is an important mediator of trade-offs during growth and development (Hall et al. 2010) we predict that supplementary antioxidants provided during early development will be preferentially allocated towards traits most important to reproductive success at the expense of investment in self-maintenance. In particular, in the light of recent work that indicates that carotenoids are relatively poor antioxidants in birds *in vivo* (e.g. Cohen and McGraw 2009), we tested whether carotenoids and vitamin E are only effective antioxidants in combination with one another (vitamin E can recycle oxidised carotenoids, for example; Catoni et al. 2008), and whether such synergistic effects during development lead to an increase in resources allocated to achieving higher reproductive success (increased sexual ornament expression and/or body size) rather than reducing oxidative damage.

3.2. Materials and Methods

3.2.1. General protocol

240 one day old ring-necked pheasants of mixed genetic stock (Holme Park Game Hatcheries, Wokingham) were used in the experiment, at the Game and Wildlife Conservation Trust, Hampshire, UK. 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 encounter 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. 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 infra red heat lamp within a semi-intensive brooder hut system. Lighting levels during the first 8 weeks were limited to the light emitted by the heat lamps and windows were painted to minimise light entering the pens as standard husbandry practice (GCT 2004). Additional birds were reared, and chicks that died (n = 8) during the experimental period (8 weeks) were replaced with non-experimental, similar-aged birds to maintain consistent rearing densities. On day 1, the birds were allocated randomly to one of four equal-sized diet treatment groups (n = 60 birds per group) :(1) Carotenoid supplemented (group C) (2) α-tocopherol (vitamin E) supplemented (group V) (3) α-tocopherol (vitamin E) with carotenoid supplemented combined (group CV) and (4) a control diet (Control). These diets were fed for the first 8 weeks. An 8 week period of dietary manipulation was chosen to include the early developmental window identified by previous studies on pheasants (Ohlsson and Smith 2001; Ohlsson et al. 2002). After 2 weeks, birds were 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 one of two outdoor single-sex pens (30m x 27m) with access to grass for the remainder of the experiment. The diet provided after 8 weeks was identical for all birds (Duke's and Botley Ltd., maintenance pellet 13% protein).

Morphometric measurements were taken at day 1 and then at weekly intervals until 10 weeks of age, then again at 21 and 22, 36 and 37, 46 and 47 weeks of age. Blood samples were taken from all birds at 8 and 47 weeks of age. At 8 weeks of age samples were used to assay plasma concentrations of vitamin E, carotenoids and oxidative damage (determined by measuring the concentration of a biomarker of lipid peroxidation, malondialdehyde (MDA) in all individuals. At 47 weeks of age oxidative damage (MDA) was assayed in all individuals again, and plasma concentrations of vitamin E and carotenoids were measured for males only. Phytohaemagglutinin was used to measure immune response of all individuals at 21 weeks of age (Smits et al. 1999; Vinkler et al. 2010). In addition, wattle colour, size and shape and spur length were measured at 21 weeks at a period when body size growth was occurring and at 47 weeks of age before the breeding season by which stage wattles were fully developed (Ohlsson and Smith 2001). A previous study on protein manipulation in ring-necked pheasants showed significant differences in

wattle size at 20 and 40 weeks of age (Ohlsson et al. 2002).

3.2.2. Dietary supplementation

All birds received a custom-made basal diet, based on standard commercial pheasant pellets, but with no added vitamin E, and low levels of vitamin A (10.0mg/kg) and selenium (0.20mg/kg) (Target Feeds Ltd., Shropshire). The basal diet was manipulated for each treatment group as follows: Carotenoid supplemented birds received 100 mg carotenoids in the form of ORO-GLO® brand 11 liquid pigmenter (lutein and zeaxanthin, 20:1 w/w) (Kemin industries Inc.) per kg of feed; birds supplemented with vitamin E received supplemental all-trans α-tocopherol (Sigma-Aldrich T36634) at a concentration of 100mg/kg of feed; birds receiving both carotenoids and vitamin E received 50mg per kg of feed; control birds received an unsupplemented diet. The concentration of vitamin E and carotenoids supplemented was consistent with concentrations (100mg/kg) used in previous studies on poultry (vitamin E: Bartov and Frigg 1992; Guo et al. 2001; carotenoids: Woodall et al. 1996; Surai et al. 2001). Supplements were added to the feed daily, by spraying with a 5 litre spray pump. α-Tocopherol was sprayed in soybean oil onto the feed and ORO-GLO® xanthophylls were mixed in water and stored in refrigerated vacuum pumped containers until given to the birds. Soybean oil was selected as a medium for α-tocopherol supplementation because it contains comparatively low levels of α-tocopherol (0.07μg/mg) compared to other naturally occuring oils (Carpenter 1979). Equal volumes of soybean oil and water were sprayed onto the other feeds. Each afternoon the feed was replenished with fresh refrigerated treatment feed stored in vacuumed pumped containers. Four diets were provided over the 8 week period of supplementation, all with medium levels of protein, in line with standard pheasant rearing practice (starter crumb 1-2 weeks: 29.8% protein, starter pellets 3-4 weeks: 25.5% protein, rearer pellets 5-6 weeks: 21.4% protein, grower pellets 7-8 weeks: 18.1% protein). Feed, grit and water were provided *ad libitum*.

3.2.3. Morphometric measurements

To calculate growth body mass, tarsus length, head to bill length and wing length was measured at each week from 0-10 weeks, at 21 and 22, 36 and 37, 46 and 47 weeks of age. Body mass was measured using a Pesola® spring balance (30g, 60g, 100g, 300g, 600g, 1000g, 2500g depending on age). Tarsus length and head to bill length was

measured using callipers (to an accuracy of ± 0.01mm) and wing length was recorded using a wing rule (accuracy ± 0.1mm). Spur length was measured 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. 2002).

3.2.4. Oxidative stress assays and α-tocopherol concentration of plasma

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 chromatography (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 ^{o}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.

3.2.5. Wattle size, shape and colour

An image of the right 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 60 fourier coefficients were then used as variables in principal component analyses. Five principal components that described over 98% of the wattle shape variation (PC1 = 42%, PC2 = 20%, PC3= 14%, PC4= 12%, PC5= 10%) were used for analyses (South and Arnqvist 2009).

Wattle reflectance data were collected using a USB2000 UV-Visible spectrophotometer and OOIBase32 Software (Ocean Optics Inc., Dunedin, FL). 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 stabilise before capture (Keyser and 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 important in female mate choice (Hillgarth and Wingfield 1997). We calculated brightness as it would be perceived by a conspecific female, using the method detailed in Endler and Mielke (2005). In galliforms, brightness is likely to be perceived by the double cones (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 pheasant 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 and 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 and Stiles 1982).

3.2.6. 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 in pro-inflammatory potential in avian studies (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 web diameters were then measured with a digimatic micrometer (0.01mm, Mitutoyo APB-2D). 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 syringes (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 using the digimatic micrometer (0.01mm) directly after injection. 24 hours (± 10 minutes)

after the injection the thickness of the patagium of the wings was measured again. The thickness measurement of the left-wing patagium was subtracted from the measurement taken from the right wing-web to identify the pro-inflammatory potential to PHA 24 hours after exposure (Vinkler et al. 2010).

3.2.7. Statistical analyses

Normality checks were carried out in SPSS and variables were log-transformed where necessary (SPSS Inc., Chicago IL). Dependent variables conforming to a normal distribution were analysed using general linear mixed models (GLMMs) with supplementary treatment group, sex and/or age included as fixed effects and hatch date as a random factor. GLMMs were run in R version 2.9.2 (R Development Core Team 2009), using the *lme* function. Treatment was included as a single factor with 4 levels and sex as a factor with 2 levels. Males and females were approximately equally represented at all levels of the factor 'treatment'. The date on which the HPLC assay was run for each sample was also included as a covariate but was dropped from all models during simplification. Where repeated measures were taken (vitamin E plasma concentration and wattle brightness) or male secondary sexual signals were measured, treatment diet and age were included in the maximal model, and two-way interactions were also included. The bird identification was included as a random factor in repeated measures analyses. Only measurements from birds that survived to a year of age were used. For model simplification, we removed the highest order interactions, followed by lower order terms in turn from the maximal model using maximum likelihood tests. Principal components produced using the coefficients calculated by elliptic fourier analysis of wattle shape and with wattle size were used in multivariate analyses of covariance (MANCOVA) the PCs were included as dependent variables.

3.3. Results

3.3.1. Concentrations of plasma antioxidants

The minimum adequate model of a GLMM with the concentration of carotenoids in plasma in males as the response variable included all explanatory variables entered into the initial model, with significant main effects of age and treatment and a

significant interaction between age and treatment (**Table 3.1, Figure 3.1a,b**). The concentration of carotenoids in the plasma decreased considerably with age from a mean across groups of 20.77 at 8 weeks to 1.99 μg/ml by 47 weeks of age, with the greatest declines shown by males in the treatment groups that received carotenoid supplements in the diet during the first 8 weeks of life (i.e. groups C and CV; **Figure 3.1.a**). GLMMs run as above but separated by age showed that males in groups C and CV had higher concentrations of plasma carotenoids at 8 weeks of age than males in the control and V groups (GLMM comparing treatment diets for males at 8 weeks of age: L.ratio=291.7 p<0.0001; **Figure 3.1.a**), but there were no differences among treatment groups in plasma carotenoid concentrations at 47 weeks (GLMM comparing treatments: L.ratio = 0.68 p=0.88; **Figure 3.1.b**). Males did not differ from females in the concentrations of carotenoids circulating in plasma at 8 weeks of age (**Table 3.1.**).

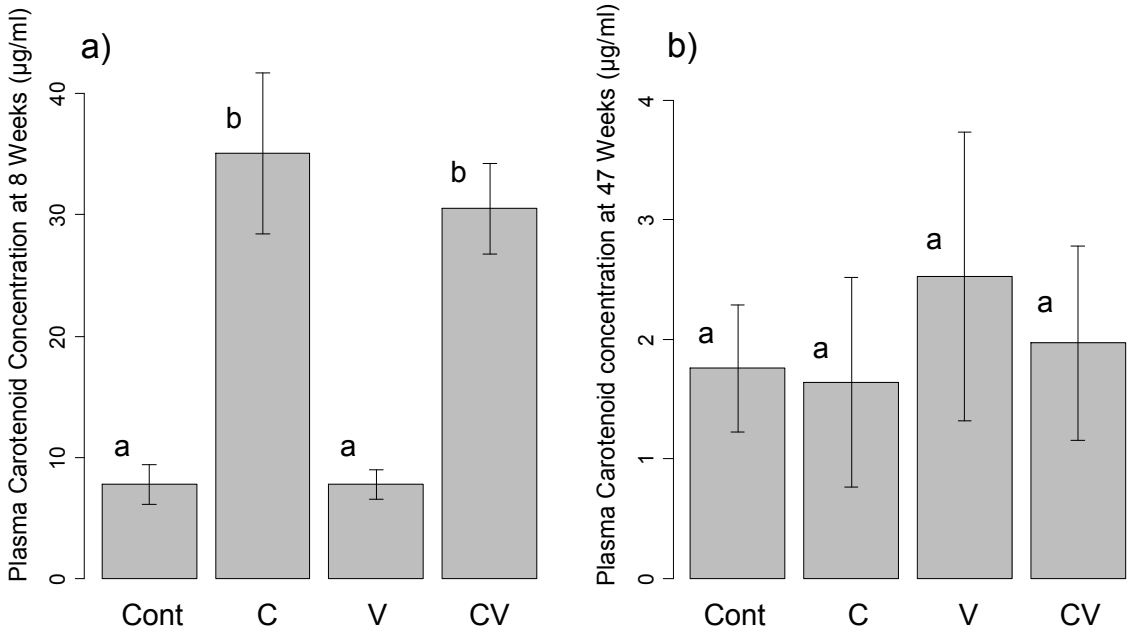

Figure 3.1.: Mean plasma carotenoid concentrations (μg/ml) in males in relation to treatment and age at a) 8 weeks of age b) 47weeks of age. Error bars show 95% confidence intervals. Results are presented on separate graphs due to scale differences. Means with the same letter do not differ significantly to one another. Carotenoid supplemented (C n=57), α-tocopherol supplemented (V n=58), α-tocopherol with carotenoid supplemented combined (CV n=54) and a control diet (Cont n=63).

There were significant main effects of treatment and age and a significant interaction between age and diet treatment on concentrations of α-tocopherol in plasma (**Table 3.1., Figure 3.2.a,b**). Males in groups V and CV had higher concentrations of plasma α-tocopherols at 8 weeks of age than males in C and control groups (GLMM

comparing treatment diets for males at 8 weeks of age: L.ratio = 59.32, p<0.0001) (**Figure 3.2.a)**. Plasma concentrations of α-tocopherols in birds that received a diet supplemented with α-tocopherol up to 8 weeks of age (i.e. groups V and CV) remained significantly higher at 47 weeks than controls and birds supplemented with carotenoids only (**Table 3.1., Figure 3.2.b**). The concentration of α-tocopherols decreased between 8 and 47 weeks of age most in those birds that received α-tocopherols in their diet up to 8 weeks of age. Males did not differ from females in the concentrations of α-tocopherol circulating in plasma (**Table 3.1.**).

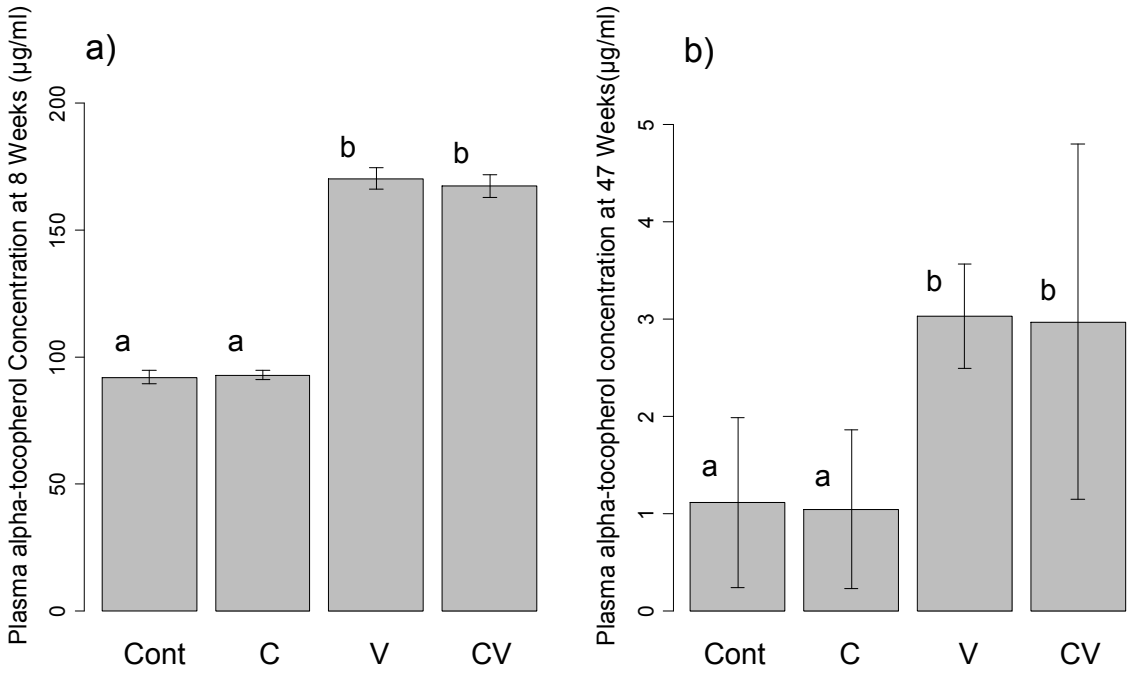

Figure 3.2.: Mean plasma α-tocopherol concentrations (µg/ml) of males in relation to treatment and age at 8 a) and 47 b) weeks of age. Error bars show 95% confidence intervals. Results are presented on separate graphs due to scale differences. Means with the same letter do not differ significantly to one another. Carotenoid supplemented (C n=57), α-tocopherol supplemented (V n=58), α-tocopherol with carotenoid supplemented combined (CV n=54) and a control diet (Cont n=63).

3.3.2. Oxidative damage

The minimum adequate model of a GLMM with plasma MDA concentration as the response variable and age, treatment and sex as main effects included significant main effects of age and treatment and a significant interaction between treatment and age (**Table 3.1.**). The mean concentration of plasma MDA across groups decreased from 5.07 µg/ml at 8 weeks to 2.28 µg/ml at 47 weeks of age (see **Figure 3.3.a,b**). GLMMs on data separated by age showed a significant main effect of treatment diet on

concentration of MDA at 8 weeks of age (treatment: L.ratio = 291.7 p<0.0001), with the birds given a control diet having a higher concentration of plasma MDA than birds given the other diets (**Figure 3.3.a**). However, by 47 weeks there were no differences in plasma MDA concentrations among treatment groups (GLMM with birds at 47 weeks from all treatments: L.ratio = 5.59 p=0.13; **Figure 3.3.b**).

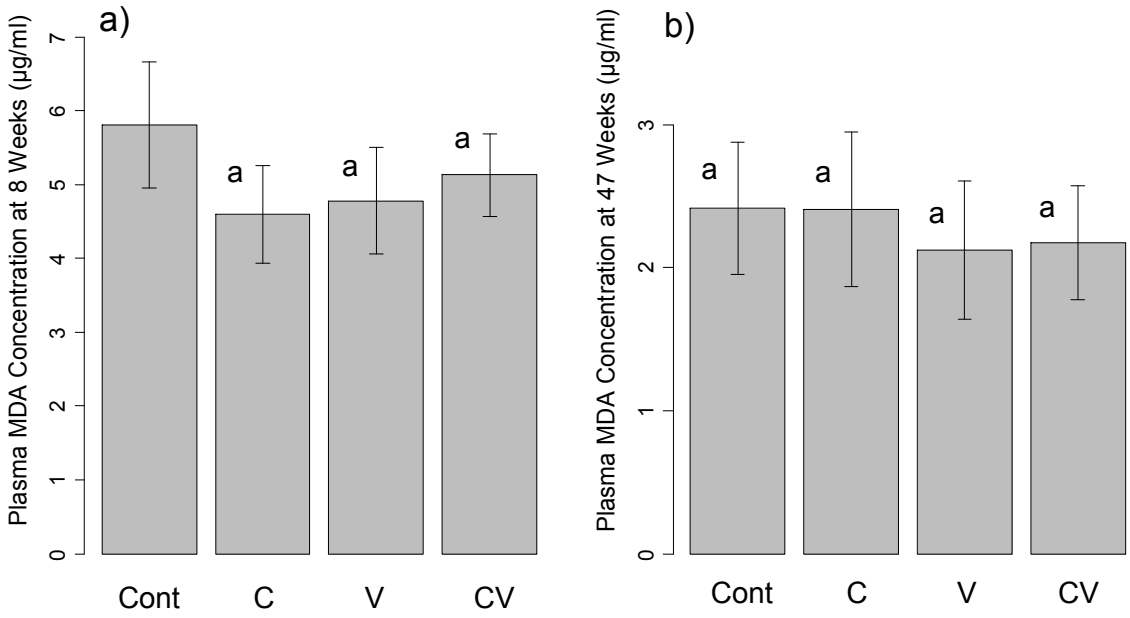

Figure 3.3.: Mean plasma malondialdehyde (MDA) concentrations (µg/ml) of both males and females in relation to treatment and age at 8 a) and 47 b) weeks of age. Error bars show 95% confidence intervals. Results are presented on separate graphs due to scale differences. Means with the same letter do not differ significantly to one another. Carotenoid supplemented (C n=57), α-tocopherol supplemented (V n=58), α-tocopherol with carotenoid supplemented combined (CV n=54) and a control diet (Cont n=63).

Table 3.1.: Results of GLMM models of total antioxidant plasma concentrations and oxidative stress at 8 and 47 weeks of age

		L.ratio	p-value
Plasma concentration of carotenoids of males at 8 and 47 weeks of age			
	Treatment x Age	134.9	<0.0001***
	Treatment	58.72	<0.0001***
	Age	346.98	<0.0001***
Plasma concentration of carotenoids at 8 weeks			
	Treatment x Sex	5.73	0.13
	Treatment	442.57	<0.0001***
	Sex	0.90	0.34
Plasma concentration of α-tocopherol of males at 8 and 47 weeks of age			
	Treatment x Age	64.18	<0.0001***
	Treatment	54.46	<0.0001***
	Age	321.33	<0.0001***
Plasma concentration of α-tocopherol at 8 weeks			
	Treatment x Sex	3.06	0.08
	Treatment	303.45	<0.0001***
	Sex	4.10	0.25
Plasma concentration of MDA at 8 and 47 weeks			
	Treatment:Age	134.9	<0.0001***
	Treatment	58.72	<0.0001***
	Age	346.98	<0.0001***

Significance: ***P<0.0001**P<0.001*P<0.01'*

3.3.3. Growth

Repeated measures GLMMs with mass at X weeks as the response variable and sex and treatment group as explanatory variables showed that males grew faster than females (**Table 3.2.**) and that there were significant differences between treatments in growth (**Table 3.2.**). Post hoc analyses showed that birds supplemented with carotenoids and vitamin E (CV) grew faster from 21 to 47 weeks of age than those birds given carotenoids only (C), vitamin E only (V) or controls (Cont) (GLMM comparing CV with C: L.ratio = 16.41, $p<0.001$, CV with V: L.ratio = 5.19, $p<0.05$, CV with Cont: L.ratio = 16.32, $p<0.001$) (**Figure 3.4.**).

As expected there were no initial differences in the mass of chicks allocated to different dietary treatments (**Table 3.2.**). However, by 8 weeks of age (i.e. the immediate end of the antioxidant supplementation period) males were heavier than females and there was a significant effect of treatment on mass (**Table 3.2.; Figure**

3.2.). Birds receiving the combined carotenoid and α-tocopherol supplement (CV group) were heavier than those supplemented with carotenoids only (GLMM comparing C and CV: L.ratio = 8.548 p=0.0035). At 21 weeks, at the time of immune response measurement, males remained heavier than females but there was no difference between diet treatment groups in mass (**Table 3.2.; Figure 3.2.**). However, by 47 weeks of age mass of birds varied according to the diet received during the first eight weeks of life. Birds supplemented with carotenoids and α-tocopherol (CV) had a significantly higher mass than birds in other groups (GLMMs comparing carotenoid and vitamin E treatment to the other treatment diets: mass: L.ratio=12.50, $p<0.01$; **Figure 3.4.**).

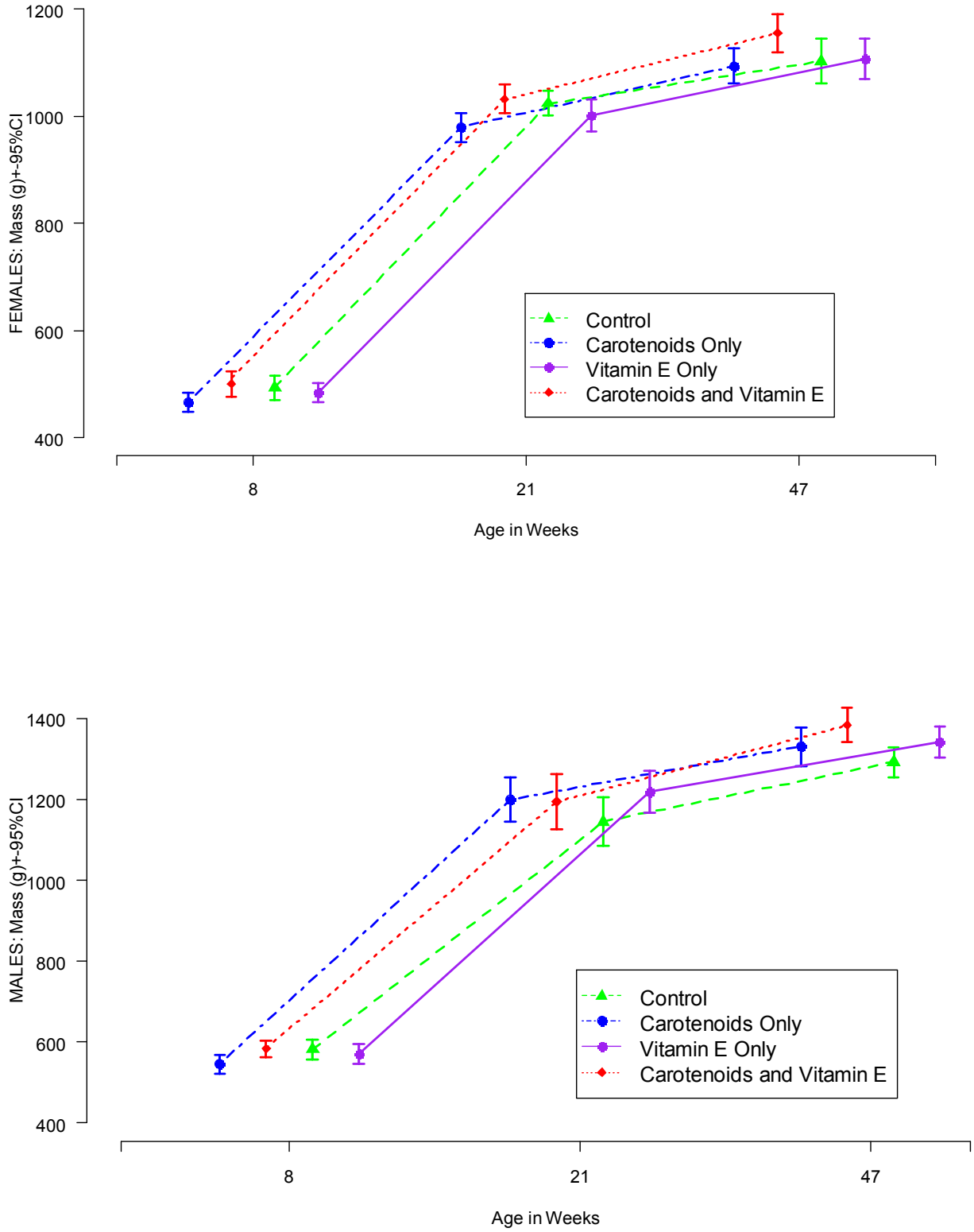

Figure 3.4.: Mean mass growth of females a) and males b) in relation to treatment and age (8, 21 and 47 weeks). Error bars show 95% confidence intervals. Carotenoid supplemented (C n=57), α-tocopherol supplemented (V n=58), α-tocopherol with carotenoid supplemented combined (CV n=54) and a control diet (Cont n=63).

Table 3.2.: Results of repeated measures GLMM models of growth and GLMM models of mass

		L.ratio	p-value
Mass growth			
	Treatment x Sex	1.20	0.33
	Treatment	2.91	0.035*
	Sex	247.70	<0.001**
Tarsus length growth			
	Treatment x Sex	0.093	0.96
	Treatment	2.27	0.082
	Sex	820.67	<0.001**
Head to Bill length			
	Treatment x Sex	0.21	0.89
	Treatment	2.88	0.037*
	Sex	30.39	<0.001**
Wing Length			
	Treatment x Sex	0.15	0.93
	Treatment	1.56	0.20
	Sex	140.88	<0.001**
Mass aged 1 day			
	Treatment x Sex	8.48	0.37
	Treatment	1.27	0.74
	Sex	0.15	0.70
Mass at 8 weeks of age			
	Treatment x Sex	1.02	0.80
	Treatment	12.66	<0.01*
	Sex	88.03	<0.001**
Mass at 21 weeks of age			
	Treatment x Sex	5.33	0.15
	Treatment	2.76	0.43
	Sex	91.00	<0.001***
Mass at 47 weeks of age			
	Treatment x Sex	2.01	0.57
	Treatment	12.50	<0.01*
	Sex	165.68	<0.001**

3.3.4. Immune function

Immune response did not vary in relation to either sex or treatment. The minimum adequate model of a GLMM with wing patagium inflammation following immune challenge as the response variable and treatment and sex as main effects included just the intercept, with all other variables dropping out of the model (GLMM Treatment:Sex L.ratio=5.87, p=0.12, Sex: L.ratio=1.48 p=0.22, Treatment: L.ratio=1.58 p=0.66).

3.3.5. Secondary sexual signals

The expression of secondary sexual signals by males also did not vary among treatment groups (**Figures 3.5. and 3.6.**). GLMMs with the sexual signal parameter as the response variable and age and treatment as explanatory variables in the initial models included only age in the minimum adequate model in all cases (**Table 3.3.**). There was a trend towards a significant interaction between treatment and age in spur length between 21 and 46 weeks of age (**Figure 3.7.**), with the mean growth (mm) of the spurs of birds that received vitamin E (i.e. CV and V males) higher than birds that received a control diet (CV = 3.08, V = 3.23, Cont = 1.63, C = 2.02), but the effect was not significant (**Table 3.3.**). There was no difference in wattle size or brightness in relation to dietary treatment (**Table 3.3.**). A MANCOVA of the 5 principal components that collectively described 75% 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 treatment (GLMM: F=0.66, p=0.82).

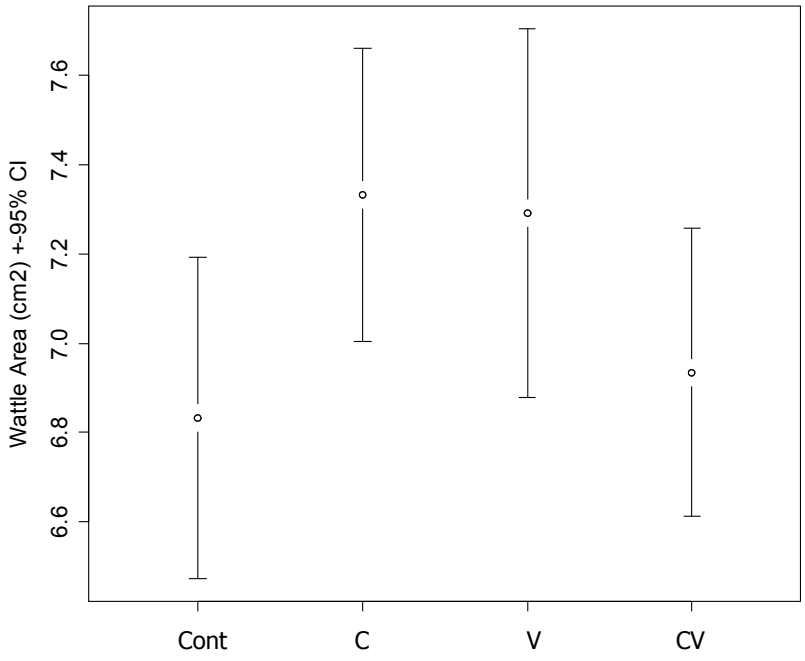

Figure 3.5.: Wattle Area at 47 weeks of age with 95 % confidence intervals. Carotenoid supplemented (C n=57), α-tocopherol supplemented (V n=58), α-tocopherol with carotenoid supplemented combined (CV n=54) and a control diet (Cont n=63).

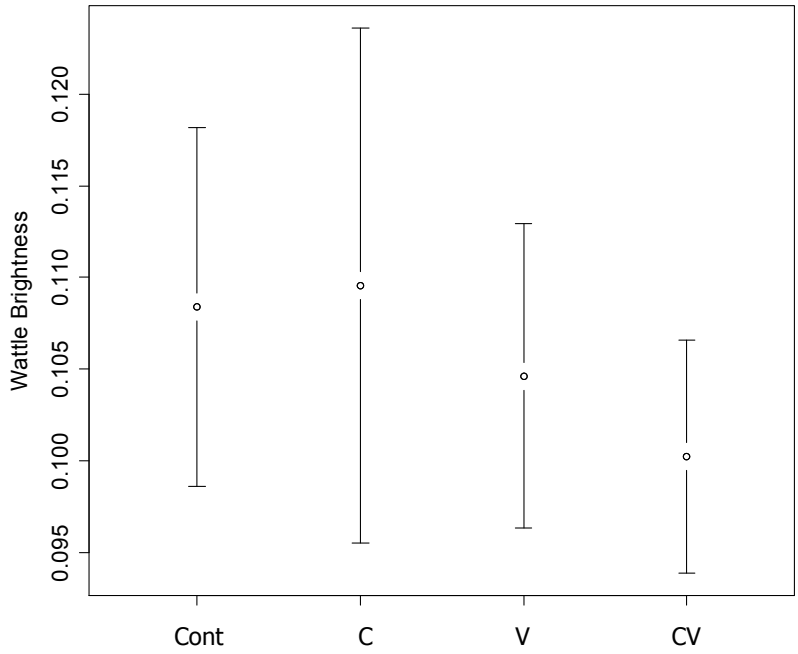

Figure 3.6.: Wattle brightness at 47 weeks of age with 95 % confidence intervals. Carotenoid supplemented (C n=57), α-tocopherol supplemented (V n=58), α-tocopherol with carotenoid supplemented combined (CV n=54) and a control diet (Cont n=63).

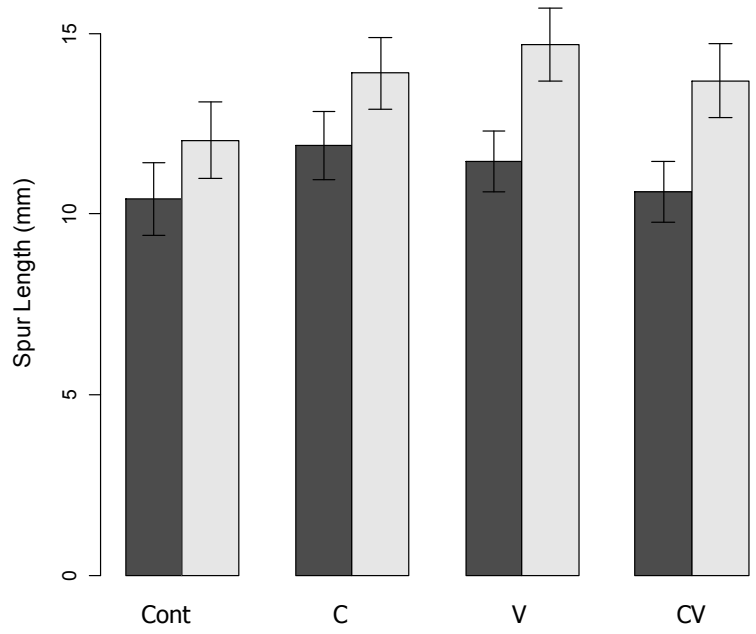

Figure 3.7.: Spur Length at 21 (dark grey) and 47 (light grey) weeks of age with 95 % confidence intervals. Carotenoid supplemented (C), α-tocopherol supplemented (V), α-tocopherol with carotenoid supplemented combined (CV) and a control diet (Cont).

Table 3.3.: Results of GLMM models of secondary sexual signals

		L.ratio	p-value
Spur Length			
	Treatment	2.38	0.50
Wattle Size			
	Treatment	6.02	0.11
Wattle Shape			
	Treatment	0.67	0.82
Wattle Brightness			
	Treatment	0.15	0.93

Significance codes <0.0001 '***' <0.001'**'<0.01'*

3.4. Discussion

The results of this study on ring-necked pheasants provide evidence that males preferentially allocated supplementary antioxidants to achieving a large size rather than to the expression of sexually-selected traits. Moreover there were synergistic combined effects of supplementation of carotenoids and α-tocopherol (vitamin E) on growth. Individuals that received a diet of carotenoids combined with vitamin E had a faster growth rate and reached a larger size than individuals given other treatment diets, including either carotenoids or vitamin E alone. We also found that supplementation with vitamin E over the early developmental period resulted in increased vitamin E plasma content at adulthood. In contrast, early supplementation with carotenoids had no effect on carotenoid plasma concentrations at adulthood. Antioxidant availability affected oxidative damage at the end of the experimental period (8 weeks), with birds that received a control diet having higher levels of oxidative stress than individuals that had received a diet supplemented with antioxidants (either alone or in combination). However, antioxidant supplementation did not reduce oxidative damage at adulthood, and secondary sexual characteristics and immune function were not influenced by the availability of antioxidants during early growth and development.

In contrast with the prediction that supplementation with both non-pigmentary and pigmentary antioxidants would result in increased allocation to sexual ornaments, extra resources were instead allocated to growth. Those birds that were supplemented with a combination of carotenoids and vitamin E showed a greater synergistic growth response than those supplemented with either carotenoids or α-tocopherol (vitamin E) alone, demonstrating that there were synergistic benefits of supplementing non-

pigmentary antioxidants along with carotenoids. Oxidised antioxidants can be recycled and regenerated to their active reduced form by reacting with other antioxidants (Catoni et al. 2008). Studies have documented the regenerative properties of vitamin E and C on carotenoids (Mortensen et al. 2001; Amorati et al. 2002) and similarly vitamin E can be recycled by carotenoids (Surai 2002). Our results provide clear evidence for synergistic effects of dietary antioxidants on somatic development, but not the expression of sexual ornaments.

Individuals supplemented with a combination of carotenoids and vitamin E were significantly heavier at 8 weeks of age, and at adulthood (47 weeks) were heavier, had a longer tarsus length, head-bill length and wing length than birds in other groups. Investment of additional antioxidant resources towards increased growth rather than sexually selected traits may appear surprising unless there are longer term benefits of achieving a large size, because increased rates of growth can result in higher levels of oxidative stress (Brown-Borg and Rakoczy 2003; Alonso-Alvarez et al. 2007). However, when resources are finite competition may lead to selection favouring traits that increase competitive ability (Wolf et al. 2008). This may explain why supplementary antioxidants were preferentially allocated to attaining a greater size rather than towards sexual ornaments. Significant competition for scarce resources would favour allocation to growth rather than self-maintenance to increase the competitive ability of an individual, thereby increasing their subsequent ability to acquire disproportionate amounts of further resources for both growth and self-maintenance (Wolf et al. 2008). In contrast, allocating resources primarily to maintenance at the expense of growth may result in these individuals being out-competed for scarce resources by larger, more competitive individuals, reducing competitiveness and therefore the relative amount of resources acquired subsequently in a negative feedback process (Hall et al. 2010). In ring-necked pheasants attaining a larger body size could have 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 adults. By maintaining a better body condition it is possible that birds may be able to capitalise on environmental fluctuations in carotenoid availability as adults (Smith et al. 2007), and it also has a positive effect on dominance (Göransson et al. 1990; Grahn and von Schantz 1994). Allocating supplementary antioxidant resources to increasing body size and therefore

dominance could lead to more reproductive benefits than allocating resources preferentially to sexually selected traits, by increasing the ability of a male to maintain control of territories and to acquire mating opportunities (Grahn and von Schantz 1994).

In contrast to treatment effects of antioxidant supplementation on growth there were no detectable effects of supplementation on immune response. Although numerous previous studies have shown that carotenoids have immunoenhancing properties (Amar et al. 2000; Saks et al. 2003; Chew and Park 2004; McGraw and Ardia 2005) we found no evidence for a greater pro-inflammatory potential following PHA injection at 21 weeks of age. This contrasts with responses to immune challenge during antioxidant supplementation of adult diets in many other species (zebra finches, *Taenopygia guttata*, (Blount et al. 2003b) guppies, *Poecilia reticulata,* (Grether et al. 2004), grey partridges, *Perdix perdix*, (Cucco et al. 2006). Hõrak et al. (2007) found no evidence for enhanced immune response to PHA injection in greenfinches following supplementation with vitamin E, but the basal diet of sunflower seeds provided to all control and supplemented birds during the experiment had a relatively high antioxidant content which may have masked the effects of antioxidant supplementation (Hõrak et al. 2007). During these studies immune response was measured during or directly after antioxidant supplementation. In contrast, in the present study immune response was measured 13 weeks after antioxidant supplementation had ceased. Our results suggest that supplemented birds did not store antioxidants for later use following the costly growth period. Smith et al. (2007) found that pheasants in better body condition, measured as residual mass, had a higher pro-inflammatory immune response to PHA injection. In our experiment there was no significant difference in mass between treatment groups at 21 weeks, so it is possible that had the immune challenge been carried out at 8 or 47 weeks of age, when there were mass differences among treatment groups, there may also have been differences in immune function among treatment groups. Alternatively it may be that the basal diet supplied sufficient resources to maintain adequate immune function, allowing supplementary antioxidants to be available for allocation to other functions, such as growth.

Circulating antioxidants were increased at the end of the 8 week period of supplementation (both carotenoids and α-tocopherol) supporting the results of previous studies in a range of avian species (e.g. Alonso-Alvarez et al. 2004; Aguilera and Amat 2007). More particularly supplementation with α-tocopherol during the first 8 weeks resulted in increased circulating α-tocopherol defences at adulthood, supporting previous work on zebra finches, *Taeniopygia guttata,* by Blount et al. (2003a). This suggests that availability of α-tocopherol during early development can have a long-term affect on the capacity of individuals to assimilate α-tocopherol in adulthood, and indicates that the lipoproteins required to assimilate antioxidants are primarily produced early in life (Blount et al. 2003a). In contrast to the long-term effects of supplementation with α-tocopherol there was no evidence that early exposure to dietary carotenoids had an effect on circulating levels of carotenoids in blood plasma at adulthood.

In addition to affecting blood plasma concentrations of antioxidants supplementation also resulted in reduced oxidative damage in the form of lipid peroxidation during the supplementation period, as levels of malondialdehyde (a robust measure of oxidative damage; Monaghan et al. 2009) were significantly higher for control birds at 8 weeks of age. However, despite the higher levels of circulating α-tocopherol (vitamin E) in birds that received a supplement of vitamin E during early life when measured at 47 weeks of age there was no difference in levels of oxidative damage in relation to treatment group. In particular supplementation with vitamin E did not provide any benefits compared to supplementation with carotenoids with respect to mitigating oxidative damage, despite the antioxidant role of carotenoids not generally being considered to be effective in birds. For example, a recent fixed effect meta-analysis study found that carotenoids could account for as little as <0.002% of antioxidant activity in birds (Costantini and Møller 2008). However, this meta-analysis included studies carried out at all periods of the life cycle. In contrast, our results indicate that carotenoids can be effective antioxidants, especially in combination with other antioxidants as supplementation with a combination of vitamin E and carotenoids during early development resulted in increased size without elevated costs of oxidative damage.

Our study aimed to address the longer term effects of early antioxidant supplementation on adult phenotype. However, it is possible that measurements over the first 47 weeks of life were still insufficient to be able to detect differences among treatment groups. For example, spur length in ring-necked pheasants is known to be the most important predictor of harem size, but continues to grow throughout the second year of life (Göransson et al. 1990), and spur length at one year of age has less influence on female mate choice than the spur length of older males (Grahn and von Schantz 1994). We found that the relative spur length of individuals was not significantly enhanced in males given vitamin E (and/or carotenoids) during development. However, it is possible that beyond one year of age significant differences in spur length could develop if there is continued growth. In addition, the effects of higher circulating vitamin E at 47 weeks found in birds supplemented with vitamin E during development on the accumulation of ROS beyond 47 weeks on the rapidity of ageing and longevity are also unknown. The greater antioxidant protection available at 47 weeks of age in individuals supplemented with vitamin E during early development could result in reduced costs of self maintenance and therefore a higher availability of antioxidant resources for allocation to the subsequent development of sexual signals. Consequently, the lack of a difference among treatments in expression of sexually-selected traits may not be indicative of longer term effects.

The results show that antioxidant availability during early development can have a substantial effect on adult phenotype, providing support for the role of oxidative stress as a unifying mechanism in life-history trade-offs (e.g. Costantini 2008), and a synergistic effect of non-pigmentary and pigmentary antioxidants supplementation on growth and development. However, there was no support for the 'carotenoid protection theory' as there were no synergistic effects of antioxidants on the expression of secondary sexual traits (Hartley and Kennedy 2004). Future studies should further explore the role of synergistic effects of ingesting and absorbing dietary antioxidants during development on the expression of adult phenotypes in other species as it is likely to provide considerable insights into the evolution of life-history traits among species.

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Chapter 4: Effects of neonatal nutrition on ornamentation and sperm quality in ring-necked pheasants

4.1. Introduction

The nutritional conditions experienced during development can have profound effects on adult phenotype. Sexually-selected traits are particularly susceptible to environmental stress during development (Hunt and Simmons 1997; David et al. 2000). For example, horned beetles, *Onthophagus taurus,* provided with a higher quality diet during development invested extra resources in increasing the length of the developmental period to grow longer horns at the increased risk of predation and susceptibility to parasitic infection (Hunt and Simmons 1997). In stalk-eyed flies, *Cyrtodiopsis dalmanni,* increased nutritional quality during development resulted in the preferential allocation of resources to increasing sexual trait size at a cost to increasing body size (David et al. 2000). The environment experienced during development can also affect primary sexual traits. Nutritional stress during development in the meal moth, *Plodia interpunctella,* results in the production of lower numbers of sperm at adulthood (Gage and Cook 1994), for example, and the protein content of spermatophores in adult male comma butterflies, *Polygonia c-album* has been shown to be affected by the species of host plant they developed on as larvae (Wedell 1996). Allocation of limited resources to primary and secondary sexual traits can come at the expense of allocation to other traits involved in self-maintenance, such as immune function or antioxidant defences. Following a period of nutritional stress during development, zebra finches, *Taenopygia guttata*, for example, appear to allocate available resources to both primary and secondary reproductive traits, at the expense of allocation to immune function and longevity (Birkhead et al. 1999).

The relative allocation of resources to sexual traits has important consequences for fitness because females often prefer to mate with the most elaborately ornamented males (Andersson and Simmons 2006) and secondary sexual characteristics may provide information about the functional fertility of males (Sheldon 1994; Merilä and Sheldon 1999). If male ornamentation provides an indication of 'direct benefits' for a female such as avoiding infectious diseases or the quantity of resources, including food, territories or parental care that a male can offer, the benefits of female mate choice are clear (Houle and Kondrashov 2002). Sheldon (1994) proposed that females seek extra-pair copulations as insurance against the functional infertility of their mates

(Gibson and Jewell 1982; Wetton and Parkin 1991), and that functional fertility correlates with male phenotype. Studies have since identified a positive relationship between the degree of male sexual ornamentation and aspects of sperm quality, quantity or testes size in some species (European greenfinches, *Carduelis chloris* Merilä and Sheldon 1999; guppies, *Poecilia reticulate,* Pilastro et al. 2002; Locatello et al. 2006; Pitcher et al. 2007; mallard ducks, *Anas platyrhynchos,* Peters et al. 2004; red deer, *Cervus elaphus hispanicus,* Malo et al. 2005, and great tits, *Parus major,* Helfenstein et al. 2010), but not in others (e.g., zebra finches, Birkhead and Fletcher 1995; sedge warblers, *Acrocephalus schoenobaenus,* Birkhead et al. 1997).

There is growing evidence that one of the unifying mechanistic links between male ornament expression and functional fertility is oxidative damage (Blount et al. 2001; Velando et al. 2008). Oxidative damage results from an imbalance between the production of damaging reactive oxygen species (ROS) and antioxidant defences, in favour of the former resulting in lipid peroxidation, DNA and protein damage (Sies 1997). For example, oxidative damage to sperm can result in DNA damage that reduces offspring fitness (Blount et al. 2001), and if the degree of oxidative stress is also reflected in the expression of male phenotype, this would allow females to choose not to mate with males with high levels of oxidative damage and thereby to increase their 'indirect benefits'. A study by Helfenstein et al. (2010) tested the role of oxidative damage as a mechanistic link between sexual signals and sperm quality by artificially increasing the workload and oxidative stress experienced by male great tits, *Parus major*, using brood size manipulation. Males with a greater workload and duller breast plumage had higher ejaculate lipid peroxidation levels than more colourful males. Supplementation with carotenoids enabled paler males to reduce lipid peroxidation damage in the ejaculate, which were similar to the levels of lipid peroxidation in unsupplemented bright males (Helfenstein et al. 2010).

Male ornamentation can be negatively affected by increased levels of ROS and improved by antioxidant supplementation (von Schantz et al. 1999). Sperm are believed to be particularly susceptible to oxidative damage and ROS have a role in contributing to between 30-80% of cases of human male infertility (Tremellen 2008). At low concentrations ROS assist with normal key processes in spermatozoa including capacitation, acrosome reaction, fertilisation and motility (Agarwal 2004;

Desai et al. 2010). However, excessive amounts of ROS caused by the heightened production of ROS by morphologically and/or functionally abnormal or immature spermatozoa (Plante et al. 1994), excessive leukocyte infiltration into the semen (Agarwal 2004) and leakage from the mitochondria within the midpiece of the spermatozoa (Desai et al. 2010) can be highly damaging. The reason for the particularly high susceptibility of sperm to oxidative damage is the high content of polyunsaturated fatty acids in the cell membrane (Desai et al. 2010) and the condensed DNA in sperm, together with the lower concentration of DNA transcription proteins, which reduce the ability of sperm to repair damaged DNA (Tremellen 2008).

The antioxidant system is diverse and includes endogenous enzymatic and non-enzymatic antioxidants as well as dietary derived, exogenous antioxidants. Vitamin E is a colourless and highly potent antioxidant (Surai 2002), whereas fat-soluble carotenoids, which also have antioxidant properties, are highly pigmented, responsible for many sexually-selected traits and have a number of other physiological functions such as immunoenhancing properties (McGraw and Ardia 2003). However, the ROS-scavenging properties of carotenoids *in vivo* have been suggested to be relatively weak compared to some other antioxidants (Costantini and Møller 2008). As a result of the multiple properties of carotenoids, there may be trade-offs in the deployment of carotenoids to immune function versus their expression in colourful displays (Lozano 1994). The presence of carotenoid-based signals may, instead, signal the availability of the more efficient non-pigmentary antioxidants ('carotenoid protection theory'; Hartley and Kennedy 2004). This is because oxidation, a consequence of ROS scavenging, results in the structural alteration of carotenoids that leaves them colourless and unavailable for use in sexual signalling (Peters 2007). Non-pigmentary antioxidants may therefore protect carotenoids from oxidation, retaining them for use in sexual signalling. While evidence has indicated that vitamin E may be present in the semen at sufficient levels to reduce oxidative stress in the domestic fowl (Surai et al. 1997), it remains unclear whether carotenoids are also present in other bird species and in sufficient quantities to reduce oxidative stress (Rowe and McGraw 2008).

In the current study, we manipulated antioxidant availability in the diet of ring-necked pheasants, *Phasianus colchicus*, during early life and then quantified the effects of

treatment diet on sexual ornamentation, concentration of vitamin E in the testes, oxidative damage in the ejaculate, sperm motility and testes mass and volume at adulthood. Testes size is positively correlated with sperm production in many birds (Møller 1988). Ring-necked pheasants have a harem polygyny mating system and females choose mates based on multiple sexual ornaments (Hill and Robertson 1988), including facial wattles (Hillgarth 1990) and spurs on the tarsus (Göransson et al. 1990). The bright wattle of males is expanded during sexual displays to attract females (Hill and Robertson 1988) and females have been shown to prefer larger wattles (Hillgarth 1990). Female pheasants are monandrous (Hill and Robertson 1988) and therefore choosing a fertile male is important. Previous work has shown that a low intake in dietary protein during early growth and development resulted in reduced brightness of the wattle of males at adulthood (Ohlsson et al. 2002), demonstrating that the expression of at least one sexual ornament in pheasants is sensitive to early environmental conditions experienced during development.

More specifically, in this study we supplemented pheasants with either carotenoids or vitamin E, a diet with both carotenoids and vitamin E or provided a control diet during the first 8 weeks of life and then quantified the effects of variation in availability of antioxidants during early life on the expression of primary and secondary sexual traits at adulthood. We predict that the degree of sexually selected trait expression will accurately indicate the quality of primary reproductive traits (Blount et al. 2001). In addition, we predict that if oxidative stress is an important mediator of trade-offs during growth and development, vitamin E will positively affect both secondary and primary sexual traits when compared to birds receiving a control diet. We expect to see the same pattern in birds receiving carotenoids during development but to a lesser degree due to their comparatively poor antioxidant ability. However, if hen pheasants do not choose males for fertility assurance we would not expect to measure a positive relationship between secondary sexual trait expression and primary sexual traits. We also expect that if the 'carotenoid protection theory' (Hartley and Kennedy 2004) is correct individuals receiving both vitamin E and carotenoids will have higher sperm quality and greater corresponding expression of secondary sexual traits resulting from the protection of supplemented carotenoids from oxidation for use in carotenoid-mediated ornament expression.

4.2. Materials and methods

4.2.1. General Protocol

240 day-old ring-necked pheasants of mixed genetic stock (Holme Park Game Hatcheries, Wokingham) were allocated randomly to one of four treatment groups at the Game and Wildlife Conservation Trust HQ, Hampshire, UK in May 2008. 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 encounter 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) Carotenoid supplemented (C) (2) α-tocopherol (i.e., vitamin E) supplemented (V), (3) Carotenoid and α-tocopherol supplemented (CV) and (4) a control diet (unsupplemented) (Cont). An 8-week period of dietary manipulation was chosen to include the early developmental window identified by previous studies on pheasants (Ohlsson 2001, 2002). The diet provided after 8 weeks was identical for all birds (Duke's and Botley Ltd., maintenance pellet 13% protein). At 47 weeks of age, 86 males were euthanized, measured (mass, wattle brightness, wattle size and spur length) and dissected. To measure investment in primary sexual characteristics oxidative damage in the testes and the semen, vitamin E concentration in the testes and the motility of sperm were measured. Oxidative stress was determined the concentration of a biomarker of lipid peroxidation, malondialdehyde (MDA). Due to small volumes of semen collected, vitamin E levels were not measured in the semen.

4.2.2. Animal Husbandry

For the first 8 weeks (commencing in early May), birds were housed in groups of 30 in indoor pens (1.8m × 1.5m) under infra red heat lamps within a semi-intensive brooder hut system (GCT 2004), with lighting levels limited to the light emitted by the heat lamps and windows painted red to minimise light entering the pens. Additional birds were reared and introduced to maintain experimental rearing densities during the first 8 weeks following 8 mortalities. After 2 weeks, birds were

given daily access to outdoor pens with wire floors (3m × 1.5m). At 8 weeks of age, the birds were sexed and then transferred to one of two outdoor single-sex pens (30m × 27m) with access to grass for the remainder of the experiment.

4.2.3. Dietary Supplementation

All birds received a custom-made basal diet, based on standard commercial pheasant pellets, but with no added vitamin E, and low levels of vitamin A (10.0mg/kg) and selenium (0.20mg/kg) (Target Feeds Ltd., Shropshire). The basal diet was manipulated for each treatment group as follows: Carotenoid supplemented birds received 100 mg carotenoids in the form of ORO-GLO® brand 11 liquid pigmenter (lutein and zeaxanthin, 20:1 w/w) (Kemin industries Inc.) per kg of feed; birds supplemented with vitamin E received supplemental all-trans α-tocopherol (Sigma-Aldrich T36634) at a concentration of 100mg/kg of feed; birds receiving both carotenoids and vitamin E received 50mg per kg of feed; control birds received an unsupplemented diet. The concentration of vitamin E and carotenoids supplemented was consistent with concentrations (100mg/kg) used in previous studies on poultry (vitamin E: Bartov and Frigg 1992, Guo et al. 2001; carotenoids: Woodall et al. 1996, Surai et al. 2001). Supplements were added to the feed daily, by spraying with a 5 litre spray pump. α-Tocopherol was sprayed in soybean oil onto the feed and ORO-GLO® xanthophylls were mixed in water and stored in refrigerated vacuum pumped containers until given to the birds. Soybean oil was selected as a medium for α-tocopherol supplementation because it contains comparatively low levels of α-tocopherol (0.07μg/mg) compared to other naturally occuring oils (Carpenter 1979). Equal volumes of soybean oil and water were sprayed onto the other feeds. Each afternoon the feed was replenished with fresh refrigerated treatment feed stored in vacuumed pumped containers. Four diets were provided over the 8 week period of supplementation, all with medium levels of protein, in line with standard pheasant rearing practice (starter crumb 1-2 weeks: 29.8% protein, starter pellets 3-4 weeks: 25.5% protein, rearer pellets 5-6 weeks: 21.4% protein, grower pellets 7-8 weeks: 18.1% protein). Feed, grit and water were provided *ad libitum*.

4.2.4. Morphometric measurements

The morphometric measurements of individuals were recorded each week up to 10 weeks of age and at 21, 22, 36, 37, 46 and 47 weeks of age. Body mass was measured

using a pesola® spring balance (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 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. 2002).

4.2.5. Semen and testes sample collection

Immediately post-mortem semen samples were collected using a pipette from the distal end of the *vas deferens* following dissection. Ten microliters of semen were immediately diluted in 200µl of Dulbecco's modified Eagle medium (Invitrogen Ltd) and 15µl of the diluted sample was placed under a phase-contrast microscope on a heated stage (40°C) and video recorded at 200× magnification (Lüpold et al. 2009). The remainder of the undiluted semen samples were collected for lipid peroxidation analysis. Testes were removed and the length (*L*), width (*W*) and height (*H*) of each testis was measured using a sliding calliper (±0.01mm). These measurements were used to calculate the volume of each testis as the volume of a regular ellipsoid ($1/6\pi \times L \times W \times H$). The mass of each testis was measured (±0.01g). Sections of testes were taken for laboratory analysis of vitamin E concentration. All samples were immediately snap-frozen in liquid nitrogen and stored at –80°C.

4.2.6. Vitamin E concentration in the testes

α-Tocopherol was measured within a month using high-performance liquid chromatography (HPLC). Samples (150mg) of the left testis were saponified in the presence of ice before 5ml of ethanolic pyrogallol 5% solution (Sigma-Aldrich®) was added and the samples were homogenised for 30 seconds. For saponification 0.4ml of 50% aqueous KOH was added and the vial was capped under nitrogen. Samples were heated in a water bath (Clifton NE-14) at 70ºC for 30 min, and then chilled on ice for 5 min. Five millilitres of hexane (Sigma-Aldrich-HPLC grade) were added and the sample was gently rocked for 1 min followed by the addition of 10ml of Milli-Q water and further gentle rocking (1 min). Samples were then centrifuged for 4 minutes at 1000 × *g* rpm. Three millilitres of the non-aqueous layer were removed and dried under a vacuum. The sample was then redissolved in 0.5ml methanol and 10µl injected into for HPLC (Dionex Corporation) analysis. α-Tocopherol plasma

concentrations were measured using a 3µm 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 v/v) with 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.

4.2.7. Lipid peroxidation of the semen

Semen samples were placed in a water bath (with ice) and sonicated (Grant Ultrasonic bath) for 10 minutes before homogenisation with a motorised pestle for one minute. Samples were then centrifuged at 13,000 rpm for 4 min at 4ºC. Butylated hydroxytoluene (BHT) solution 5µl (0.05% w/v in 95% ethanol), 40µl of phosphoric acid (0.44*M*) and 10µl of 2-thiobarbituric acid (TBA) was added to 5µl of sample. The solution was vortexed for 5s and heated in a dry bath incubator for 1hr at 100ºC. Samples were then cooled in ice for 5 minutes. *n*-butanol (80µl; HPLC grade) was added to each sample and the mixture was vortexed for 20s and centrifuged for 3 min at 4°C (12,000 rpm) to separate the MDA-TBA complex phase. A 20µl aliquot of the *n*-butanol phase was injected into the HPLC and measured using fluorescence detection. Potassium monobasic phosphate (50mM) with a pH of 6.8 (adjusted using 5M potassium hydroxide) was mixed with methanol (HPLC grade) 60:40 v/v and filtered to form the mobile phase. A flow rate of 1.1ml per minute was used with a Hewlett-Packard Hypersil 5µm ODS 100 × 4.6 mm column and 5µ ODS guard column to determine concentrations of sample MDA. Chromeleon software produced chromatograms (version 6.80 © 1994-2007 Dionex). Known concentrations of MDA were then used to calculate a standard curve.

4.2.8. Sperm motility and sperm number

Videotapes of semen samples were analysed using computer-aided sperm analysis (Hobson Tracking Systems Ltd. UK). Sperm motility was then analysed using principal components analysis on three descriptors of sperm performance: (i) curvilinear, (ii) average-path and (iii) straight-line velocities. This analysis collated information from the speed parameters (i–iii) into a principal component (Lüpold et al. 2009). Sperm concentration in the semen was measured by completing counts of sperm present in the semen from videotape recordings. Five counts were used per

individual at randomly selected times of the video footage to produce a mean sperm count.

4.2.9. Wattle colour measurement and size measurements

An image of the right wattle at 47 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 60 Fourier coefficients were then used as variables in principal component analyses. Two principal components that described over 95% of the wattle shape variation (PC1 = 78%, PC2 = 20%) were used for analyses (South and Arnqvist 2009).

Wattle reflectance data were collected using a USB2000 UV-Visible spectrophotometer and OOIBase32 Software (Ocean Optics Inc., Dunedin, FL). 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 stabilise before capture (Keyser and 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 important in female mate choice (Hillgarth 1990). We calculated brightness as it would be perceived by a conspecific female, using the method detailed in Endler and Mielke (2005). In Galliforms, brightness is likely to be perceived by the double cones (Osorio et al. 1999). Because no data on photoreceptor spectral sensitivity have been collected for ring-necked pheasants, we used data for a closely-related pheasant 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). This template incorporates the transmittance spectra of the combined ocular media for peafowl (Hart 2002) and estimated oil droplet transmission spectra calculated using the equations of Hart and Vorobyev (2005) and data from Hart (2002). Since the birds were reared outdoors, a standard daylight-simulating illumination spectrum (D65) was used in the model (Wyszecki and Stiles 1982).

4.2.10. Statistical analyses

Statistical analyses were chosen to investigate factors that may increase levels of oxidative stress in primary sexual characteristics, to examine the effects of oxidative stress levels on primary sexual characteristics and to explore the role of oxidative stress as a potential mechanism linking fertility and ornament expression.

To examine the effect of diet during development on primary sexual traits and the relationship between the degree of ornament expression and primary sexual traits at adulthood, I used general linear mixed models (GLMMs) with supplementary treatment group, wattle size, wattle brightness, final body mass and spur length included as explanatory variables and the primary sexual trait as the response variable. Day-old individuals were delivered on two days and randomly assigned in equal numbers to treatment groups. Therefore, hatch date was included in all models as a random effect. To determine the role of diet during development on the levels of oxidative damage in the semen, I included final body mass, treatment diet and sperm number, MDA in the testes, mean sperm motility and all two-way interactions as fixed effects within a GLMM with concentration of MDA in the semen as the response variable. Sperm are motile within the *vas deferens* (Howarth 1983), so sperm motility measurements were included as an explanatory variable in the model with MDA in the semen as the response variable.

To examine the effects of diet and exposure of sperm to oxidative stress within the semen on determining sperm motility, we used GLMMs with mean sperm motility of either the total measured sperm population or the fastest 20% thereof as response variables and with supplementary diet, final body mass and MDA concentration in the

semen and all two-way interactions as fixed effects. A further model with sperm concentration, treatment diet and mass was included in a GLMM with concentration of MDA in the testes as the response variable.

Normality of the data was examined and variables were log-transformed where necessary. GLMMs were conducted using the *lme* function in the statistical package R version 2.9.2 (R Development Core Team 2009). Treatment was included as a single factor with 4 levels. For model simplification, I removed the highest order interactions, followed by lower order terms in turn from the maximal model using maximum likelihood tests. Body mass, spur length and wattle size were correlated. To avoid errors due to multicollinearity 2-way interactions between these variables were not used. Only significant interactions are presented in the tables. *Post hoc* pairwise comparisons were carried out using comparisons of the original GLMMs with models in which treatment groups were paired. Mass at 47 weeks of age was included in all models due to the higher growth rates and differences in final mass identified in CV individuals as part of the wider study (see **Chapter 3**).

4.3. Results

4.3.1. The effect of diet during development on primary sexual traits

Concentrations of α-tocopherol were higher in the testes of both V and CV birds compared to C or Cont diet birds but did not differ between V and CV or between C and Cont birds, respectively (**Table 4.1.**, **Figure 4.1.**). No carotenoids were found in the testes at 47 weeks of age. Testicular concentrations of MDA were higher in C and Cont birds compared to CV and V birds but did not differ between CV and V birds or between C and Cont birds, respectively (**Table 4.1.**, **Figure 4.2.**). Treatment diet over the first 8 weeks had no effect on the concentration of MDA in the semen sample at adulthood (**Table 4.1.**, **Figure 4.3.a**). Furthermore, antioxidant supplementation had no effect on combined testes mass or volume (**Table 4.2.**, **Figure 4.3.b**), sperm motility (**Table 4.3.**, **Figure 4.3.c**) or sperm concentration (**Table 4.3.**, **Figure 4.3.d**).

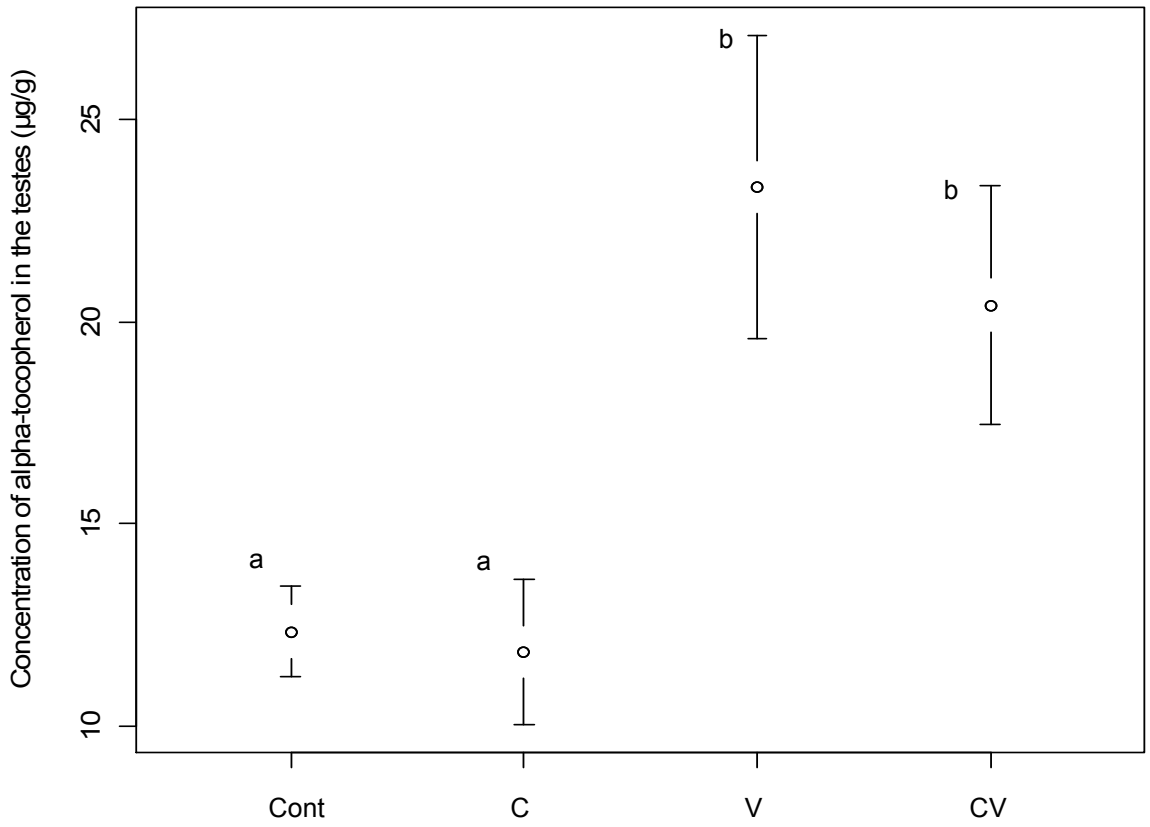

Figure 4.1.: Mean concentration of α-tocopherol in testes (µg/g) in relation to treatment at 47 weeks of age. Error bars show 95% confidence intervals. Means with identical letters do not differ significantly (p>0.05). Carotenoid supplemented (C n=22), α-tocopherol supplemented (V n=21), α-tocopherol with carotenoid supplemented combined (CV n=22) and a control diet (Cont n=21).

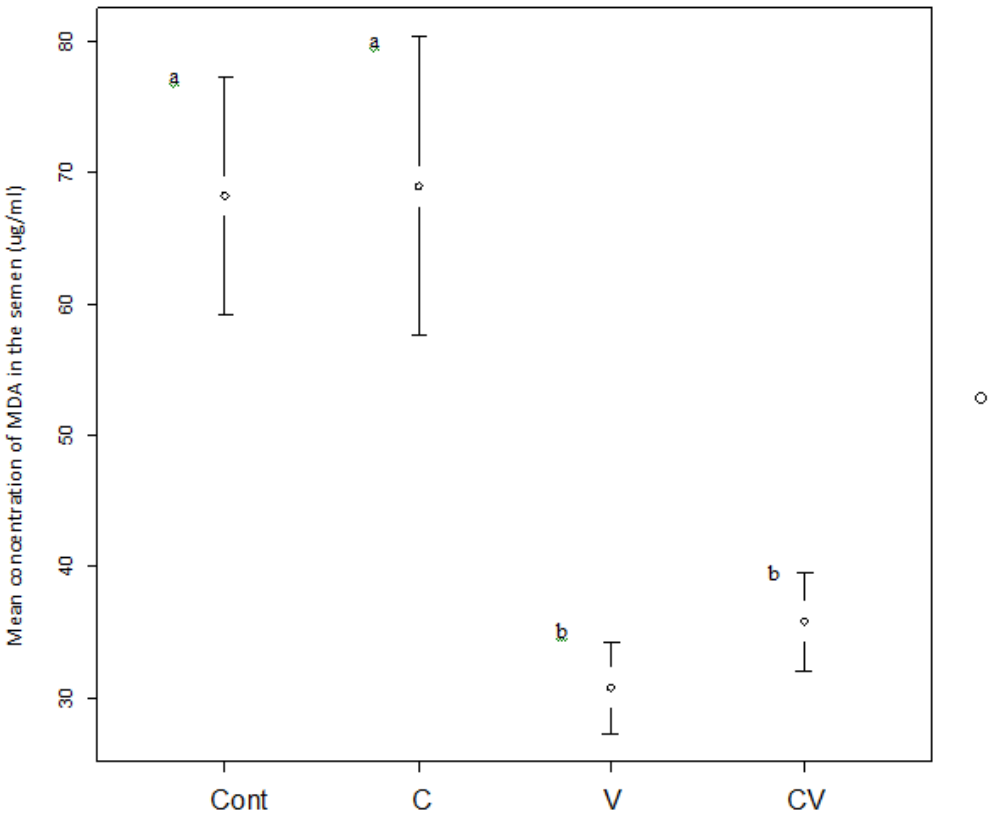

Figure 4.2.: Mean concentration of MDA in semen (µg/g) in relation to treatment at 47 weeks of age. Error bars show 95% confidence intervals. Means with the same letter do not differ significantly (p>0.05). Carotenoid supplemented (C n=22), α-tocopherol supplemented (V n=21), α-tocopherol with carotenoid supplemented combined (CV n=22) and a control diet (Cont n=21).

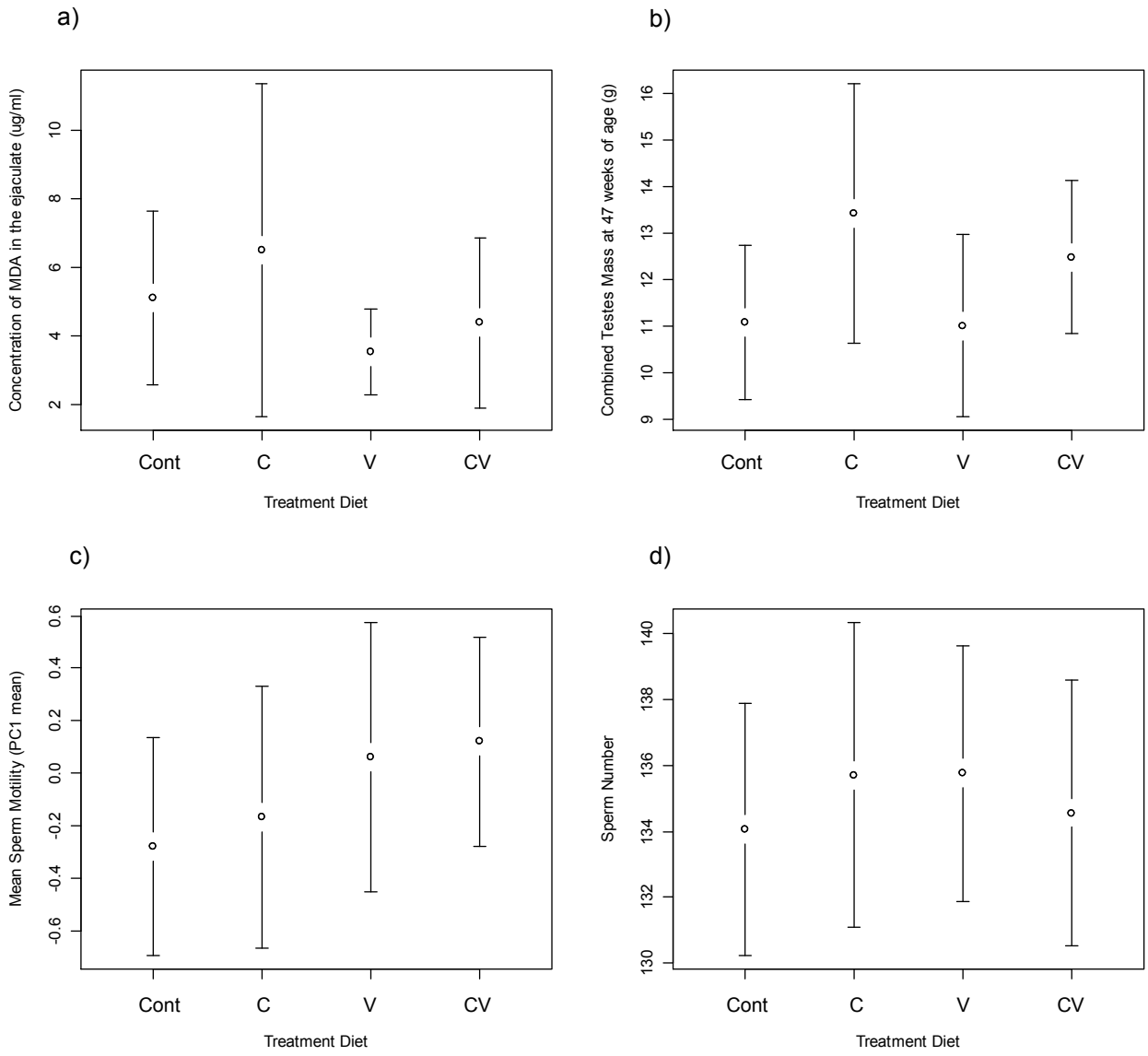

Figure 4.3.: Effects of treatment diet on a) Mean concentration of MDA in the semen b) Mean Combined Testes Mass c) Mean sperm motility and d) Mean Sperm concentration per frame at 47 weeks of age. Error bars show 95% confidence intervals. Carotenoid supplemented (C n=22), α-tocopherol supplemented (V n=21), α-tocopherol with carotenoid supplemented combined (CV n=22) and a control diet (Cont n=21).

Table 4.1.: Results of the GLMM models of vitamin E concentration of testes and MDA concentration of the semen. L.ratio denotes the maximum likelihood ratio. The slope and standard error of each variable is presented in the tables.

			Slope	SE	L.ratio	p-value
Concentration of α-tocopherol in the testes						
	Treatment				80.89	<0.001***
		Cont	4.1761	0.6181		
		C	4.1713	0.0668		
		V	3.3960	0.0618		
		CV	3.5487	0.0578		
	Final Mass		0.0006	0.0003	0.06	0.81
	Spur Length		-0.0071	0.0056	1.68	0.20
	Wattle Size		-0.0186	0.0388	0.29	0.59
	Wattle Brightness		0.3595	1.4527	0.07	0.79
Concentration of MDA in the testes						
	Treatment				47.71	<0.001***
		Cont	12.2971	1.6137		
		C	11.6995	1.7029		
		V	23.6672	1.6137		
		CV	20.5074	1.5471		
	Final Mass		0.0058	0.0052	1.15	0.28
	Spur Length		-0.0745	0.1197	0.41	0.52
	Wattle Size		0.2339	0.7259	0.12	0.72
	Wattle Brightness		12.9176	27.2217	0.27	0.60
Concentration of MDA in the semen						
	Treatment				1.42	0.70
		Cont	0.1569	1.1126		
		C	-0.0528	1.0999		
		V	-0.0536	1.0836		
		CV	-0.1518	1.0973		
	Final Mass		0.0008	0.0008	1.19	0.28
	Spur Length		0.0076	0.0200	0.17	0.68
	Wattle Size		0.1160	0.1183	1.13	0.29
	Wattle Brightness		3.2381	4.4987	0.58	0.45

Table 4.2.: Results of the GLMM models of combined testes mass and volume. The slope and standard error of each variable is presented in the tables. The slope and standard error of each variable is presented in the tables.

			Slope	SE	L.ratio	p-value
Combined Testes Mass						
	Treatment				2.86	0.41
		Cont	13.6282	4.7216		
		C	14.8418	4.6679		
		V	13.5349	4.5985		
		CV	15.0464	4.6569		
	Final Mass		-0.0019	0.0033	0.33	0.53
	Spur Length		0.0045	0.0867	0.03	0.95
	Wattle Size		0.3352	0.5115	0.51	0.48
	Wattle Brightness		12.2092	19.1932	0.45	0.50
Combined Testes Volume						
	Treatment				1.59	0.66
		Cont	-3870.3061	5642.6117		
		C	-2716.4182	5575.5661		
		V	-3110.2651	5432.1542		
		CV	-2646.9243	5472.4024		
	Final Mass		3.1657	1.8571	1.89	0.17
	Spur Length		-103.4951	74.4130	2.17	0.14
	Wattle Size		832.1236	356.5150	4.83	0.28
	Wattle Brightness		-3930.731	18175.5801	0.05	0.82

Table 4.3.: Results of GLMM models of sperm motility and sperm number. The slope and standard error of each variable is presented in the tables.

			Slope	SE	L.ratio	p-value
Sperm Concentration						
	Treatment				1.18	0.97
		Cont	155.1023	11.2312		
		C	156.1895	11.1330		
		V	156.0919	10.9674		
		CV	155.1984	11.1067		
	Final Mass		-0.0159	0.0078	3.88	0.14
	Spur Length		0.0412	0.1972	0.05	0.82
	Wattle Size		0.8995	1.1565	0.66	0.42
	Wattle Brightness		-57.8777	43.7015	2.05	0.15
Sperm motility (mean of all sperm)						
	Treatment				2.45	0.48
		Cont	-0.0733	0.3795		
		C	0.0783	0.3831		
		V	0.2916	0.3792		
		CV	0.3512	0.3395		
	Final Mass		0.0005	0.6059	0.27	0.61
	Spur Length		-0.0080	0.0079	1.31	0.25
	Wattle Size		0.1437	0.1335	1.36	0.24
	Wattle Brightness		3.5207	5.2126	0.57	0.45
Sperm motility (mean of top 20%)						
	Treatment				12.18	0.16
		Cont	0.5747	0.2112		
		C	-0.4341	0.2282		
		V	0.1004	0.2132		
		CV	-0.2300	0.1976		
	Final Mass		-0.0009	0.2675	1.23	0.27
	Spur Length		0.0039	0.8392	0.04	0.83
	Wattle Size		0.1943	0.1224	2.64	0.10
	Wattle Brightness		2.9776	4.7790	0.46	0.50

4.3.2. The relationship between primary and secondary sexual traits

There was no relationship between wattle brightness, wattle size or spur length and final body mass nor were there any interactions with treatment diet on concentration of vitamin E in the testes, concentration of MDA in the semen (**Table 4.1.**), testes mass or volume (**Table 4.2.**), sperm concentration or sperm motility (**Table 4.3**.).

4.3.3. The effects of oxidative damage on primary reproductive traits

The concentration of MDA in the semen was not associated with sperm number, treatment diet or final body mass (**Table 4.4.**), and there was no interaction between these traits on the concentration of MDA in the semen. In addition, I found that the concentration of MDA in the semen was not affected by the testicular MDA concentration, treatment diet or final body mass and there was no interaction between these traits on concentration of MDA in the semen (**Table 4.4.**). There was no significant relationship between the concentration of MDA in the semen, treatment diet and their interaction on mean sperm motility or the motility of the fastest 20% of the measured sperm population. There was also no significant relationship between the concentration of MDA in the testes, treatment diet and their interaction on sperm motility or sperm concentration (**Table 4.4.**).

Table 4.4: Results of GLMM models of MDA concentration in the semen and primary reproductive traits

			Slope	SE	L.ratio	p-value
Concentration of MDA in the semen	Treatment				3.41	0.27
		Cont	1.1243	1.2789		
		C	0.6953	1.2901		
		V	1.0078	1.1071		
		CV	0.7978	1.1482		
	Final Mass		-0.0002	0.0005	0.36	0.52
	MDA in the testes		1.7496	0.6037	0.04	0.94
	Sperm number		1.0419	1.0044	1.32	0.12
	Sperm motility		-0.9362		0.46	0.20
Mean Sperm Motility						
	Treatment				2.38	0.43
		Cont	-0.3024	0.2493		
		C	-0.1101	0.2363		
		V	0.0608	0.2564		
		CV	0.1441	0.2421		
	Final Mass		0.0015	0.0008	0.24	0.58
	MDA in the semen		0.0329	0.1671	0.08	0.81
	MDA in the testes				0.07	0.64
Sperm Motility (top 20%)						
	Treatment				4.28	0.23
		Cont	0.1542	0.1567		
		C	0.1245	0.1645		
		V	0.0841	0.0942		
		CV	0.0994	0.0854		
	Final Mass		-0.0002	-0.0005	0.84	0.36
	MDA in the semen		0.0140	0.1485	0.01	0.92
Sperm Number	Treatment				0.97	0.81
		Cont	93.8945	120.3041		
		C	95.2193	120.7391		
		V	93.2879	119.9519		
		CV	91.9335	120.0545		
	Final Mass		-0.0141	0.0080	3.20	0.07
	MDA in the testes		0.0004	0.0008	4.18	0.24

4.4. Discussion

The results showed significant effects of vitamin E supplementation on concentration of vitamin E in the testes and the concentration of MDA in the testes. These results suggest that vitamin E levels can be a limiting factor in the allocation of antioxidant resources to testes to reduce oxidative damage but that the lower concentrations of antioxidants does not appear to result in adverse effects on semen quality. To investigate the functional importance of antioxidants during development this study measured a range of primary and secondary sexual traits. The results indicated that the greater allocation of vitamin E to testes had no effect on key primary reproductive traits, including sperm concentration, sperm motility or testes size. In addition, in contrast with numerous recent studies (Merilä and Sheldon 1999; Pilastro et al. 2002; Peters et al. 2004; Malo et al. 2005; Locatello et al. 2006; Pitcher et al. 2007; Helfenstein et al. 2010), and despite the measurement of both morphological and carotenoid-mediated sexual signals and a wider range of primary sexual traits, there was no evidence for a role of oxidative damage as a potential mechanism to link ornament expression and male reproductive quality.

Supplementation with vitamin E during development resulted in higher concentrations of vitamin E in the testes at sexual maturity. These findings support previous experiments in the domestic fowl by Surai et al. (1997, 1998), where testicular vitamin E concentrations were found to be highly responsive to supplementation for a 2-month period between 10 weeks and 6 months of age. Our study further indicates that a short window of vitamin E supplementation during development, in this case even earlier (for 8 weeks post-hatch), can have sustained effects on concentrations in the testes at adulthood. In zebra finches, neonatal nutrition had a long-term effect on the capacity of individuals to assimilate α-tocopherol in adulthood (Blount et al. 2003). In ring-necked pheasants, supplementation with α-tocopherol for 8 weeks post-hatch resulted in higher circulating α-tocopherol levels at adulthood (**chapter 3**). These results indicate that the lipoproteins required to assimilate antioxidants are primarily produced early in life (Blount et al. 2003), which may have resulted in the higher α-tocopherol concentrations found in the testes of males that had received vitamin E during development.

However, despite the presence of elevated α-tocopherol concentrations and reduced oxidative stress in the testes at adulthood, oxidative damage in the semen at adulthood did not differ depending on antioxidant supplementation. Previous vitamin E supplementation studies have documented a reduction in lipid peroxidation in the semen at adulthood in a range of species, including wild boar *Sus scrofa* (Brezenzinska-Slebodzinska et al. 1995), rabbits *Oryctolagus cuniculus* (Castellini et al. 2003; Yousef et al. 2003), humans (Suleiman et al. 1996), Arctic char, *Salvelinus alpinus* (Mansour et al. 2006) and domestic fowl (Surai et al. 1997; Danikowski et al. 2002). However, these studies measured lipid peroxidation during or immediately after antioxidant periods of supplementation. Our results indicate that early antioxidant supplementation during development does not affect lipid peroxidation levels in semen at adulthood, suggesting that vitamin E availability is not a limiting factor. Carotenoid (lycopene) dietary supplementation in humans results in increased carotenoid concentrations in the semen but not in antioxidant capacity (Goyal et al. 2007). Small concentrations of carotenoids have been found in avian seminal fluid (Rowe and McGraw 2008). However, while we did not measure carotenoid concentration in the semen, we did not observe the presence of carotenoids in the testes or a reduction in lipid peroxidation in the testes following early carotenoid supplementation, providing support for a limited role of carotenoids in protecting the semen from the effects of oxidation.

One limitation of this study is that the concentration of vitamin E in the semen could not be measured due to the small volumes of semen samples available. However, oxidative damage was significantly lower in the testes of individuals that received vitamin E and the results indicate that increased levels of oxidative damage resources are likely to be preferentially allocated to the semen.

Antioxidant supplementation during development had no affect on testes size at adulthood in this study. This contrasts with a study that supplemented vitamin E in Japanese quail (*Coturnix coturnix japonica*) from 5 weeks of age to 10 weeks of age at similar concentrations (75μg per kg of feed and 150μg per kg feed respectively) our experiment and found a significant increase in testes mass with vitamin E supplementation (Hooda et al. 2007). However, Biswas et al. (2007) found no such effect in the same species if males were supplemented with 150μg per kg of feed from

1 day old to 25 weeks of age. These conflicting results suggest that the period of supplementation during development may be critical and a developmental window during spermatogenesis may exist during which higher vitamin E can affect testes mass. Kirkpatrick and Andrews (1944) described the main period for testes development in ring-necked pheasants as approximately between day 236 (average testes mass 0.21g) and day 334 (average testes mass 10.40g) post-hatch. The first phase from post-hatch until between day 56 and day 81 was described as very gradual growth (from 0.03g to 0.31g) and the presence of only seminiforous tubules and a few scattered primary spermatocytes in the developing testes (Kirkpatrick and Andrews 1944). It therefore appears that despite the supplementation of antioxidants at such an early stage, the greater concentrations of vitamin E in the testes and greater circulating vitamin E concentrations (**chapter 3**) measured that there is no effect on sperm quality in pheasants.

In this study, we found no correlations between sperm motility and oxidative damage in the semen despite previous observations that ROS-induced lipid peroxidation of the sperm membrane can decrease the flexibility of the sperm and reduce the tail motion, and that damage to mitochondria can decrease the energy availability to power motility (Tremellen 2008). In addition, we found no relationship between oxidative damage and primary sexual traits to indicate a role for oxidative stress in determining the fertility of individuals or for the expression of secondary sexual traits in influencing the allocation of antioxidants and therefore indirectly affecting the susceptibility of semen to oxidative stress.

Studies testing the phenotype-linked fertility hypothesis have produced inconsistent results. In greenfinches, *Carduelis chloris*, individuals with brighter plumage exhibit larger testes, which may be related to the intensity of sperm competition resulting from extra-pair copulations (Merilä and Sheldon 1999). Sperm competition should select for greater sperm production if the fertilisation success of a male is proportional to the relative number of sperm inseminated. Therefore, sperm competition could drive the evolution of testes size because males producing more sperm have higher fertilisation success (Hosken and Ward 2001). High levels of extra-pair paternity (EPP) are also observed in great tits that show a relationship between carotenoid-mediated plumage colouration and lipid peroxidation in the semen (Helfenstein et al.

2010). However, the sedge warbler, which also exhibits a high incidence of EPP, showed no relationship between song complexity, number of sperm, proportion of abnormal sperm but experiences a trade-off of with immune response (Folstad and Karter 1992). In the ring-necked pheasant, sperm competition may be less important as their mating system has been described as territorial defence polygyny, and females are believed to be monandrous (Hill and Robertson 1988), however territorial males are known to guard mates against harassment by non-territorial males. Sperm quality fluctuated rapidly following social challenge in domestic fowl (Pizzari et al. 2007). Sperm quality dropped in males that won two social challenge contests when paired with other males in contact with females. However, sperm quality remained constant in individuals that lost both contests, suggesting that there is a trade-off between pre- and post- reproductive traits (Pizzari et al. 2007). During the current experiment males were housed together in one single-sex pen from which the females were visible. Aggressive interactions between males were frequently observed and therefore it is possible that the dominance of males could have resulted in rapid fluctuations in the parameters that we measured. The degree of extra-pair paternity in ring-necked pheasants has not yet been documented and if females are monandrous, pre-copulatory sexual selection might be more important than post-copulatory sexual selection and male investment in ornaments might be more important than traits related to sperm competition. In addition, the study by Pizzari et al. (2007) indicates that sperm parameters can fluctuate rapidly (within 14 days). Therefore, spermatogenesis may respond to environmental fluctuations too quickly unless there are long-term effects of dietary availability during development.

We found no relationship between the degree of male ornamentation following the measurement of both morphological and carotenoid-mediated sexual traits and testes mass/volume, semen lipid peroxidation, sperm motility or number to suggest honest signalling of fertility. However, it is possible that the relationship between male quality and ornament expression in males is more pronounced during the second breeding season. For example, spur length in ring-necked pheasants is known to be the most important predictor of harem size, but continues to grow throughout the second year of life (Göransson et al. 1990). Consequently, spur length at one year of age has less influence on female mate choice than the spur length of older males (Grahn and von Schantz 1994). In addition, we found no greater effect of the combined supplementation of carotenoids and vitamin E on ornament expression and

primary sexual traits to provide support for the 'carotenoid protection theory' or an effect of growth rate on primary sexual traits at adulthood. However, despite a large body of research on the honesty of secondary sexual ornaments it is still unclear what information is being transmitted.

In conclusion, the results show that vitamin E availability during early development affects antioxidant defences in the testes in adulthood and that carotenoids appear to have no role in shaping the primary reproductive quality of males. There was also no evidence for a synergistic effect of carotenoids and vitamin E on primary sexual traits. Despite the measurement of a wide range of traits there was no evidence for oxidative damage as a proximate link between ornament expression and male primary reproductive characteristics.

4.5. References

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Chapter 5: Antioxidant supplementation during early development reduces parasite load but does not affect sexual ornament expression in adult ring-necked pheasants

5.1. Introduction

Females in many animal species prefer to mate with the most elaborately ornamented males (Andersson and Simmons 2006). In species in which males contribute nothing beyond their genes (Kirkpatrick and Ryan 1991; Andersson 1994) females are expected to choose mates based on 'indirect benefits' (Borgia 1979; Reynolds and 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 and 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 and Poulin 1997). Experiments with controlled infections show that sexual ornaments are more sensitive to parasite infection than other morphological traits (Zuk et al. 1990; Houde and 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 and Zuk 1982). Tests of Hamilton and Zuk's idea have produced equivocal results however (Hamilton and 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 variation in 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 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 (Blount et al. 2003). Carotenoids are dietary derived, highly pigmented antioxidants that have immuno-enhancing properties (McGraw and 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 and Bakker 1990; Zuk et al. 1990; Houde and Torio 1992; Thompson et al. 1997; Brawner et al. 2000; McGraw and Hill 2000; Baeta et al. 2008; Mougeot et al. 2010). As a result of the multiple properties of carotenoids organisms may experience a trade-off between allocating carotenoid resources to immune function and allocation to colourful display (Blount et al. 2003). 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 diminish 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 (von Schantz et al. 1999), 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 et al. 2009; Hall et al. 2010). Under this scenario early exposure to parasite infection can be viewed as a contributory factor influencing the oxidative status of individuals, 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 and Møller 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 and 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 and Kennedy 2004). The majority of studies testing the carotenoid protection theory and the effects of antioxidants on sexually selected traits have been conducted on adults (e.g. Bertrand et al. 2006; Pike et al. 2007; Pérez et al. 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 exposure to antioxidants can determine the ability to assimilate and metabolise antioxidants in adulthood (Kim et al. 1996; Blount et al. 2003; Koutsos et al. 2003) for example, and somatic growth results in the production of higher levels of ROS (Stoks et al. 2006). As a result the availability of dietary antioxidants, and the degree of environmental insult (e.g. exposure to parasite infection) can 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* as a model 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 and 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 and Robertson 1988) and females have been shown to prefer males with larger wattles (Hillgarth 1990).

Body mass has also been found to be an important determinant of success in mating (Göransson et al. 1990). An increased number of studies have identified the role of multiple cues by females 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 as potential signal components in mate choice. 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 within the caecum (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 increase 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 underlying mechanism behind trade-offs during development then males supplemented with dietary antioxidants will be able to trade-off lower costs of self-maintenance (oxidative damage) by increasing resource allocation to sexually selected traits. 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.

5.2. Materials and methods

5.2.1. General methods and experimental design

240 day old ring-necked pheasants of mixed genetic stock (Holme Farm Hatcheries, Wokingham) were allocated randomly to one of four treatment groups 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 encounter 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) α-tocopherol supplement with addition of *Heterakis* nematode parasites (P-VitE) (2) α-tocopherol supplement without parasites (NP-VitE) (3) control diet with *Heterakis* parasites (P-Ctrl) (4) control diet without parasites (NP-Ctrl). An 8 week period of dietary manipulation was chosen to include the early developmental window identified by previous studies on pheasants (Ohlsson and 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 and Quinn 1999), oxidative stress was measured by assay of the concentration of a biomarker of lipid peroxidation, malondialdehyde (MDA). 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. Previous studies have shown that the expression of ornaments is responsive to dietary quality manipulation during development (Ohlsson et al. 2001) and in adulthood (Smith et al. 2007) when young adult males are a year old.

5.2.2. 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.

5.2.3. Dietary Supplementation

The treatment diets were (1) α-tocopherol supplemented (100mg/kg) (Sigma-Aldrich T36634) with *Heterakis* infection (2) α-tocopherol supplemented 100mg/kg with no infection (3) control diet with *Heterakis* infection and (4) a control diet with no infection. 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 and Frigg 1992). Supplements were added to a basal diet made to specification with no introduced vitamin E, low levels 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 (treatments 1 and 2) - α-tocopherol 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 α-tocopherol 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 (treatments 3 and 4). 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 protein availability masking any effects of antioxidant supplementation (Orledge et al. *unpublished*). 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).

5.2.4. 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 and 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).

5.2.5. Morphometric measurements

The morphometric measurements of individuals were recorded each week up to 10 weeks of age and at 21, 22, 36, 37, 46 and 47 weeks of age. Mass at 0, 8, 21 and 47 weeks was used to indicate growth in statistical analyses. 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).

5.2.6. Measurement of plasma antioxidants and oxidative stress

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 20µl of plasma. The mixture was vortexed for 10s and heated in a dry bath incubator for 1hour at 100ºC. Samples were then cooled in 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 the HPLC and measured using fluorescence detection. The mobile phase was 50mM potassium monobasic phosphate (pH 6.8 adjusted using 5M potassium hydroxide) mixed with methanol (HPLC grade) 600:400ml. A flow rate of 1.1ml per minute was used with a Hewlett-Packard Hypersil 5µm ODS 100 x 4.6 mm column and 5µ ODS guard column. Known concentrations of MDA and α-tocopherol were then used to calculate standard curves (Hall et al. 2010).

5.2.7. 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 stabilise before capture (Keyser and 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 and 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 and Stiles 1982).

5.2.8. 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. . Five principal components that described over 98% of the wattle shape variation (PC1 = 42%, PC2 = 20%, PC3= 14%, PC4= 12%, PC5= 10%) were used for analyses (South and Arnqvist 2009).

5.2.9. 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™ needles (Fisher Scientific UK Ltd.) and BD Plastipak™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 then measured using callipers (0.01mm) directly after injection. 24 hours (± 10 minutes) after the injection the thickness of the patagium of the wings was measured. . The thickness measurement of the left-wing patagium was subtracted from the measurement taken from the right wing-web to identify the pro-inflammatory potential to PHA 24 hours after exposure (Vinkler et al. 2010).

5.2.10. Statistical analyses

Normality checks were carried out in SPSS (SPSS Inc., Chicago IL) and data was log-transformed where necessary. Data were analysed using general linear mixed models (GLMMs) with treatment group, sex and/or age included as fixed effects and hatch date as a random factor. Bird identification was included as a random effect in repeated measures analyses. Treatment was included as a single factor with 4 levels and sex as a factor with 2 levels. GLMMs were completed in R version 2.9.2 (© R Development Core Team 2009). Generali linear mixed models were tested using the *lme* function. All relevant 2-way 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 (Crawley 2008) to identify the minimum adequate model (MAM). Only measurements from birds that survived to a year of age were used in each model. Principal components produced using the coefficients calculated by elliptic fourier analysis of wattle shape and wattle size were used in multivariate analyses of covariance (MANCOVA) as dependent variables.

5.3. Results

5.3.1. 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. Males had a significantly higher mean parasitic burden than females in all treatment groups, but there was no sex*treatment interaction (**Figure 5.1.**, **Table 5.1.**). Individuals infected with parasites and given a control diet had more parasites at 47 weeks of age than individuals from other treatment groups (**Figure 5.1.**). Birds that were infected with parasites and supplemented with vitamin E had fewer parasites at 47 weeks than individuals that were infected and received a control diet (GLMM comparing P-Ctrl with P-VitE; L.ratio = 4.39 p=0.03). Control birds infected with parasites had a higher number of parasites at 47 weeks than those control birds that had not been infected (GLMM comparing P-Ctrl and NP-Ctrl, L.ratio = 5.11 p=0.02). There was no difference in the number of parasites at 47 weeks between individuals that received a vitamin E diet with parasites and vitamin E with no infection (GLMM comparing P-VitE and NP-VitE, L.ratio=2.83, p=0.09).

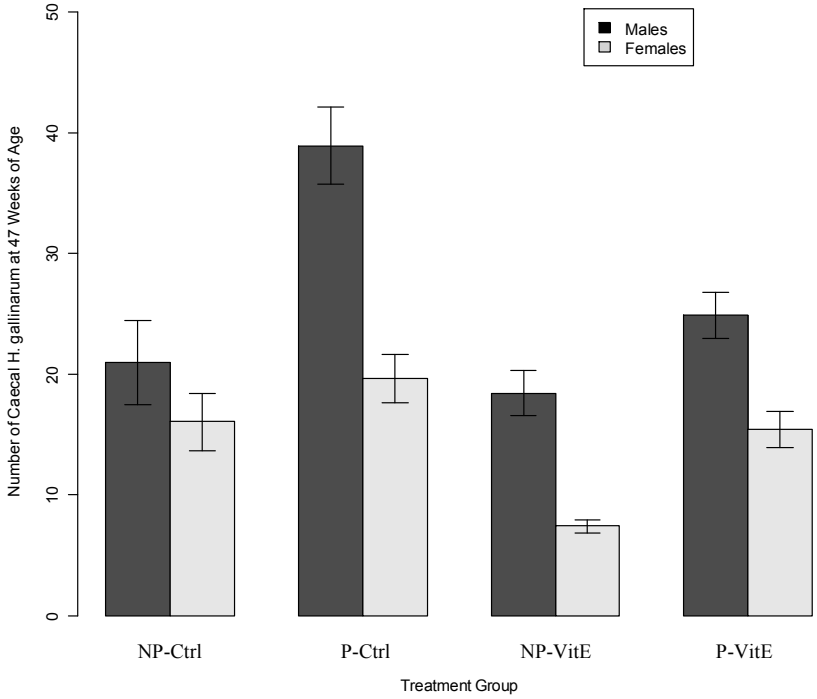

Figure 5.1.: Parasitic burden at 47 weeks of age in relation to sex and treatment group with 95% confidence intervals. α-tocopherol supplement with addition of *Heterakis* nematode parasites (P-VitE n=62), α-tocopherol supplement without parasites (NP-VitE n=58), control diet with *Heterakis* parasites (P-Ctrl n=55), control diet without parasites (NP-Ctrl n=57).

Table 5.1.: Results of GLMM models of parasitic burden at 47 weeks of age

	Explanatory term	L.ratio	p-value
Parasitic burden at 47 weeks of age	Treatment:Sex	2.65	0.45
	Treatment	14.83	**<0.01****
	Sex	13.07	**<0.001*****

Significance: ***P<0.0001**P<0.001*P<0.01'

5.3.2. Concentrations of plasma antioxidants

The concentration of α-tocopherols decreased from a mean across groups of 87.66 at 8 weeks to 2.59 μg/ml by 47 weeks of age, with the greatest decrease in those birds that received α-tocopherol in their diet up to 8 weeks of age (**Table 5.2.**, **Figure 5.2.**). Males in treatment groups that were supplemented with α-tocopherol had higher concentrations of plasma α-tocopherol at 8 weeks of age than males given a control diet (GLMM comparing treatment diets for males at 8 weeks of age: L.ratio = 59.32, p<0.0001). Plasma concentrations of α-tocopherol in birds that received a diet

supplemented with α-tocopherol up to 8 weeks of age remained significantly higher at 47 weeks than birds given a control diet (GLMMs comparing males at 47 weeks: P-VitE vs Ctrl groups: L.ratio = 22.24, p<0.0001, NP-VitE vs Ctrl groups: L.ratio = 37.23 p<0.0001). Infection with parasites did not affect the concentration of α-tocopherols in the plasma at 8 or 47 weeks of age, and males did not differ from females in the concentrations of α-tocopherol circulating in the plasma at 8 weeks of age (see Table 1). There were no effects of treatment, age (**Table 5.2.**, **Figure 5.3.**) or sex on the concentrations of carotenoids circulating in plasma (see Table 5.2).

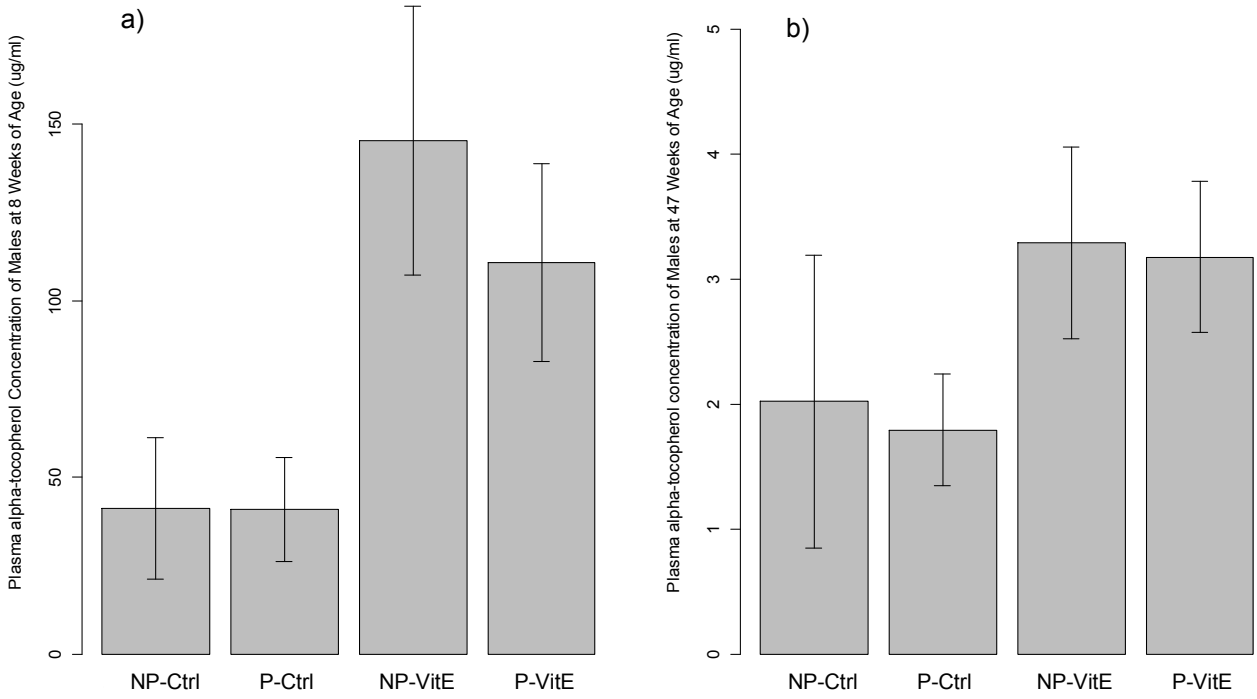

Figure 5.2.: Plasma α-tocopherol concentrations (µg/ml) in relation to treatment and age at 8 a) and 47 b) weeks of age with 95% confidence intervals. Note that scales differ considerably between 8 and 47 weeks of age. α-tocopherol supplement with addition of *Heterakis* nematode parasites (P-VitE n=62), α-tocopherol supplement without parasites (NP-VitE n=58), control diet with *Heterakis* parasites (P-Ctrl n=55), control diet without parasites (NP-Ctrl n=57).

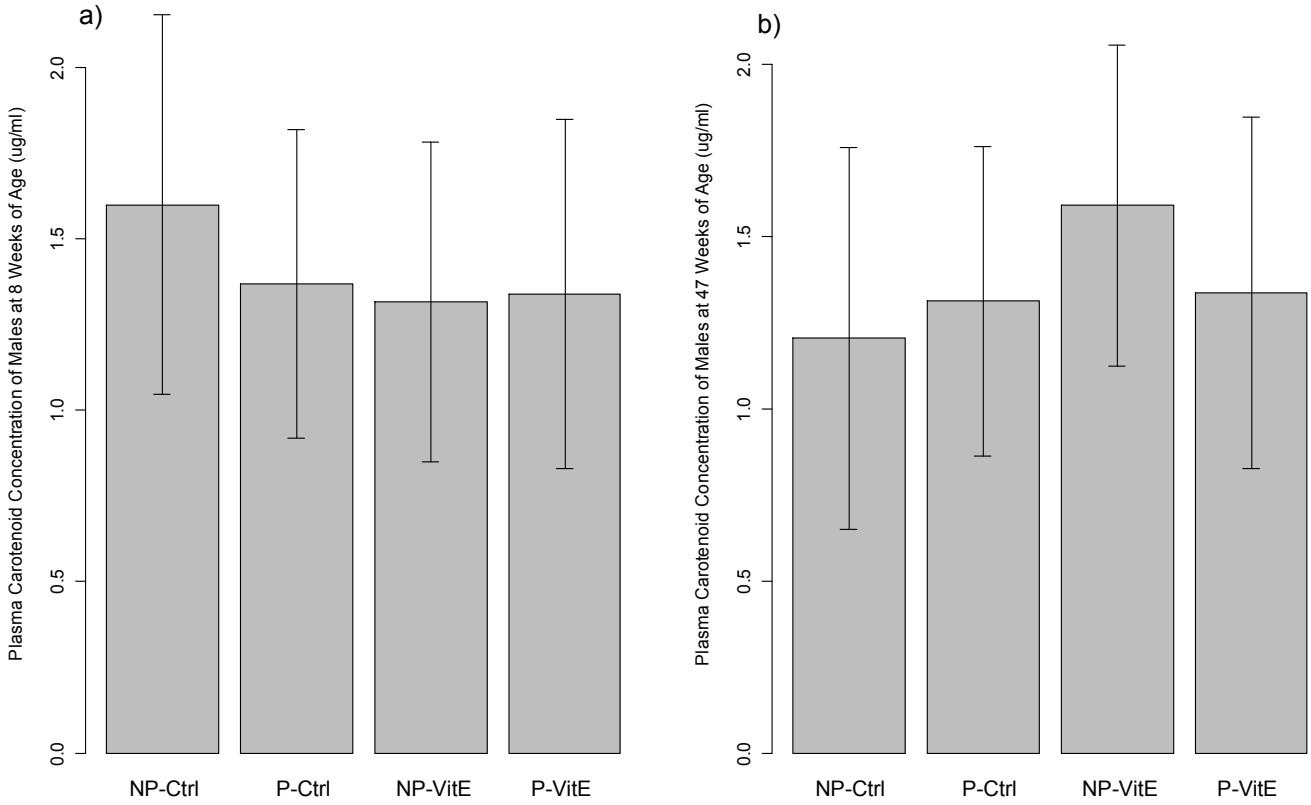

Figure 5.3.: Plasma carotenoid concentrations (µg/ml) in relation to treatment and age at 8 a) and 47 b) weeks of age with 95% confidence intervals. Scales differ at 8 and 47 weeks of age. α-tocopherol supplement with addition of *Heterakis* nematode parasites (P-VitE n=62), α-tocopherol supplement without parasites (NP-VitE n=58), control diet with *Heterakis* parasites (P-Ctrl n=55), control diet without parasites (NP-Ctrl n=57).

Table 5.2.: Results of GLMM models of plasma α-tocopherol and carotenoids in males at 8 and 47 weeks of age

	Explanatory term	L.ratio	p-value
Plasma concentration of α-tocopherol of males at 8 and 47 weeks of age	Treatment:Age	132.34	**<0.001*** **
	Treatment	85.74	**<0.001*** **
	Age	206.65	**<0.001*** **
Plasma concentration of α-tocopherol for males and females at 8 weeks	Treatment:Sex	0.94	0.81
	Treatment	223.53	**<0.0001*** **
	Sex	3.122e-07	1.00
Plasma concentration of carotenoids of males at 8 and 47 weeks of age	Treatment:Age	7.32	0.62
	Treatment	1.29	0.73
	Age	0.03	0.86
Plasma concentration of carotenoids of males and females at 8 weeks	Treatment:Sex	2.35	0.50
	Treatment	5.59	0.13
	Sex	0.10	0.75

Significance: ***P<0.0001**P<0.001*P<0.01'

5.3.3. Oxidative Stress

The concentration of MDA in plasma did not differ between males and females (**Table 5.3.**), 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; see **Figure 5.4.**). Birds given a control diet and infected with parasites had a higher concentration of MDA at 8 weeks than birds in other treatment groups (GLMM comparing the P-Ctrl to other treatments: NP-Ctrl: L.ratio=6.58 $p<0.01$, NP-VitE: L.ratio=10.15 $p<0.001$, P-VitE: L.ratio=7.46 $p<0.01$). However, by 47 weeks there were no differences in plasma MDA concentrations among treatment groups (GLMM with birds at 47 weeks from all treatments: L.ratio = 5.81 $p=0.12$).

Table 5.3.: Results of GLMM models of plasma MDA in males at 8 and 47 weeks of age

	Explanatory term	L.ratio	p-value
Plasma concentration of MDA of males at 8 and 47 weeks of age	Treatment*Age*Sex	1.24	0.74
	Age*Sex	0.25	0.62
	Treatment*Sex	2.51	0.47
	Treatment*Age	18.51	**<0.001*****
	Treatment	11.42	**<0.01****
	Age	251.11	**<0.001*****

Significance: ***p<0.001 **p<0.01 *p<0.05

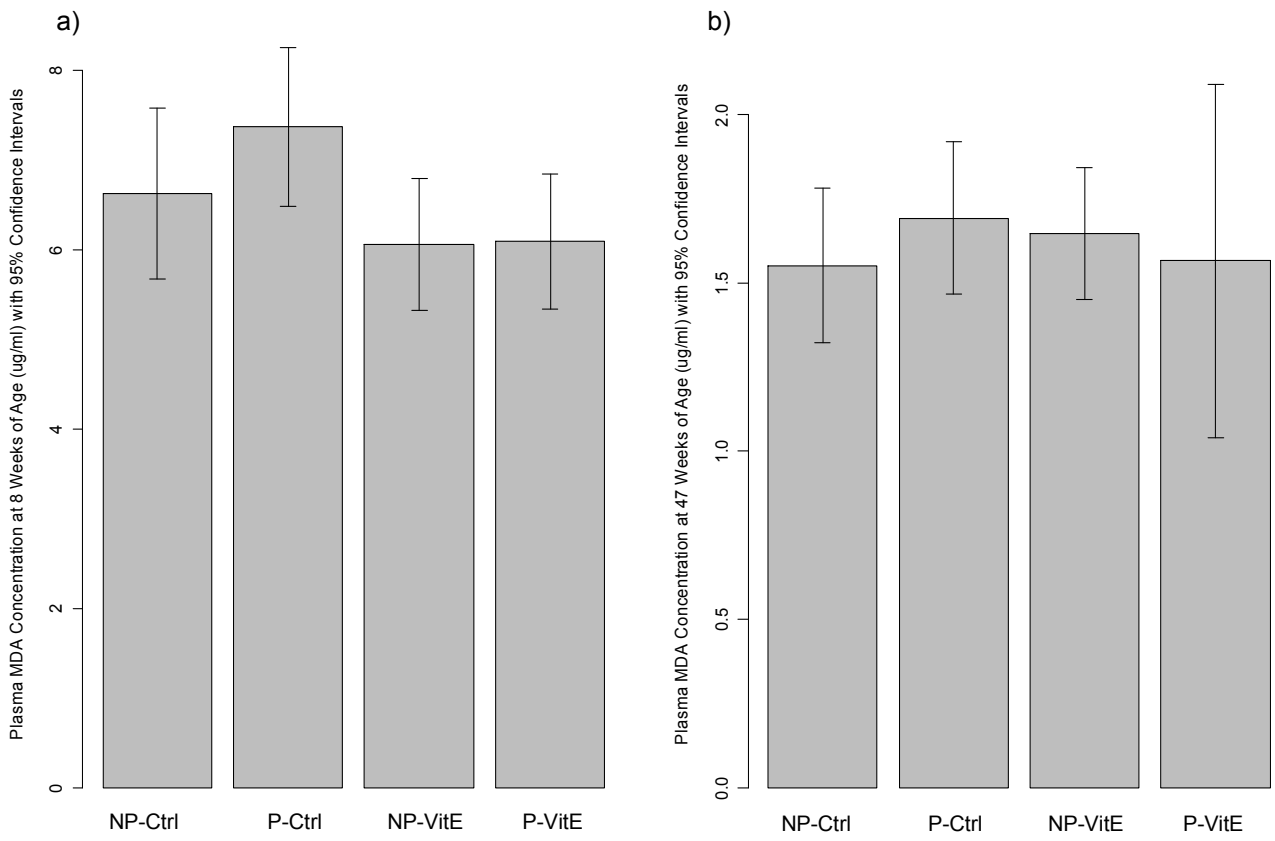

Figure 5.4.: Plasma MDA concentrations (μg/ml) in relation to treatment and age at 8 a) and 47 b) weeks of age with 95% confidence intervals. Scales differ at 8 and 47 weeks of age. α-tocopherol supplement with addition of *Heterakis* nematode parasites (P-VitE n=62), α-tocopherol supplement without parasites (NP-VitE n=58), control diet with *Heterakis* parasites (P-Ctrl n=55), control diet without parasites (NP-Ctrl n=57).

5.3.4. Morphometric measurements

There were no initial differences in the size of chicks allocated to different dietary treatments. Repeated measures GLMMs including mass as the response variable and sex and treatment group as explanatory variables indicated that males were faster growing than females (**Table 5.4.**) but that there were no significant differences between treatments in growth (**Figure 5.5.**).

Table 5.4.: Calculated L.ratios and p-values following GLMM analyses of mass measurements.

	Sex	Treatment:Sex	Treatment
Mass growth	L.ratio=3.61, p<0.001***	L.ratio=0.15, p=0.99	L.ratio=0.24 p=0.98
Head to Bill growth	L.ratio=14.4, p<0.001***	L.ratio=0.24, p=0.98	L.ratio=0.56 p=0.92
Tarsus Length growth	L.ratio=11.44, p=<0.001***	L.ratio 0.26, p=0.98	L.ratio=0.12, p=0.99
Wing length growth	L.ratio=1.34, p<0.001***	L.ratio=4.92, p=0.18	L.ratio=2.75, p=0.43

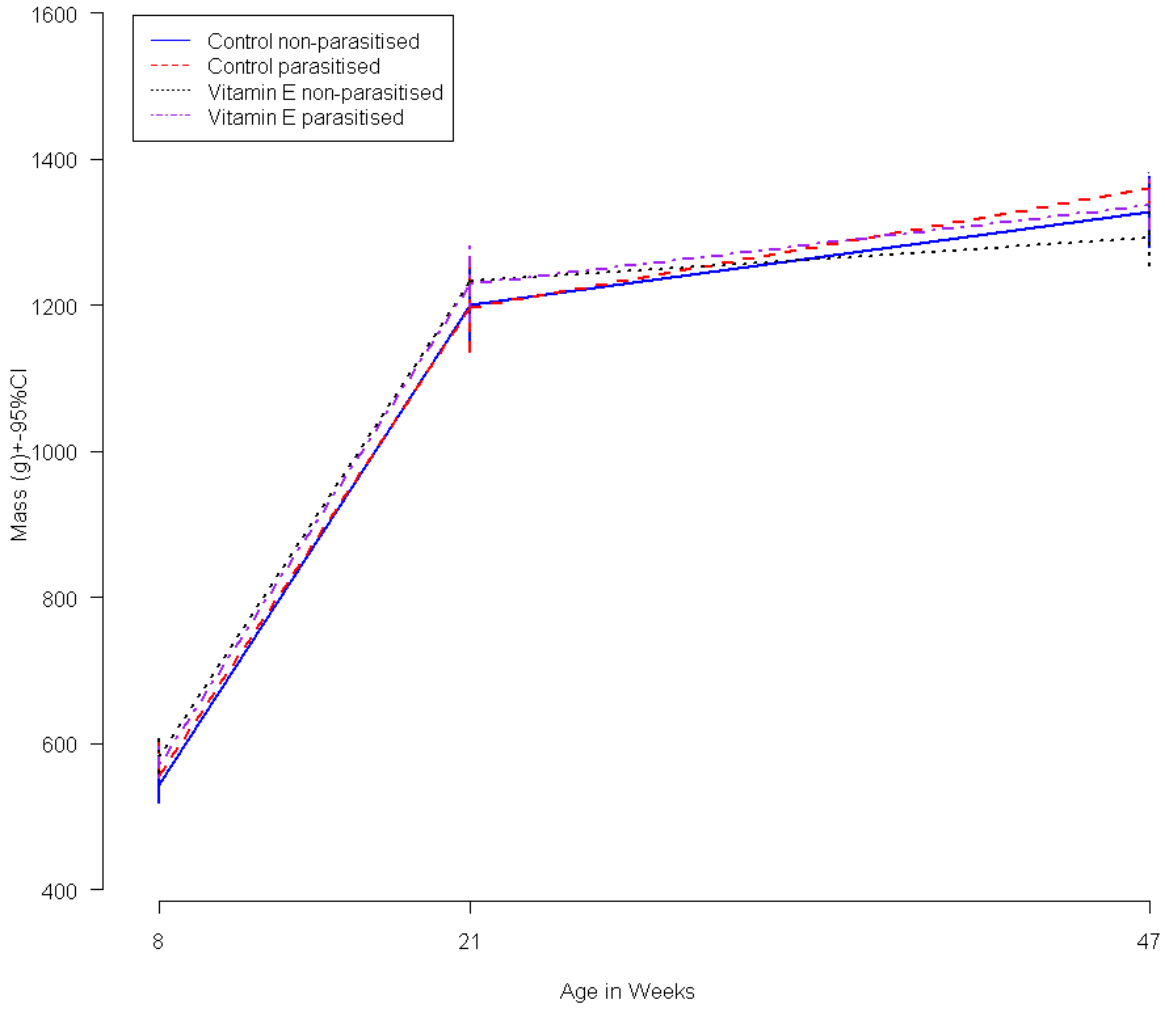

Figure 5.5.: Mass of males aged 8, 21 and 47 weeks of age with 95% confidence intervals.

5.3.5. Immune function

Immune response did not vary in relation to either sex or treatment. The minimum adequate model (MAM) of a general linear mixed model (GLMM) with wing patagium inflammation following immune challenge as the response variable and treatment and sex as main effects included just the intercept, with all other variables dropping out of the model (**Table 5.5.**).

Table 5.5.: Results of GLMMs of immune response at 21 weeks of age.

	Explanatory Term	F-value/L.ratio	2-tail p/p
Immune response to PHA at 21 weeks of age	Treatment:Sex	L.ratio =0.66	p=0.88
	Sex	L.ratio = 0.54	p=0.46
	Treatment	L.ratio = 1.95	p=0.58

5.3.6. Secondary Sexual Signals

The expression of sexual signals by males did not vary among treatment groups (**Table 5.6.**, **Figures 5.6. and 5.7.**). There was no difference in spur length or wattle brightness in relation to dietary treatment. There was no difference in wattle size in relation to dietary treatment. 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 treatment. GLMMs with the sexual signal as the response variable and parasite burden as an explanatory variable found that there were no significant relationships between sexual signals and current parasite burden (**Table 5.6.**).

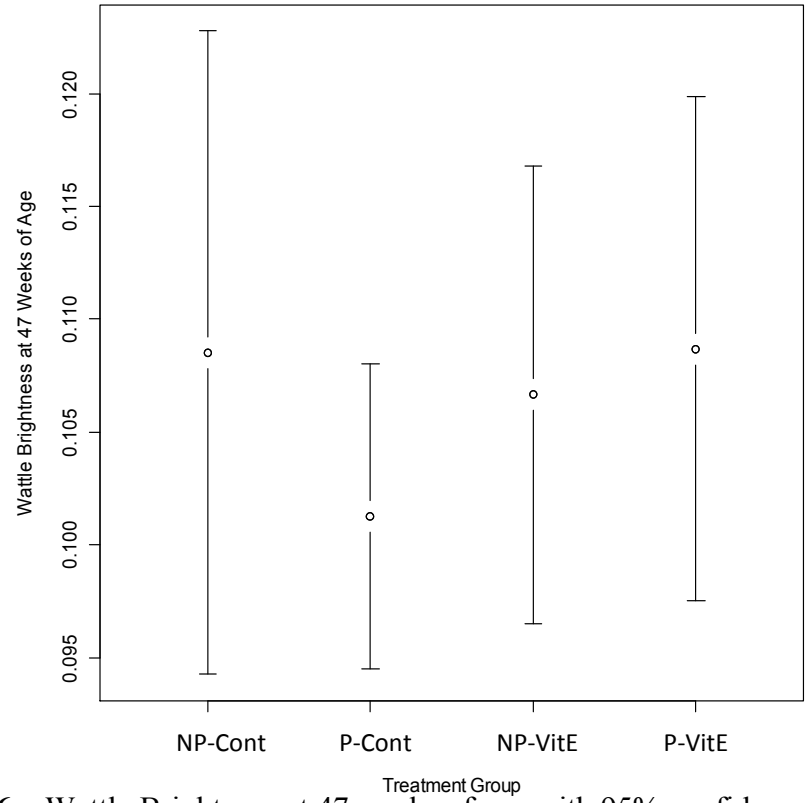

Figure 5.6.: Wattle Brightness at 47 weeks of age with 95% confidence intervals. α-tocopherol supplement with addition of *Heterakis* nematode parasites (P-VitE n=62), α-tocopherol supplement without parasites (NP-VitE n=58), control diet with *Heterakis* parasites (P-Ctrl n=55), control diet without parasites (NP-Ctrl n=57).

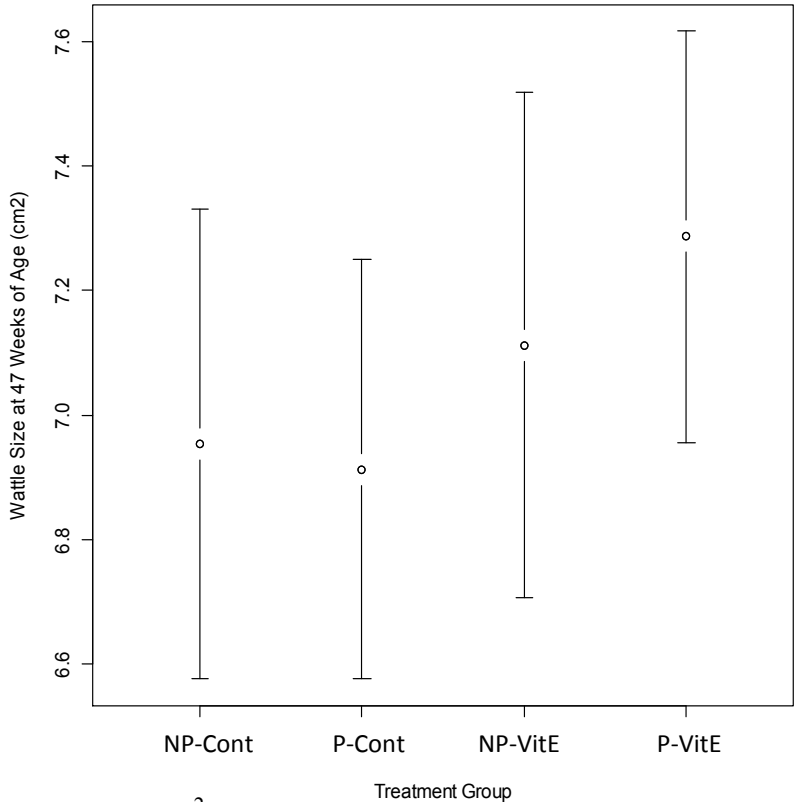

Figure 5.7.: Wattle Size (cm^2) at 47 weeks of age with 95% confidence intervals. α-tocopherol supplement with addition of *Heterakis* nematode parasites (P-VitE n=62), α-tocopherol supplement without parasites (NP-VitE n=58), control diet with *Heterakis* parasites (P-Ctrl n=55), control diet without paraites (NP-Ctrl n=57).

Table 5.6.: Results of GLMM analyses for secondary sexual signals at 46 weeks of age.

	Explanatory Terms	L.ratio	p
Spur Length	Treatment	L.ratio = 3.37	p=0.34
Wattle Size	Treatment	L.ratio= 2.62	p =0.45
Wattle Shape (MANCOVA)	Treatment	L.ratio = 0.88	p =0.74
Wattle Brightness	Treatment	L.ratio = 5.47	p = 0.14
Spur Length	Number of Parasites	L.ratio = 2.34	p = 0.13
Wattle Size	Number of Parasites	L.ratio = 0.11	p = 0.74
Wattle Shape (MANCOVA)	Number of Parasites	L.ratio = 0.39	p = 0.51
Wattle Brightness	Number of Parasites	L.ratio = 0.75	p = 0.39

5.4. Discussion

The results show that, contrary to expectations, the expression of sexually-selected traits 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. However, 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 controls, 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 numbers at adulthood (47 weeks) of individuals that received supplementary vitamin E during the first 8 weeks of growth may still have had positive 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 and 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

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 affects 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, in contrast with some previous studies (Møller et al. 1999) 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 and 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; Hõrak 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 and Holmes 1996). In the current study birds infected with parasites but 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 were 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 (von Schantz et al. 1999). 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-vitE 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 infected control 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 and 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. **chapter 3**). 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, or it may be that unless vitamin E is supplemented in conjunction with carotenoids it is effectively unavailable for preferential allocation towards growth (Orledge et al. **chapter 3**).

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 and 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 only one component of the immune system during at 21 weeks of age, the pro-inflammatory immune response following PHA injection, so 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 did not find that extra antioxidant resources during development were allocated towards increasing sexual signalling when infected with nematode parasites, or that the degree of ornamentation in pheasants reflected either the parasite load of *H. gallinarum* or oxidative status of males. However 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. 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 if it is only expressed when individuals have experienced very high parasite loads. In such circumstances signals 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.

5.5. References

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Chapter 6: Feather odour changes in response to antioxidant status, but not intestinal parasite levels, in ring necked pheasants

6.1. Introduction

An increasing number of recent studies have characterised the volatile compounds present on the feathers of birds (Burger et al. 2004; Douglas 2006; Soini et al. 2007; Bonadonna et al. 2007; Haribal et al. 2009; Whittaker et al. 2010), and it has been suggested that these odour profiles may provide important information about individuals that may be utilised by both members of the same species and different species (Hagelin and Jones 2007; Balthazart and Taziaux 2009). For example, recent studies have shown that in some avian species individuals may be able to utilise odours in social situations, for example in the recognition of unrelated conspecifics (Hagelin et al. 2003; Whittaker et al. 2010), close relatives (Whittaker et al. 2010) or social partners (Bonadonna and Nevitt 2004; Jouventin et al. 2007; Mardon et al. 2010). However, while it is generally acknowledged that odours are undoubtedly complex (Soini et al. 2007) and may differ significantly between individuals (Bonadonna et al. 2007), it is often unclear what information is being transmitted.

In red grouse, *Lagopus lagopus* (Hudson et al. 1992; Isomursu et al. 2008) and ring-necked pheasants (Woodburn 2000; Millán et al. 2002), there is some evidence that infection with parasitic nematodes makes them more vulnerable to predation by mammals. For example, Hudson et al. (1992) found that dogs trained to locate birds by scent found significantly fewer incubating female grouse that had been treated with an anthelmintic drug than untreated individuals that were infected with a caecal nematode (*Trichostrongylus tenuis*), while pheasants with higher natural levels of *Eucoleus contortus* infection were found to be more susceptible to depredation than individuals with lower parasite levels (Millán et al. 2002). Hudson et al. (1992) hypothesised that increased vulnerability to mammalian predation may arise from a reduced ability to regulate scent emission, causing the volatile concentration or composition of odour to change, thereby rendering the birds more detectable to predators hunting by smell.

It has been proposed that preen oil, a waxy lipid secretion from the uropygial gland has a role in delaying feather wear, keeping feathers flexible (Stettenheim 1972), waterproofing (Elder 1954) and antimicrobial properties (Jacob et al. 1997). Preen oil

is also thought to contribute largely to the odour of birds' feathers (Reneerkens et al. 2002). Females of some species experience a compositional shift in uropygial secretions during courtship and incubation indicating an additional function for preen oil (Piersma et al. 1999). There is evidence for dietary effects on preen oil composition. For example, the composition of the preen oil of white throated sparrows, *Zonotrichia albicollis*, differed between individuals that received a diet enriched with sesame oil or enriched with fish oil (Thomas et al. 2010). In addition, the toxins found on the surface of the skin and feathers of birds from the genus *pitohui* are believed to be derived from melyrid beetles in the diet (Dumbacher et al. 2004). Preen oils have a high number of polyunsaturated fatty acids which are highly susceptible to oxidative damage providing a potential role for dietary antioxidants on feather surface volatiles to signal antioxidant capacity. During incubation females of some sandpiper species (from the Charadriiform family Scolopacidae) reduce the relative amount of highly volatile compounds in preen oil, which may be an adaptation to make them less susceptible to depredation by olfactory predators (Reneerkens et al. 2008). Parasites may disrupt this regulation of the production of volatiles on feathers possibly through effects on the composition of preen oil.

As a first empirical step towards addressing whether exposure to parasites and/or diet during early development can affect feather odour in birds, I manipulated the intestinal parasite burden (the nematode *Heterakis gallinarum*) and availability of dietary antioxidants (vitamin E) of female pheasants during early development and quantified the effects on the volatile compounds present on the feathers and in the contents of the caecum at adulthood. Vitamin E supplementation during early development in pheasants was found to reduce parasite load in adults (see **Chapter 5**), so it is possible that there may also be additive or independent effects of antioxidant availability on the odour profiles of pheasants.

6.2. Material and methods

6.2.1. General methods

The methods used in this experiment are identical to **chapter 5** with the exception of the odour analysis methods which are outlined below.

240 day-old ring-necked pheasants of mixed genetic stock (Holme Park Game Hatcheries, Wokingham) were allocated randomly to one of four equal-sized treatment groups at the Game and Wildlife Conservation Trust HQ, Hampshire, UK, that received vitamin E supplementation and/or parasite manipulation. Two of the groups received supplemental vitamin E during the first 8 weeks, and the other two groups received a control diet with trace amounts of vitamin E. An 8 week period of dietary manipulation was chosen to include the early developmental window identified by previous studies on pheasants (Ohlsson and Smith 2001; Ohlsson et al. 2003). Of these groups, two (one vitamin E supplemented and one control with trace amounts of vitamin E) were infected with *Heterakis* nematodes at 21 days of age, the optimal age for successful infection in chickens (Olsen 1974).

For the first eight 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 birds were reared and introduced to the pen on mortality of an experimental bird to maintain experimental rearing densities during the first eight weeks following eight mortalities across the treatment groups. After two weeks birds were given daily access to outdoor pens with wire floors (3m x 1.5m), and at eight weeks of age the birds were given access to grass for the remainder of the experiment, in line with standard rearing practice (The Game Conservancy 2006). In line with standard rearing practice, four different basal (i.e. unsupplemented) diets were provided over the first 8 weeks, 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*. The diet provided after 8 weeks was identical for all birds. Further details regarding dietary supplementation can be found in **chapter 5**.

At sexual maturity (47 weeks), we took feathers from the mantle of a randomly selected sample of ten birds in each group. We also took a small blood sample for determination of plasma vitamin E levels, and dissected the caecum so that the number of parasites could be counted.

6.2.2. Experimental manipulations

(i) Vitamin E manipulation

Further details regarding dietary supplementation can be found in **chapter 5**.

(ii) Heterakis infection and counts

Further details regarding *H. gallinarum* infection and counts can be found in **chapter 5**.

6.2.3. GC/MS analysis of feather volatiles

Samples of feathers taken from the mantle (mean ± SD: 0.28 ± 0.10 g) or caecal contents (0.41 ± 0.15 g) of each individual were weighed (± 0.01 g) and placed in 25 ml autosampler GC MS screw top glass vials with silicone/PTFE septum lid. Quantitative analyses were performed on a GCMS (Agilent 7890 GC coupled with an Agilent 5975 Mass spectrometer) fitted with a J and W DB1-MS, 30m x 0.25mm, film thickness 0.25µm, 0.5µm film thickness, using helium as a carrier gas. The inlet was set at 250 ºC, and the injection was in pulsed splitless mode. Headspace volatiles were collected by solid-phase microextraction (SPME) using 60-µm polydimethylsiloxane/ divinylbenzene (PDMS/DVB) fibres (Supelco Ltd). A general purpose fibre was used to detect a broad range of compounds (Buszewski et al. 2007). Vials were heated to 37ºC and volatiles allowed to collect in the headspace of the vial for 5 mins; fibres were then exposed to the headspace for 15 mins and immediately transferred to the GC's injector port where volatiles were desorbed for 1 min. Separation of the extract was optimized by using a GC column profile which began at 70ºC for 1 min, rising by 10ºC /min to 200ºC, over a run time of 15 minutes.

Peaks identified following the quantitative analyses of blank vials are likely to have resulted from contamination from the fibre or laboratory environment, and so these

peaks were removed from feather and faeces samples (Bryant and McClung 2011). Volatile compounds were not identified due to low library matches (typically < 50%); however, this does not pose a problem as I am not interested in the identity of the peaks *per se*, but rather the overall composition of the volatile profile. This is because (i) I had no *a priori* hypotheses for which volatiles would be present, nor which volatiles would be likely to be altered by the experimental manipulations, and (ii) receivers of the odour would be exposed to a suite of volatiles simultaneously, not individual compounds, and work on other galliform species has shown that birds are much better at detecting mixtures of compounds than the compounds on their own (T. Pike, *unpublished*). This approach has been widely used to analyse complex, novel odour profiles (D'Amico et al. 2007).

6.2.4. Statistical analyses

Total ion chromatogram peak areas were log-transformed to reduce contrasts and to ensure that each sample profile was not dominated by a few large peaks (Xu et al. 2007). A pair wise distance matrix was then constructed to compare peak chromatogram profiles for all individuals using a quantitative distance matrix of Xu et al. (2007), in which the distance d between any two chromatogram profiles, i and j, is given by

$$d_{ij} = 1 - \frac{\sum_{k=1}^{n}(x_{ik} \cdot x_{jk})}{\|\mathbf{x}_i\| \cdot \|\mathbf{x}_j\|},$$

where x_{ik} is the normalised log intensity of peak k in chromatogram i, and $\mathbf{x}_i$ is the vector containing all n chromatogram peaks (feather sample data n=118, caecal sample data n=85).

I used a permutation (non-parametric) multivariate analysis of variance to examine differences in the volatile composition between treatment groups, using the FORTRAN program PERMANOVA (Anderson 2001; McArdle and Anderson 2001; Anderson 2005) which has been used in previous studies to analyse large numbers of unidentified compounds (Mardon et al. 2010; Mardon et al. 2011). This method analyses the variance of multivariate data explained by a set of fixed explanatory factors, in this case 'diet' (i.e. whether or not they received supplementary vitamin E) and 'parasite infection' (i.e. whether or not they were experimentally infected with

parasites), random factors and covariates based on the chromatogram distance matrix. *F*-ratios and *p*-values were calculated using 9999 permutations of the residuals under a reduced model (Anderson and Legendre 1999), and integral *post-hoc* tests were used by calculating the multivariate version of the t-statistic (Andersson 2001). To visualize the multivariate patterns among observations, metric multidimensional scaling (MDS) was performed using the MASS package in the R statistical program. The amount of volatiles was estimated as the sum of all peaks in a chromatogram (Degen et al. 2004), and the effect of treatment group was analysed using one-way analysis of variance (ANOVA). Post hoc analyses were performed using Tukey's multiple comparison procedure to identify differences between treatment groups.

6.3. Results

Parasite treatment during early development resulted in higher parasite numbers at adulthood when compared to non-treated birds ($F_{1,36} = 5.95$, $p = 0.020$), and this was unaffected by vitamin E supplementation (diet: $F_{1,36} = 0.98$, $p = 0.33$; interaction: $F_{1,36} = 0.24$, $p = 0.63$) (**Figure 6.1.a**).

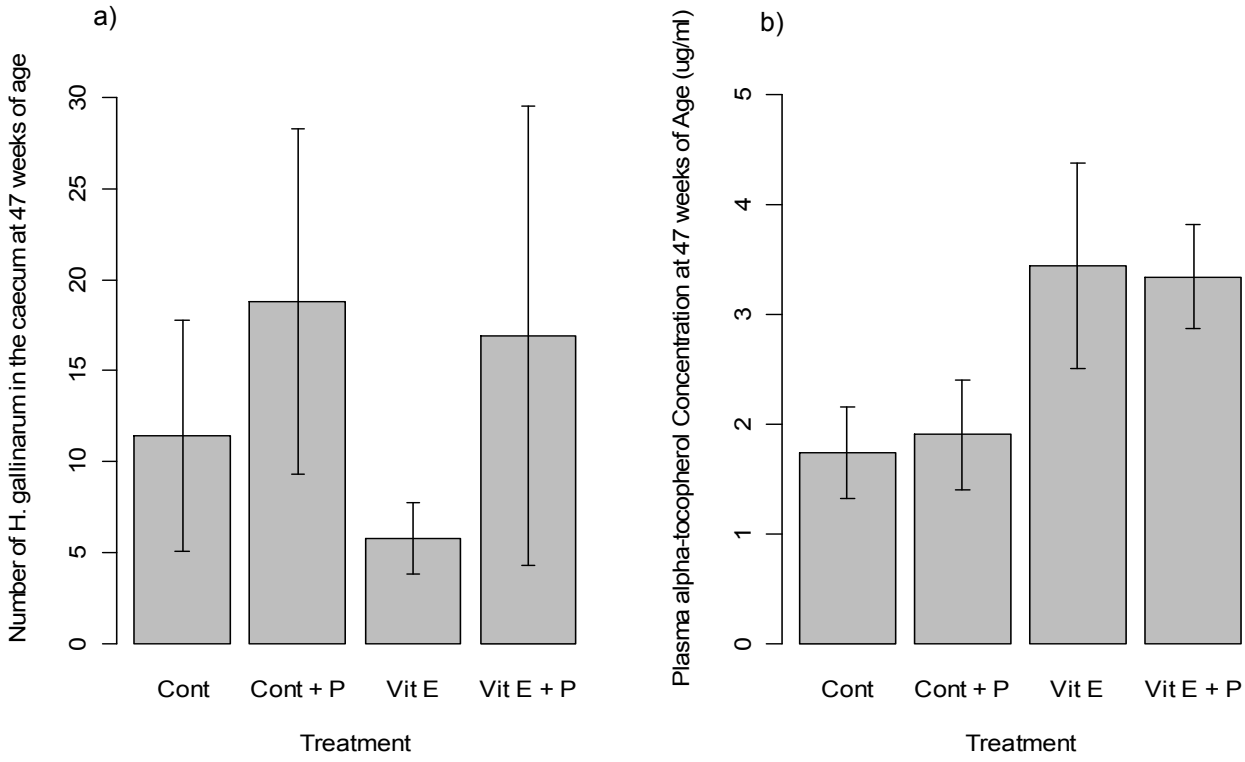

Figure 6.1.: **a**) Mean number of parasites present in the caecum at adulthood for each treatment group **b**) Mean plasma concentration of α-tocopherol at adulthood (μg/ml) and treatment group. Error bars show 95% confidence intervals.

As expected, birds supplemented with vitamin E during early development had significantly higher plasma vitamin E levels in adulthood than unsupplemented birds ($F_{1,36}$ = 33.05, p < 0.001), irrespective of their parasite treatment (parasites: $F_{1,36}$ = 0.02, p = 0.90; interaction: $F_{1,36}$ = 0.25, p = 0.62) (**Figure 6.1.b**).

6.3.1. Caecal odour

There were no differences between individuals in relation to parasite treatment during early development ($F_{1,36}$ = 1.58, p = 0.19) or between individuals that received supplementary vitamin E compared to those that did not ($F_{1,36}$ = 0.75, p = 0.57) on the composition of volatiles derived from caecal samples at adulthood. However, there was a significant interaction between parasite exposure and vitamin E supplementation ($F_{1,36}$ = 6.21, p < 0.01) on caecal volatile profiles.

6.3.2. Feather odour

There were no differences between parasitised and non-parasitised individuals in the composition of volatile profiles derived from feather samples ($F_{1,36}$ = 1.01, p = 0.42; Figure 2 and 3). However, the vitamin E levels that individuals received during the first 8 weeks post-hatch had a highly significant effect on the volatile composition of their feathers at adulthood ($F_{1,36}$ = 5.93, p < 0.001; **Figure 6.2 and 6.3b,d**). There was no significant interaction between the two treatments ($F_{1,36}$ = 1.99, p = 0.34).

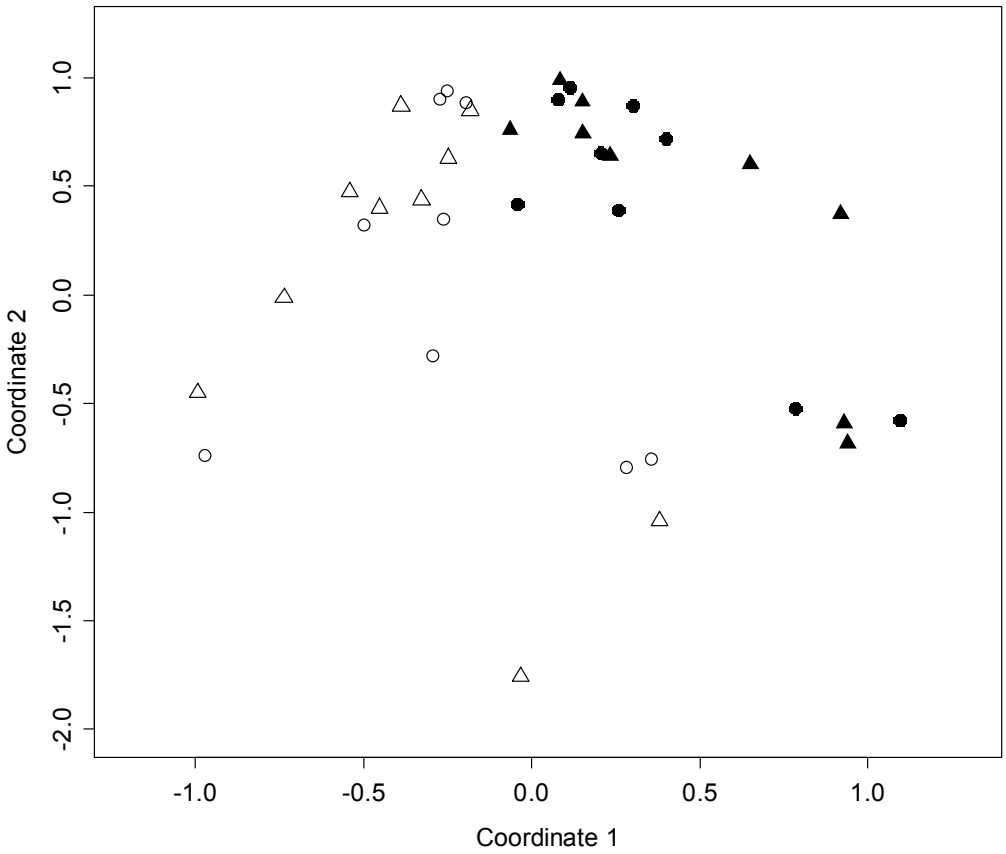

Figure 6.2.: Metric multidimensional scaling (MDS) plot illustrating the similarity between odour profiles of birds in the different treatment groups. Each data point denotes one individual that received either vitamin E supplementation (black points) or trace vitamin E levels (white points), and/or parasite manipulation (circles) or not (triangles).

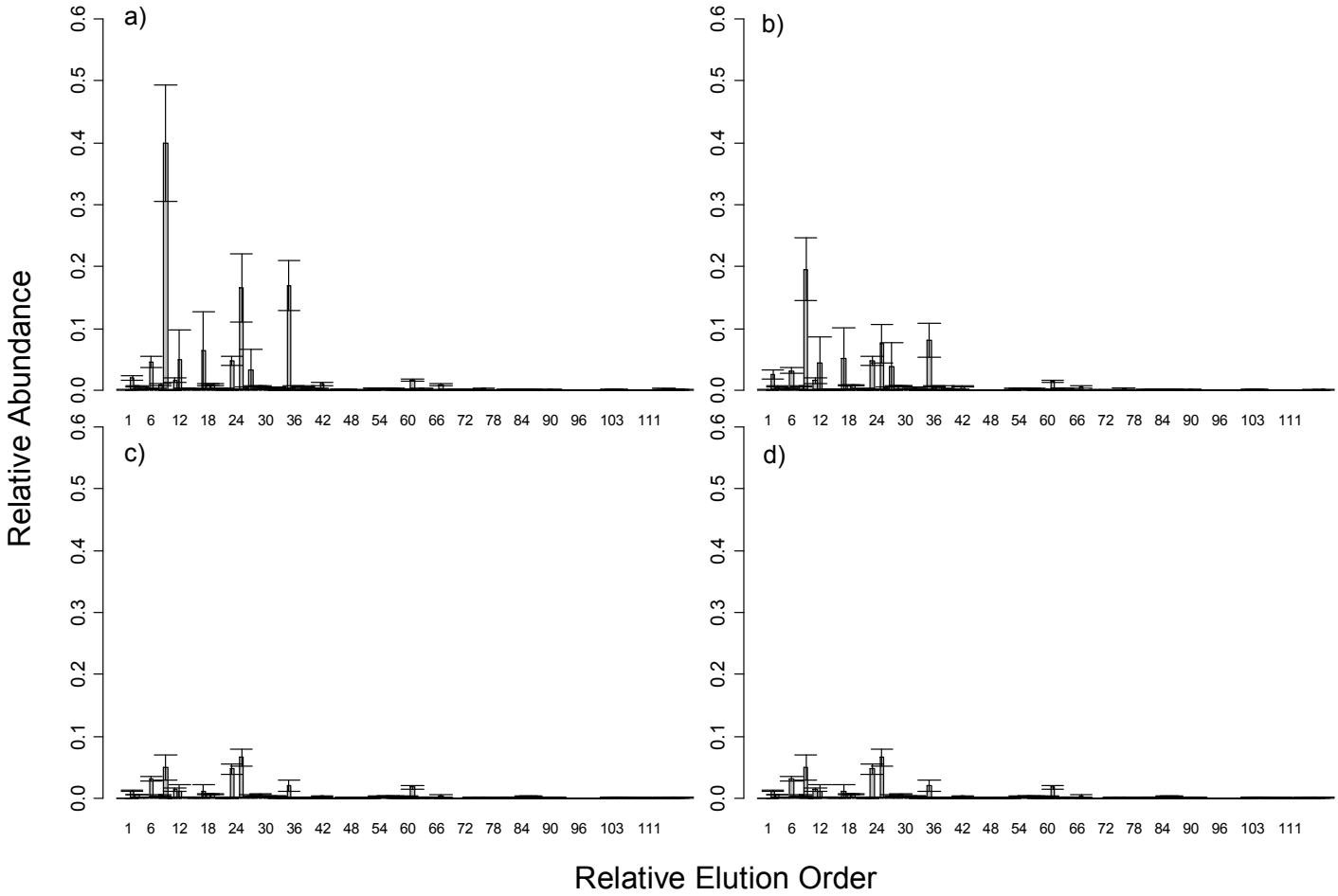

Figure 6.3.: Feather chromatograph profiles of all individuals given a) vitamin E diet with parasites, b) vitamin E diet with no parasites, c) control diet with parasites d) control diet with no parasites. Error bars show 95% confidence intervals.

6.3.3. Concentration of volatiles present on the surface of feathers

The concentration of all the volatiles present on the surface of feathers differed between treatment diets ($F_{1,36}$ = 12.65, p < 0.01) but not parasite treatment group ($F_{1,36}$ = 3.99, p= 0.053). The interaction between diet and parasite treatment was non-significant ($F_{1,36}$ = 0.96, p = 0.33) (**Figure 6.4.a**).

6.3.4. Concentration of volatiles measured in caecal samples

The concentration of volatiles measured in caecal samples did not differ significantly between treatment diets ($F_{1,36}$ = 0.62, p = 0.43), or parasite treatment groups ($F_{1,36}$ = 0.11, p = 0.74), and there was no interaction between treatment diet and parasite infection treatment ($F_{1,36}$ = 0.007, p = 0.93) (**Figure 6.4.b**).

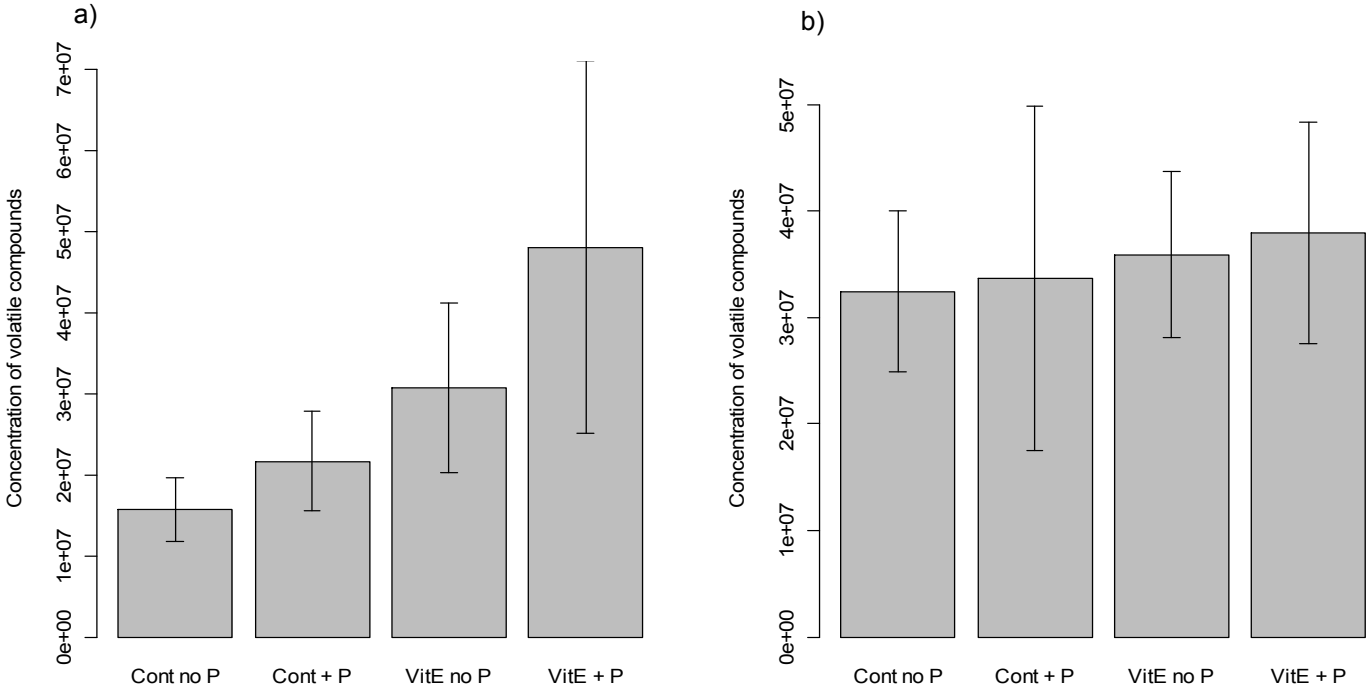

Figure 6.4. Concentration of volatile compounds from a) feathers and b) caecal content and treatment group. Error bars show 95% confidence intervals.

6.4. Discussion

The results of this study provide no support in pheasants for the hypothesis that parasite burden affected odour, which was put forward to explain why parasitised birds were more susceptible to depredation by mammals than unparasitised birds (Hudson et al. 1992). However, we found that supplementation of vitamin E during the first 8 weeks of life significantly altered the composition and concentration of volatile compounds (odour) on the feathers, but not the caecal matter, of adult birds.

Our results therefore suggest that it is unlikely that higher vulnerability to predation results from a change to the composition of volatile compounds present on feathers (Hudson et al. 1992), but instead suggest that it is the behavioural responses of birds to parasite infection that increases their susceptibility to predators. Hudson et al. (1992) suggested that parasites compete with the host during incubation for a female's reserves forcing her to leave the nest more frequently, and resulting in a greater chance of heavily parasitized individuals being detected by a predator around the nest.

Hens supplemented with vitamin E during development had greater concentrations of volatiles present on their feathers at adulthood. Given the need for birds to minimise their detectability to olfactory predators, this suggests that there may be a long-term cost to the availability of vitamin E early in development. However, as Vitamin E supplementation resulted in decreased numbers of *H. gallinarum* at adulthood (**Chapter 5**), if the increased importance of behavioural responses postulated above is correct then vitamin E will also reduce the competition from *H. gallinarum* for internal reserves. This reduced competition for host reserves would decrease the frequency that a hen needs to leave the nest and in turn reduce the vulnerability of the hen and her eggs to predation. However, we must be cautious when interpreting the data in this case because the individuals measured were not incubating at the time of measurement.

A recent review concluded that the functional role of the avian olfactory system and odour perception has been underestimated in avian communication (Balthazart and Taziaux 2009). Experimental evidence indicates that olfactory signals may be important in food location, orientation and nest localisation in a range of species, including galliformes (Balthazart and Taziaux 2009). Several studies have also identified a role for olfactory signals in mate choice (Balthazart and Schoffeniels 1979; Hagelin et al. 2003; Bonadonna and Nevitt 2004; Soini et al. 2007). It is possible that males could detect the differences in odour associated with females on the different diets in this study. If this were the case then the difference in scent composition and volume of volatiles emitted by hens may signal the quality of their antioxidant system to males. Studies in fowl, which have a similar harem polygyny mating system to pheasants, have shown that males adjust sperm number transferred in the ejaculate based on female attractiveness (Cornwallis and Birkhead 2006; 2007a; 2007b;). Male mate choice experiments have also identified male preferences for traits that correlate with female fecundity and maternal investment such as body size and mass (e.g. red-spotted newt, *Notophthalmus viridescens*, Verrell 1985; fruit fly, *Drosophila melanogaster*, Byrne and Rice 2006) and comb size in fowl (*Gallus gallus*, Pizzari et al. 2003; Cornwallis and Birkhead 2006). It is therefore plausible that in female ring-necked pheasants, which have a dull cryptic plumage and lack ornamentation, scent emission could provide a sexual signal of fecundity.

Vitamin E is essential for normal hatchability and embryonic development (Wilson 1997) and poor antioxidant resources at hatching can result in increased tissue damage in chicks (Surai 2000). Vitamin E, as a dietary antioxidant, is believed to be scarce in the environment and therefore a limited resource (Surai 2002) and maternal investment through the deposition of antioxidants in the egg can improve the potential fitness of offspring (Royle et al. 1999, 2001; Blount et al. 2002). As a result, when investing vitamin E in their offspring females might incur significant costs and compromise their own antioxidant defence. The variation in maternal investment of nutrients in eggs will depend on the condition of hens during egg production.

Vitamin E may affect the bacterial fauna present within uropygial secretions resulting in changes to feather odour. The secretions of the uropygial gland which are spread over the skin and feather surface can have a defensive role against external pathogens (Bandyopadhyay and Bhattacharyya 1996; Shawkey et al. 2003; Reneerkens et al. 2008). For example, the mixture of volatile compounds isolated from the uropygial secretions of the European woodhoopoe, *Upupa epops,* showed antagonistic capacity towards feather degrading bacteria and several potentially pathogenic bacteria. Interestingly, the mixture of volatile compounds in woodhoopoes that received an antibacterial treatment did not have this antagonistic capacity towards the feather degrading bacteria (Martín-Vivaldi et al. 2010). In this study, symbiotic bacteria present in the uropygial gland of pheasants may account for the differing compositions or total concentrations of volatile compounds observed on the surface of feathers and dietary intake and antioxidant provision may affect the bacterial communities present in uropygial gland.

In conclusion, the results provide evidence that early exposure to nematode parasites does not affect the odour of female pheasants, but vitamin E availability during the first 8 weeks of life can affect the composition and concentration of volatile compounds found on the surface of the feathers in adults. Further research is required to determine whether this may have an adaptive significance either by providing a means for males to assess female antioxidant levels or results from a difference in the secretions or presence of symbiotic bacteria within the uropygial gland.

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Chapter 7 General Discussion

7.1. General Discussion

In **Chapter 1** I introduced and reviewed the current scientific literature linking oxidative stress, early life history-effects and resource allocation trade-offs including studies in which antioxidant supplementation during development resulted in increased growth rates (e.g. Cucco et al. 2006; de Ayala et al. 2006; O'Brien and Dawson 2008; Hall et al. 2010) and in which antioxidant supplementation at adulthood positively affected sexual signal expression (Pike et al. 2007; Pérez et al. 2008). However, little is known about the longer-term effects of variation in dietary antioxidant availability during early life on the expression of traits at adulthood. The evolution of life history strategies is believed to result from trade-offs in the allocation of limited resources to multiple traits (Zera and Harshman 2001). However, as highlighted in a recent review, reconciling empirical observations of functional linkages between traits that are separated in time and therefore not expected to compete for the same resources is difficult given the current bias towards snapshot studies that focus on single estimates of costs at single points in time (Isaksson et al. 2011). The aim of this thesis was to examine the potential role of antioxidants in mediating resource allocation trade-offs during growth and development and their long-term effects on adult traits.

Predicted outcomes

Previous research has shown that nutrition during development can have a greater impact on sexual signals than morphological traits (David et al. 2000), that there are synergistic interactions between antioxidants (e.g. vitamin E can recycle oxidised carotenoids; Catoni et al. 2008) and that interactions between vitamin E and carotenoids when supplemented at adulthood result in the enhanced expression of sexual traits (Pike et al. 2007; Pérez et al. 2008). I therefore predicted that antioxidants supplemented during development would primarily be allocated towards reproductive traits at adulthood at the expense of self maintenance. In addition if there are synergistic effects of carotenoid and vitamin E supplementation during development that the expression of secondary sexual traits would be greater in individuals that had received a combination of antioxidants (**chapter 3**). Previous experiments indicated that the environment experienced during development can also

affect primary sexual traits (Gage and Cook 1994; Wedell 1996) and that secondary sexual characteristics may provide information about the functional fertility of males (Sheldon 1994; Merilä and Sheldon 1999). In **chapter 4** I measured the early life history effects of antioxidant supplementation on the expression of primary sexual traits at adulthood, predicting that if oxidative stress is an important mediator of trade-offs during growth and development, that vitamin E would positively affect both secondary and primary sexual traits when compared to birds receiving a control diet. Furthermore, I predicted that the degree of secondary sexually selected trait expression would accurately indicate the quality of primary reproductive traits (Blount et al. 2001). Research by 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') and as a result that females could improve the fitness of their offspring by choosing males with the most exaggerated ornaments (Hamilton and Poulin 1997). In **chapter 5** I measured the effects of early exposure to parasites on the allocation of antioxidant resources during development predicting that males supplemented with dietary antioxidants would be able to trade-off the lower costs of self-maintenance and antioxidant defence against oxidative damage by increasing resource allocation to sexually selected traits. A recent review by Balthazart and Taziaux (2009) suggested that the role of avian olfaction had been overlooked in avian reproduction. Finally, studies showing the higher vulnerability to predation of heavily parasitised ground nesting birds by scent hunting mammals (Hudson et al. 1992; Woodburn 2000; Millán et al. 2002; Isomursu et al. 2008) had led to the suggestion that parasites affected the ability of birds to regulate scent emission (Hudson et al. 1992). In **Chapter 6** I measured the effects of early antioxidant supplementation and exposure to intestinal parasites on feather odour and caecal odour and predicted that these odour profiles could transmit important information about the parasite burden of individuals.

What are the early life-history effects of antioxidant supplementation?

The results of the experiments detailed in this thesis indicate that antioxidant availability during early development has no long-term effects on secondary sexual trait expression, immune function, levels of plasma lipid peroxidation (**chapters 3 and 5**) or primary sexual traits such as sperm motility, sperm number or lipid peroxidation in the semen (**chapter 4**). However, supplementation of vitamin E

during development can have long-term affects on circulating vitamin E levels in plasma (**chapters 3 and 5**), parasite burden (**chapter 5**), vitamin E concentration in the testes (**chapter 4**), lipid peroxidation in the testes (**chapter 4**) and feather odour (**chapter 6**). Vitamin E availability during development can also affect growth but only when supplemented in combination with carotenoids (**chapter 3**). When supplemented alone carotenoids had no long term effects on the traits measured (**chapter 3**). However, there were short term affects on plasma lipid peroxidation levels at 8 weeks of age following carotenoid and vitamin E supplementation when supplemented as a combined treatment (carotenoids and vitamin E) or supplemented separately (carotenoids or vitamin E). These differences in levels of lipid peroxidation were not visible at adulthood (**chapters 3 and 5**). Vitamin E supplementation during development has significant long-term effects at adulthood on body size, circulating antioxidants and feather odour which are likely to have considerable effects on fitness. Reaching a greater body size at adulthood has been associated with a range of benefits including an increased immune response (Smith et al. 2007), increased success in the male-male antagonistic interactions associated with increased harem size (Göransson et al. 1990; Grahn and von Schantz 1994) and a greater ability to capitalise on fluctuations in carotenoid availability (Smith et al. 2007). A greater ability to assimilate vitamin E at adulthood may increase the availability of antioxidant resources in these individuals for self-maintenance (i.e. reducing the accumulation of cellular damage that results from oxidative stress). Finally, the ecological implications of differing feather odour at adulthood are currently unknown and further research is required. However, feather odour could potentially provide a cue by which an individual could assess the antioxidant status of a potential mate.

Why do carotenoids and vitamin E have synergistic effects on growth and not sexually selected traits?

The oxidative stress levels experienced by individuals vary with age, changing activity levels and environmental conditions (Monaghan et al. 2009). The early developmental period is believed to be a period of high exposure to ROS and the comparative functional importance of the different classes of the antioxidant system may not be constant over the early life history of an individual. The results of **chapters 3 and 5** support the findings of Blount et al. (2003) which, following the manipulation of diet quality during early development, found that zebra finches that

received a low quality diet (lower levels of protein, vitamins E and A and carotenoids) early in their development had lower levels of circulating dietary antioxidants at adulthood than those that received a standard quality diet. Vitamin E (**chapters 3 and 5**) and carotenoid levels (**chapter 3**) were manipulated over the first 8 weeks post-hatch in pheasants. In contrast with the findings of Blount et al. (2003), however, we found that only vitamin E levels were affected at adulthood by early vitamin E availability and that carotenoid levels did not differ at adulthood depending on early carotenoid availability. Reduced antioxidant availability of vitamin E during development may have a negative effect on the formation of the lipoproteins required for the assimilation and transport of lipophilic antioxidants (Blount et al. 2003) which are known to be affected by early nutritional quality (Nutting et al. 2002). The results presented in this thesis indicate that in the case of vitamin E it is likely that it was the early availability of vitamin E and not the experimental manipulation of protein levels that caused the effects on vitamin E levels observed at adulthood by Blount et al. (2003). Monaghan et al. (2009) suggested that the measurement of oxidative damage during development and at adulthood would have provided a greater indication of the impact on the overall antioxidant defences in the Blount et al. (2003) study. Measurement of lipid peroxidation levels at 8 weeks and 47 weeks of age in the current study indicated that while reduced vitamin E availability during development resulted in higher levels of oxidative damage than other treatments despite similar rates of growth, that oxidative damage was not higher at adulthood, even with higher levels of circulating vitamin E (**chapters 3 and 5**).

Are dietary antioxidants limiting during development on primary sexual traits at adulthood?

In this study vitamin E supplementation during development had sustained effects on the concentration of vitamin E in the testes and oxidative damage in the testes at adulthood. However, despite greater testes vitamin E concentrations and lower oxidative damage I found no effects on key reproductive traits, including sperm concentration, sperm motility or testes size (**Chapter 4**). Vitamin E is believed to have a primary role in antioxidant protection during sperm storage which by reducing lipid peroxidation of spermatozoa membranes would improve their velocity (Catoni et al. 2008). However, these results indicate that early antioxidant supplementation during development does not affect lipid peroxidation levels in semen at adulthood,

suggesting that vitamin E is not a limiting factor. In addition, our results indicated that there was no effect of lipid peroxidation levels in the semen on sperm motility. Previous vitamin E supplementation studies have documented a reduction in lipid peroxidation in the semen at adulthood in a range of species (Brezenzinska-Slebodzinska et al. 1995; Suleiman et al. 1996; Surai et al. 1997; Danikowski et al. 2002; Castellini et al. 2003; Yousef et al. 2003; Mansour et al. 2006). However, these studies measured lipid peroxidation during or immediately after periods of antioxidant supplementation. The findings of **Chapters 3 and 5** showed that early vitamin E supplementation resulted in a greater circulating vitamin E at adulthood and that vitamin E may not have been allocated during development but result from an increased ability to assimilate vitamin E at adulthood. It would be of interest to measure vitamin E concentration in a range of tissues at adulthood to determine whether there was preferential allocation to testes or whether vitamin E concentrations were elevated following supplementation in all tissues. Unfortunately due to the limited volumes of semen samples, there are no data available to determine whether early vitamin E supplementation resulted in higher concentrations in the semen.

In the ring-necked pheasant, which has a territorial defence polygyny mating system in which females are generally believed to be monandrous (Hill and Robertson 1988), post-copulatory sexual selection may be of lesser importance than the allocation of resources to traits increasing competitive ability such as body mass (Göransson et al. 1990). A further avenue to pursue using the remaining plasma samples taken at 47 weeks of age in the study described in **chapter 4** is circulating testosterone concentrations. All the males in this study were housed within a single pen. Male pheasants are territorial; aggressive interactions between individuals were frequently observed and sperm quality is known to fluctuate rapidly following social challenge in the closely related domestic fowl (Pizzari et al. 2007). Testosterone and carotenoid-mediated signalling are believed to be part of a complex integrated physiological mechanism (Peters 2007) and testosterone data could be used to establish whether dominance hierarchies influenced sperm parameters and ornamentation in this study (**Chapter 4**).

Feather odour: A novel cue of antioxidant status?

In **chapter 6** I presented evidence for a novel route by which the antioxidant status of a conspecific may be determined in birds using olfactory cues present on the feathers. This is the first study to measure the effects of antioxidant supplementation on feather odour and to show that vitamin E availability during early life can affect the composition and concentration of volatile compounds found on the surface of the feathers in adults. In **chapter 6** I discussed the benefits to male fitness of adjusting investment of reproductive resources to favour females with a high antioxidant status. In female ring-necked pheasants, which have a dull cryptic plumage and lack ornamentation, feather volatile composition could provide a sexual signal of fecundity. Several studies have identified a role for olfactory signals in mate choice (Balthazart and Schoffeniels 1979). However, whether the differences in the volatile composition measured in **chapter 6** could be detected by a male is unknown and requires further study. In addition, the source of the differences in the composition of volatiles on the feathers is unknown. In **chapter 6** I discussed the currently limited evidence for direct dietary affects on preen oil composition, including a potential role for oxidative damage in affecting the composition of the preen wax and the possibility that bacterial fauna within the uropygial gland could affect the surface feather composition. However, despite the limitations in determining the ecological relevance of this study it does provide a novel foundation for research into the role of odour as a signal of antioxidant status in avian mate choice.

Challenges in studying the influence of oxidative stress on life-history trade-offs

The outcomes of trade-offs influenced by oxidative stress may become apparent over a range of time-scales, including immediate costs in allocating resources to combating ROS rather than the investment of resources to other traits, or over the longer term due to the accumulation of oxidative damage (Monaghan et al. 2009). In this thesis the experiments were designed to determine longer term consequences of trade-offs during growth and development than those measured in previous studies (Isaksson et al. 2011). The current study indicated that antioxidant supplementation had long term effects on body size (**chapter 3**), circulating antioxidant concentrations (**chapters 3 and 5**) and feather odour (**chapter 6**). In this thesis individuals were measured from 1 day of age to 47 weeks of age. However, as discussed in **chapters 3** and **5** the consequences of the allocation of antioxidant resources during development may only

have become really apparent beyond a year of age; for example spur length continues to grow throughout the second year of life (Göransson et al. 1990), and spur length at one year of age has less influence on female mate choice than the spur length of older males (Grahn and von Schantz 1994). Due to time constraints it was not possible to collect data beyond 47 weeks of age and to determine even longer term effects (e.g potential effects on reproductive success, accelerated senescence and longevity). Therefore, while the results of this thesis advance our understanding of the longer term effects of antioxidant supplementation they still do not provide the full picture.

Many ecological studies have measured only antioxidant levels, using these data to make inferences about oxidative damage (Monaghan et al. 2009). However, while higher antioxidant levels may suggest that oxidative stress levels are lower, individuals experiencing less oxidative stress can have lower levels of antioxidants (Costantini and Verhulst 2009) and care should be taken when interpreting results that measure antioxidant protection and not oxidative stress. One strength of the experiments described in this thesis is that both lipid peroxidation and circulating antioxidants were measured at two diffenent stages in the life history of the birds. However, measuring oxidative stress is complex and there are limitations to the available methods of measuring oxidative damage, including the measurement of MDA as a biomarker of lipid peroxidation (Monaghan et al. 2009). With greater resources available I would also have measured levels of endogenous antioxidants, oxidative damage to DNA and proteins in multiple tissues (Costantini et al. 2008).

Sexual signals are often highly complex, involving multiple components. In birds, males are often both brightly ornamented with morphological sexual traits and perform elaborate songs and behavioural displays. Research suggests that females may pay greater attention to some of these cues, vary the number of cues that they use for mate choice and that interactions between multiple cues may obscure female preferences for single cues (Candolin 2003). Determining female preference could be further complicated if feather odour provides a signal of antioxidant status in mate choice (**chapter 6**). Reaching broad evolutionary and ecological conclusions on the role of oxidative stress in mediating life-history trade-offs is therefore complicated by the intricacy of mechanisms underlying the physiology of reactive oxygen species and antioxidants (including for example, the positive effects of ROS on cell signalling and

the complex interactions between androgens and antioxidants), the differing reponses of taxa to resource fluctuations and multiple cues in mate choice.

Some implications for antioxidant supplementation studies

Catoni et al. (2008) cautioned against the focus of behavioural and evolutionary ecology studies on carotenoid supplementations at the neglect of manipulations of the more potent, common, and potentially more important non-pigmentary antioxidants. The review argued that while the focus on carotenoids has generated many valuable insights into carotenoid-mediated animal signals, it has at the same time limited our understanding of the general relevance of antioxidants in evolutionary ecology and physiology. A large number of *in vitro* studies have identified both positive and negative interactions including competitive absorption between antioxidants with similar solubility and regenerative interactions between carotenoids and vitamin E (Catoni et al. 2008). Carotenoids may therefore only be effective antioxidants in the presence of vitamin E. A few recent studies have observed positive effects *in vivo* of antioxidant interactions on sexual signal expression at adulthood (Pike et al. 2007; Pérez et al. 2008). The importance of antioxidant synergism on early life history effects is underlined by our findings in **chapter 3** of higher growth rates in individuals supplemented with both carotenoids and vitamin E during early development. Supplemented individually carotenoids (**chapter 3**) and vitamin E (**chapter 3, 5**) had no effect on growth. In **chapter 3** I suggested that if significant competition for scarce resources, for example territories, existed that the allocation of resources to growth to increase competitive ability may have been more beneficial than allocation to other traits, i.e. secondary sexual traits. The focus on a single class of antioxidants would have missed these effects. The results of **chapter 3** also indicate the need for experimental studies to supplement antioxidants at different life history stages when the differing internal environment can change resource allocation priorities.

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