ROLES OF DIETARY ANTIOXIDANTS AND OXIDATIVE STRESS IN MEDIATING FITNESS RELATED TRAITS IN BIRDS

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Candidate's declaration

I declare that the work presented in this thesis is entirely my own unless otherwise stated and that it is of my own composition. MDA analysis throughout the thesis was carried out by Joanne Coffey at WALTHAM® Centre for Pet Nutrition. Comet assay in Chapters 5 and 6 was carried out by Clive Tregaskes at WALTHAM® Centre for Pet Nutrition. Molecular sexing was carried out by Aileen Adam. Analysis of heterophil to lymphocyte ratio in Chapter 2 was carried out by Lotta Ducaroir as part of her MRes thesis "Does diet during egg laying affect egg production and indices of chronic stress?"

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General abstract

Trade-offs occur where different physiological systems compete for the same limited resource. Reactive oxygen species (ROS) can potentially damage all endogenous cells and processes, and antioxidants help prevent this oxidative stress. Since dietary acquired antioxidants are a limited resource, there is growing interest in the roles of dietary antioxidants in mediating life-history trade-offs. During reproduction, mothers must trade-off investing antioxidants in reproduction, and retaining them for self maintenance. Improving maternal antioxidant condition may allow increased investment of antioxidants in chicks and/or in self maintenance during breeding. Outwith the breeding season animals may also trade-off antioxidants between roles in physiological systems associated with large amounts of ROS production. During exercise, energy metabolism increases production of ROS, and dietary antioxidants may remove these, protecting tissue associated with exercise and potentially improving exercise performance. Most previous studies of antioxidants in birds have focused on carotenoids, but the antioxidant functions of carotenoids have recently been questioned. In this thesis I studied the effects of antioxidants, with proven antioxidant function *in vivo* on a range of fitness related traits in birds. Specifically, I assessed the effects of dietary antioxidant availability on maternal and offspring phenotype and fitness in blue tits *Cyanistes caeruleus*. I also investigated the role of dietary antioxidants in reducing oxidative stress produced during exercise in adult captive budgerigars *Melopsittacus undulatus*.

Supplementing blue tit parents with α-tocopherol, a potent dietary antioxidant, before and during egg laying did not affect maternal condition, nor reproductive performance. Mothers supplemented with α-tocopherol produced significantly more daughters than control mothers. I initially predicted α-tocopherol supplementation may result in male-biased sex ratios, since an improvement in maternal condition may allow increased investment in the larger, more costly sex. I discuss potential reasons for this difference in sex ratio between control and α-tocopherol supplemented broods. Of course, the true test of maternal allocation decisions is in effects on phenotype and fitness of mothers and offspring alike.

Next, I cross-fostered half broods from α-tocopherol treated pairs with control treated pairs, to separate effects due to egg quality from those of rearing environment. Chicks from α-tocopherol treated egg parents were smaller on day three, than control chicks.

However, chicks from α-tocopherol eggs grew faster than controls, and by day 14 there was no difference in mass. The α-tocopherol chicks did not pay the expected oxidative cost of this increased growth rate, indicating efficient antioxidant systems in these chicks. There was no obvious benefit of this increased growth rate in α-tocopherol chicks, in terms of phenotype at fledging. Despite the difference in sex ratio previously described, daughters from α-tocopherol supplemented egg parents were not in better condition than control daughters. There was some evidence that male chicks reared by control parents had longer tarsi than male chicks reared by α-tocopherol parents. Interestingly, retrapping adults and F1 chicks the year following experimental supplementation suggested increased survival, and future reproduction of α-tocopherol supplemented mothers. The effect on F1 offspring was less clear, but we captured more chicks from α-tocopherol supplemented egg parents in winter two years following the experiment. There was no evidence that survival of adults was related to the sex ratio of their broods.

In the next experiment, I tested the Red Herring hypothesis. The Red Herring hypothesis suggests that carotenoids are unlikely to be used as antioxidants in nature. However, carotenoid mediated plumage traits may still reveal antioxidant levels as non-pigmentary antioxidants may increase expression of carotenoid mediated plumage colour, through antioxidative protection of carotenoid pigments. By supplementing chicks within a brood with either; α-tocopherol, carotenoids or control, I hoped to assess the roles of different antioxidants during development in blue tits, with particular reference to plumage colouration and oxidative stress. Contrary to the Red Herring hypothesis, α-tocopherol supplemented chicks did not have reduced oxidative stress compared to controls or carotenoid treated birds, as measured by malonidialdehyde (MDA), a by-product of lipid-peroxidation. Only chicks supplemented with carotenoids increased in plumage colour, thus I found no evidence that non-pigmentary antioxidants protect carotenoid pigments from oxidation and bleaching.

In addition to growth, another process that can lead to increased ROS production is physical activity. In captive adult budgerigars, I found a relationship between the propensity to engage in active behaviours during undisturbed observations, and a measure of DNA damage. This suggests individual differences in oxidative stress may be partly mediated by differences in activity rates. It was not possible to rule out a role for stress-related behaviour mediating this relationship. Interestingly, I also found a positive

relationship between DNA damage, and selection of an antioxidant rich food item. This has two implications; firstly, that birds may detect oxidative damage levels and react accordingly. Secondly, that dietary antioxidants may play an important role in alleviating oxidative stress. Of particular note, was the fact that three different measures of oxidative stress carried out for each individual did not correlate with one another. Different measures may therefore reveal specific types of oxidative damage, and selection of an appropriate test will be important when interpreting results relating to oxidative stress.

Flight is the most metabolically expensive behaviour in birds, and the most likely to result in oxidative stress. By subjecting relatively sedentary budgerigars to measured exercise, I manipulated an oxidative cost for each bird. I assessed individual differences in oxidative stress and take-off escape time in birds following both an enhanced or reduced quality diet. Birds always had reduced levels of oxidative stress on the enhanced quality diet, rich in antioxidants, regardless of diet order. This shows that flight activity can increase oxidative stress in birds, and that dietary antioxidants may ameliorate this. All three indices of oxidative stress used showed an effect of flight exercise. There was no difference in escape time on different diets, but differences in exercise performance may only be detectable in tests of stamina, or with longer experimental manipulation of diet and activity.

Using a captive population of wild-type budgerigars, I assessed the role of exercise training on oxidative stress. I found that birds had reduced levels of MDA after long-term flight training than after one day of flight training. This result was independent of diet quality. This shows that performing the same exercise task repeatedly attenuates oxidative stress, perhaps mediated by an upregulation of endogenous antioxidant enzymes. Whether or not such an upregulation in antioxidant activity is possible in wild birds is unclear; antioxidant activity may operate maximally at all times. This has important implications for all studies of antioxidants in life-history trade-offs.

I conclude that dietary acquired antioxidants are important nutrients, mediating a range of trade-offs in birds. The multifaceted effects of dietary antioxidants merit further investigation, to elucidate their precise role in determining oxidative stress, and ultimately fitness.

Chapter 1: General Introduction

1.1 Introduction

In all animals, trade-offs arise when a single resource is required for two or more physiological systems. How an organism manages these trade-offs will ultimately impact upon its fitness: the relative ability to survive and pass on genes to future generations (Jenkins, 1990). In the field of behavioural ecology there is considerable interest in identifying limited resources involved in fitness determining trade-offs. In order to achieve this, the factors affecting survival and reproduction in animals must be addressed. Oxidative stress has recently garnered much attention in this regard, and detrimental effects of oxidative stress have been implicated in almost all aspects of an animal's life, including ageing (Finkel and Holbrook, 2000), disease processes (Cui et al., 2004), cognition (Gotz et al., 1994), exercise performance (Urso and Clarkson, 2003) and reproduction (Alonso-Alvarez et al., 2004). Oxidative stress may thus represent a key fitness determinant. Differences in individual susceptibility to oxidative stress are likely to be mediated in large part by an individual's antioxidant capacity; a combination of endogenous antioxidant systems, augmented by exogenous, dietary acquired antioxidants, which remove damaging oxygen species (see below). An individual's ability to acquire and assimilate antioxidants may have widespread implications for its fitness.

An individual's fitness will be determined by the genes inherited from its parents and its environment, particularly the environment experienced early in development. An effect of maternal environment or behaviour influencing offspring phenotype or fitness, holding genetic sources of variation constant, is known as a maternal effect (Kirkpatrick and Lande, 1989). Such maternal effects are gathering much interest, as growing evidence suggests that early environment can have pervasive long-term effects on an animal's fitness (Lindström, 1999). Since oxidative stress will impact upon adults and offspring alike, mothers face a trade-off between retaining antioxidants for self maintenance, and providing antioxidants for their offspring. Mothers may provision antioxidants to both embryos, via the egg or placenta, and neonates via parental feeding. Therefore antioxidants offer an example of how a mother's condition (in this case her antioxidant availability) may determine the amount of resources available for her offspring. Revealing how these differences in maternal antioxidant conditions alter the fitness of both offspring and mothers, will have implications for our understanding of life history trade-offs.

Besides the trade-off between allocation of antioxidants between self maintenance and offspring provisioning, antioxidants may be involved in trade-offs between physiological systems at the level of the individual. Damaging oxygen radicals are ever present in all physiological systems, and there is a growing understanding of the roles of antioxidants in maintaining function of cellular and genetic processes. Despite this interest, the various roles of antioxidants within animals have only begun to be addressed. In this thesis I will investigate how dietary derived antioxidants are involved in mediating fitness determining traits in different life history stages.

In this PhD, I have focussed on two main themes of interest. Firstly, the effects of maternal antioxidant levels and antioxidant conditions in early development, on offspring phenotype and fitness. Secondly, the roles that dietary antioxidant availability play in determining individual differences in behaviour and exercise performance. In this introduction the nature of oxidative stress, and mechanisms of antioxidant protection will be described. Furthermore, the roles of antioxidants at different life history stages, and in different physiological systems will be discussed. Each data chapter has been written in the form of a manuscript, some of which (Chapters 4 and 6) have been submitted for publication. The aim of this general introduction is to provide a broad overview of the topics and species covered in the thesis. The general discussion will attempt to highlight key findings, and provide a synthesis of my conclusions.

1.2 Oxidative stress and reactive oxygen species

Before any discussion of oxidative stress, it is important to understand the mechanisms and nature of Reactive Oxygen Species (ROS), such as free radicals, and their effects. A free radical is the name given to any atom, molecule, or compound containing unpaired electrons. Free radicals are unstable and cause damage to lipids, proteins, DNA, and other biological complexes essential for normal body maintenance (Diplock, 1994). Exposure to ROS is inevitable for most organisms, since atmospheric oxygen crucial to most life, is also toxic (Knight, 1998). Within the body, mitochondria used for energy production, phagocytes in the immune system, and transition metal reactions, are just a few sources of radical production. External sources of radicals for many species include UV light, radiation and pollution (Furst, 1996). These sources of free radicals, ensure that the body is constantly under free radical attack. The greatest effect of free radicals is that of lipid

peroxidation (Surai, 2002). During lipid peroxidation, a peroxyl radical, produced by a carbon centred radical reacting with oxygen, will react with any peroxidizable molecule present in order to produce a more stable hydroperoxide molecule, and a new carbon centred radical. It follows that this newly produced carbon centred radical may in turn react with any oxygen, thus producing a chain reaction of lipid peroxidation, providing there is available oxygen and peroxidazable material. Polyunsaturated fatty acids (PUFAs), major constituents of cell membranes, are a major group of peroxidazable compounds. Thus a chain reaction, causing damage to PUFAs, can result in damage to cell membranes, affecting fluidity and permeability (Surai, 2002). In a similar fashion, free radicals can attack proteins and DNA. In fact, it has been estimated that ROS are responsible for around 10,000 base modifications a day, and oxidation is one of the important factors affecting DNA damage (Diplock, 1994). Genomic integrity is vital for all animals, thus oxidative damage is potentially a major fitness component. The effects of oxidative stress are gaining much attention, particularly in medicine, where a huge literature is available on the effects of oxidative stress in ageing, neurodegenerative disorders, cancers and other illnesses (Cui et al., 2004). However, it is important to stress that ROS are actually necessary for some physiological processes. ROS are produced by immune cells to destroy pathogenic organisms and cells as a normal part of the immune response (Bogdan et al., 2000). They may also function as modulators of internal processes: Nitrogen oxide (NO), itself a free radial, is an important transducer in smooth muscles, neurones and other cells (Lamas, 2004). ROS are involved as signal molecules during RNA transcription, and as second messengers in activation of transcription factors involved in apoptosis (Cui et al., 2004). During exercise a certain amount of ROS production is actually necessary for optimum muscle performance (Reid, 2001). Thus, it is not always the case that the body requires to rid itself entirely of ROS, since they can be important messengers and signallers in normal physiological systems. In summary, ROS can have positive effects, but the body requires defences to prevent oxidative stress and damage to DNA, proteins, and cells.

1.3 Antioxidants

There are a wide variety of antioxidants, acquired both from the diet, and synthesised internally: fat soluble antioxidants (e.g. vitamin E, vitamin A, carotenoids); water soluble antioxidants (e.g. Ascorbic acid, uric acid); antioxidant enzymes (glutathione peroxidase (GSP-Px), catalase (CAT), superoxide dismutase (SOD)); and thiol redox system (glutathione system, thioredoxin system) (Surai, 2002). In general, antioxidants can fulfil

several roles: as vitamins, as pigments, and as the name suggests, as protectors against oxidative stress. In preventing oxidative stress, antioxidants form a three-step defence system within animals to protect cells and processes: 1. prevention of ROS production, 2. removal of ROS already produced, and 3. the repair of oxidative damage caused by ROS (Surai, 1999). The second step, halting those free radicals that are produced, is the focus of this PhD. Fat and water-soluble antioxidants inhibit peroxidation by scavenging peroxyl radical intermediates. The most important of the antioxidants responsible for the second level of defence within cells is vitamin E, although other important antioxidants include vitamin A and C, carotenoids, and glutathione (Surai, 2002).

Vitamin E is the collective name given to several compounds sharing similar biological functions and molecular structures. Vitamin E molecules include α-, β-, γ-, and δ – tocopherol, and α-, β-, γ-, and δ – tocotrienol. The first to be discovered, α-tocopherol (see Figure 1.1) is largely seen as the reference for vitamin E, and also has the greatest biological activity (Machlin, 1991). Since its discovery, the function of vitamin E was elusive, though keenly investigated. Only recently has it been generally accepted that the main role of vitamin E *in vivo*, is as a free radical scavenging antioxidant, particularly in the protection of lipids by preventing the chain reaction of lipid peroxidation (Burton and Ingold, 1981). Thus, α-tocopherol is generally found within cell membranes *in vivo*. Importantly, it is now known that vitamin E can be recycled after oxidation, by a range of compounds (Kagan et al., 1992). Vitamin C (ascorbic acid), a water soluble antioxidant, appears particularly important in regenerating α-tocopherol, from an oxidised tocopherol radical (Kagan et al., 1992). Vitamin E is synthesized in plants and is found in leaves and seed, while animals cannot synthesize vitamin E, and must acquire it through the diet Despite the increasing understanding of some of the key benefits of vitamin E, and the recognition that vitamin E is the main membrane-bound antioxidant in nature, its exact role in all organisms remains unclear. There is a notable lack of information concerning vitamin E requirements and metabolism in birds.

Carotenoids, like vitamin E, are a class of lipophillic antioxidants, synthesized by plants, algae and some microorganisms, which animals must acquire through the diet. Carotenoids are the most numerous pigments in nature, with some 600 described, and are responsible for yellow, orange or red colouration in many plants and animals (Armstrong, 1997). They are grouped into two classes depending on their structure: xanthophylls, and carotenes. Major xanthophylls include lutein and zeaxanthin (see Figure 1.1), β-cryptoxanthin,

canthaxanthin and astaxanthin, and carotenes include β-carotene (See Figure 1.1), α-carotene and lycopene. Carotenoids are particularly interesting to avian biologists and behavioural ecologists since they are involved in several capacities *in vivo*. Their roles include plumage colouration, antioxidant systems, immunomodulation, vitamin A precursors, and cell signalling, amongst others (Blount, 2004). The antioxidant function of carotenoids has been demonstrated both *in vitro* and *in vivo* (for review see Surai, 2002), though the efficiency and relevance of carotenoids as antioxidants is unclear. As with all antioxidants, the antioxidant activity of carotenoids will depend on the cellular environment (Palozza et al., 1995), and interactions with other compounds (Polozza and Krinsky, 1992). There is increasing evidence that it is unlikely that carotenoids are used as primary antioxidants in nature, though this standpoint remains controversial (Hartley and Kennedy 2004; Costantini and Møller, 2008). Despite this lack of clarity, in avian studies carotenoids are often used to study the effects of "antioxidants" on various life-history parameters (Costantini and Møller, 2008).

1.4 Antioxidants and reproductive investment

An individual's phenotype is the result of its genotype plus the environmental conditions experienced throughout life, particularly during development (Mousseau and Fox, 1998). Environmental factors include food availability, temperature and climate, but will also include what is known as "maternal effects". A maternal effect is the name applied to any effect of maternal environment or behaviour that influences the phenotype or fitness of her offspring, holding genetic sources of variation constant (reviewed in Mousseau and Fox, 1998). In birds for example, maternal deposition of lipids, proteins, antioxidants, antibodies and hormones into the egg by mothers, are all capable of altering the offspring phenotype in some way (e.g. Schwabl, 1993; Blount et al., 2002; Grindstaff et al., 2003). Maternal investment into chick provisioning is another maternal effect, as is choosing a mate who is more (or less) likely to engage in parental care (Mousseau and Fox, 1998).

It has long been recognised that egg production by female birds must be costly, since resources required by the female for self maintenance will be diverted to supply the embryo for development within the egg. This theory has been supported by egg-removal experiments, in which one or more eggs are removed from a clutch in order to stimulate further egg production by the female (e.g. Nager et al., 2001). Females who lay more eggs have been shown to suffer increased parasitism, decreased ability to rear chicks and

decreased survival (e.g. Monaghan et al., 1998; Nager et al., 2001). It has recently been suggested that egg production may be limited by not only overall nutritional or energetic requirements, but also by the need for specific nutrients e.g. antioxidants. Antioxidants may protect and nourish the developing embryo, ensuring normal development. Since egg yolk is composed of a high concentration of polyunsaturated fatty acids, and is also necessary for the development of the embryo, antioxidants are necessary in order to prevent lipid peroxidation of yolk material (Cherian and Sim, 1997). Thus antioxidants are particularly important constituents of the eggs. Indeed, embryo growth will be associated with ROS production, and post-hatching there is likely to be a large hike in oxygen pressure and hence exposure to ROS (Vleck and Butcher, 1998). Antioxidant levels in eggs predict the levels in chick tissue (Surai et al., 1998) therefore yolk antioxidant concentration will protect against ROS, post-hatching as well as within the egg. Moreover, antioxidant levels may also influence gene expression during development (Allen and Venkatraj, 1992).

Perhaps the most striking feature of egg yolks is their intense yellow/orange colour, directly indicative of carotenoid levels. Carotenoids are a class of lipophillic antioxidants, and the large investment in egg carotenoids by birds has been suggested to indicate the high requirements for antioxidants during embryonic growth (Blount et al., 2000). Experiments have shown that during egg laying, the plasma concentration of carotenoids in female plasma decreases, and also that the concentration of carotenoids within the egg yolk is higher than that of the female's plasma (Blount et al., 2002). This suggests that carotenoids are specifically selected for deposition in yolk. To demonstrate that carotenoid deposition in eggs indicates an adaptive early maternal effect, first it must be shown that increasing carotenoids in the diet of the female will similarly increase carotenoid levels in the egg. Such a hypothesis has been tested and verified in a range of commercial and wild bird species (e.g. Surai and Speake, 1998; Blount et al., 2002) including in blue tits *Cyanistes caeruleus* and great tits *Parus major* (Biard et al., 2005; Berthouly et al., 2007). Such increased yolk carotenoids are capable of enhancing offspring quality in a range of ways (but see Remeš et al., 2007). For example, in blue tits, nestlings from carotenoid rich eggs had longer tarsi, enhanced plumage colour and a greater immuno-responsiveness than nestlings from control eggs. Similarly, in great tits, nestlings from carotenoid enhanced mothers were better able to mount an immune response following cross fostering, than nestlings of control females (Berthouly et al., 2007). Great tits chicks from carotenoid supplemented eggs also had enhanced growth rates compared to controls (Berthouly et al.,

2008). The effects of early environment may have important implications for long term phenotype (Lindström 1999; Metcalfe and Monaghan, 2001; Rhind et al., 2001), and thus antioxidant supply in eggs and chicks may represent an important fitness determinant. Indeed, early exposure to antioxidants appears to affect the long term ability to assimilate them (Blount et al., 2003; Karadas et al., 2005a). Thus conditions during early development may predict long term antioxidant levels.

All of the studies mentioned above have demonstrated apparently beneficial effects of increased carotenoid availability to birds. There can be no doubt that carotenoids are vital yolk constituents, improving several aspects of chick health and development. In one study it was demonstrated that increased carotenoid concentration of yolk led to decreased peroxidative susceptibility *in vitro* (Surai and Speake, 1998). However, there may be a problem in attributing all observed benefits of carotenoids during development to antioxidant function. Also, *in vitro* tests of oxidative susceptibility, or antioxidant capacity, do not discern the intended role of the carotenoids in tissue, even where increased carotenoid concentrations equates to an increased antioxidant capacity. As discussed above, carotenoids have several roles within birds, and few studies are able to separate their antioxidant effects from their other impacts. Thus, when investigating the roles of antioxidants in life history, nutrients with clearer antioxidant function, but fewer other roles in the body might be more suitable. Vitamin E is the major lipophillic antioxidant in most animal species, and although vitamin E can be used in other roles in some circumstances (for review see Azzi and Stocker, 2000) its primary function is in quenching ROS, and preventing lipid peroxidation. Indeed, vitamin E is another major constituent of egg yolk (Surai, 2002), though perhaps its lack of colour masks its ubiquity. For example, Biard et al's (2005) study of carotenoid availability on blue tit *Cyanistes caeruleus* reproduction, found carotenoid supplementation significantly increased carotenoid levels in yolk, but levels of vitamin E were still 10x higher than those of total carotenoids. Also, Surai et al. (1998), analysed yolk concentrations of various antioxidants in four commercial avian species and found considerably higher levels of vitamin E than all carotenoids in each species. Despite the wealth of information about the effects of carotenoids in reproduction, there are few studies of the effects of vitamin E availability during breeding in wild birds. Interestingly, data from commercial avian species has shown links between vitamin E during development and growth rate (Surai et al., 2002) and oxidative status (Lin et al., 2005). In chapters 2-4, I address the role of vitamin E availability during reproduction on maternal condition, reproductive effort and chick

development and phenotype, in a wild population of blue tits. Firstly, by supplementing adults with either a control or α-tocopherol enriched supplement, I will assess the effect of maternal α-tocopherol status on reproductive output, egg quality and maternal condition in chapter 2. In chapter 3, I will then assess the effects of the antioxidants via the egg on chick rearing, growth and phenotype, and on F1 recruitment and adult survival. In chapter 4, by feeding chicks within the same brood with either a control, or a α-tocopherol or carotenoid supplement, I will separate the different effects of the two different nutrients on chick development, phenotype and oxidative stress.

1.5 Antioxidants and somatic maintenance

Reasons why antioxidant supply may be of key importance during breeding have been addressed above, although the benefits of dietary antioxidants are not limited breeding birds. The roles of antioxidant in protecting somatic function, for example by reducing the deleterious effects of ROS that promote senescence and degenerative illnesses are of key importance throughout life (Finkel and Holbrook, 2000). Since antioxidants will protect cellular integrity, they may assist in multiple physiological systems in the body, especially those associated with a high metabolic rate, and therefore ROS production. For example, the brain is an organ extremely vulnerable to attack by free radicals since it has high levels of c20 and c22 polyunsaturated fatty acids and despite its size, consumes much of the oxygen inspired, in turn producing many ROS (Surai, 2002). In spite of this, in birds, the brain is known to have particularly low levels of some antioxidants: both vitamins A and E, as well as carotenoids and selenium are apparent in much lower concentrations in the brain than in other tissues (Surai et al., 1996). Research with rats has previously revealed that the extent to which lipid peroxidation occurs in the brain, depends on vitamin E (Meydani et al., 1988). Since oxidative damage can reduce brain function, and antioxidant supply appears to be capable of ameliorating this (Rao and Balachandran, 2002), antioxidant availability may mediate some cognitive capabilities. Brain function will be vital in animals whose life history depends on learning and memory. Indeed, in species where birdsong is a sexually selected trait, antioxidant protection of brain regions associated with song production may be an important fitness determinant (Nowicki et al., 2002).

Another source of increased ROS production occurs during exercise, whereby metabolism is increased. During exercise there are several more sources of ROS production; prostanoid

metabolism, xanthine oxidase, and exercise released catecholamines can lead to radical production, in addition to secondary sources such as macrophages used in tissue repair (Jackson, 2000). Although a certain amount of ROS production is actually necessary to maximise muscle performance (Jackson 1999, Reid 2001), overproduction of ROS may induce oxidative stress. Increases in oxidative stress caused by strenuous exercise have been reported in many species, including mammals and fish (Hartmann et al., 1995; Aniagu et al., 2006). Recently, experimental evidence showed an increase in oxidative stress with long flights in homing pigeons *Columba livia* (Costantini et al., 2008), though data on the oxidative costs of flight are generally scarce. In just one other study, indirect evidence was provided for an oxidative cost of prolonged flights in two migratory species (Costantini et al., 2007a). Although short term increases in ROS production are inevitable during exercise, there may be costs associated with chronic high oxidative stress. Owing to the damaging nature of ROS, it has long been supposed that exercise induced oxidative stress may eventually lead to a decrease in exercise performance. ROS may reduce muscle contractility, or alter the ability to generate muscle contractions through oxidative damage to ATPase pumps (Morris and Sulakhe, 1997; Lawler et al., 1998). Indeed, proteins forming muscles themselves may be susceptible to oxidative damage (Bailey et al., 2007), and oxidative stress may therefore reduce exercise performance through a variety of routes. Since antioxidants can reduce oxidative stress, there has been much interest in the effects of antioxidant supplementation on exercise performance, particularly in human athletes, though results are varied (for review see Urso and Clarkson, 2003). Flight in birds is a particularly strenuous form of exercise, associated with high energy demands and increased metabolism (Schmidt-Nielson, 1972). In birds, flight performance is likely to be a fitness determining component in many species, for example escape flight in prey species, or endurance flight in migratory species. The effects of dietary antioxidants on flight performance are far from clear, though in one study male zebra finches *Taeniopygia guttata* fed a high carotenoid diet showed improved flight performance compared with controls (Blount and Matheson, 2006).

In Chapters 5, 6 and 7 (and Appendix 1), I will use captive budgerigars to assess the role of dietary antioxidant availability on behaviour, flight performance, and oxidative stress. In chapter 5, the correlations between selection of antioxidant rich food items, levels of individual oxidative stress, and different aspects of behaviour will be addressed. In Chapter 6, I will test whether dietary antioxidant availability influences flight performance, or post-

flight oxidative stress, in adult budgerigars. In Chapter 7, I will consider the role of a training effect on oxidative stress generated by flight.

1.6 Study species

To assess the role of maternally derived antioxidants in mediating offspring phenotype, we used a wild population of nesting blue tits at Loch Lomond in Scotland (see Study Sites). To assess the effects of dietary antioxidants on adult exercise induced oxidative stress we used captive budgerigars at WALTHAM® Centre for Pet Nutrition, and at the University of Glasgow. The main features of these species, pertinent to these investigations will be discussed below.

Blue tits Cyanistes caeruleus

Blue tits belong to the family Paridae, a sub order of the oscines. Blue tits, and their close relatives, great tits (*Parus major*) are perhaps the most studied species of wild birds. Reasons for this intensive study are probably twofold: firstly, blue tits are widely distributed over much of continental Europe. Secondly, their ready use of nestboxes, and relative resistance to disturbance, make them amenable to scientific investigations. The indigenous population in Britain is recognized as a distinct subspecies (*Cyanistes caeruleus obscurus*) to the rest of continental Europe (*Cyanistes caeruleus caeruleus*). Blue tits have a widespread distribution, and are found in many habitats, including urban gardens and parks, and coniferous woodlands, although their primary habitat is deciduous woodland (Perrins, 1979). Blue tits lay large clutches, amongst the largest for an altricial bird, with an average clutch size of 10-12 eggs (Cramp and Perrins, 1993). They lay eggs at a rate of one per day, and begin incubation on clutch completion (Perrins, 1979).

Blue tits make a particularly suitable model for studies of trade offs mediated by dietary antioxidants. Although blue tits eat a wide variety of foods, they will generally eat insect and spiders where available (Cramp and Perrins, 1993). During the breeding season, their diet is entirely composed of invertebrates, particularly lepidopteran larvae. Indeed, lepidopteran larvae can comprise more than 90% of the diet during breeding in oak woodland (Betts, 1955). This reliance on caterpillars means that reproductive success of blue tits is dependent on the ability to raise chicks during times of peak caterpillar availability (Perrins, 1991). In addition to the energetic resources provided by caterpillars,

they are also rich in antioxidants (Partali et al., 1987). In particular, caterpillars are rich in carotenoids and α-tocopherol (Arnold and Ramsay, unpublished). The yellow chest plumage of blue tits is mediated by carotenoid pigments, and the trade-offs between the roles of carotenoids as pigments and in other physiological systems have attracted much attention (Olson and Owens, 2005). Many studies have demonstrated positive correlations between carotenoid availability, plumage colouration, and fitness determining traits in blue tits (e.g. Senar et al., 2002; Hidalgo-Garcia, 2006). However, despite their α-tocopherol rich diet, the effects of α-tocopherol during breeding have been untested in this, and many other avian species.

Budgerigars Melopsittacus undulatus

Budgerigars are small, broad tailed parrots, belonging to the sub family Platycercinae of the family Psittacidae. They are commonly kept as pets throughout the world, however, apart from some feral populations, in the wild they live in drier regions of Australia. Wild budgerigars live in small flocks, though in favourable conditions may form large flocks. Flocks of budgerigars live a nomadic lifestyle, travelling vast distances, dependent on water and seed availability. This nomadic lifestyle makes studies of wild budgerigars difficult, with few published studies. Most knowledge of the ecology of wild budgerigars comes from the notes of early naturalists. It was shown that the diet of wild budgerigars was almost exclusively composed of seeds (Wyndham, 1980), and there was no evidence of a requirement for other foods during breeding. However, studies of captive budgerigars have revealed birds frequently show clinical signs of deficiency of key antioxidants such as vitamin A (Earle and Clarke, 1991). Since seeds are naturally low in many antioxidants, budgerigar seed mixes often contain antioxidant supplements. Budgerigars have been bred for the pet trade since around 1850. Domestic budgerigars bred for the pet trade (aka "English" budgerigars) are typically larger than their wild-type counterparts. However, these domestic budgerigars have not been selected for elaborate traits unlike show budgerigars and their long lifespan suggests selection on an efficient antioxidant system should have been maintained.

In this project, I chose to use budgerigars as a model for the effects of exercise behaviour on oxidative stress. Since budgerigars are a long-lived species for their body size, living up to 15 years in captivity, they may have extremely efficient antioxidant systems. In addition, their natural diet appears to be relatively low in some antioxidants, thus we may

expect antioxidant absorption to be particularly efficient. Flight performance may be a potentially fitness modifying trait in this species, since wild budgerigars are required to fly long distances. In addition, as a prey species for aerial and terrestrial species, budgerigars may rely on fast vertical take-off to avoid predation. Using captive animals for this study was appropriate for several reasons. Firstly, studying captive animals allows a precise manipulation of dietary factors. Next, levels of oxidative stress are likely to be mediated by differences in age, which are often unknown in wild adult animals. Also, to assess diet related differences in exercise performance, repeated measures from one individual control for individual differences in e.g. body size, likely to affect exercise.

1.7 Study sites

The blue tit fieldwork in chapters 2-4 was carried out in woodland surrounding, and near to, the Scottish Centre for Ecology and the Natural Environment (SCENE) at Rowardennan, on the east side of Loch Lomond, in west Scotland (see Figure 1.2). All the fieldwork carried out in chapters 2 and 3 was in Ross Woods and Sallochy Woods, in an area of woodland directly around SCENE. In this location used a nestbox population comprising 251 boxes. These nestboxes were deployed between 1991 and 1993. The majority of nestboxes used are “woodcrete” boxes (Schwegler Vogel- & Naturschutzprodukte GmbH, Schorndorf, Germany) with a domed cylinder shape, and 32mm diameter entrance holes. There are additional larger nestboxes, rectangular in shape though also with a 32mm entrance hole. The average height above ground of these nestboxes is 2.78m, and all nestboxes are hung close to the trunk of a tree, and face a variety of directions. The woodland used in this study are close to Loch Lomond itself, and also a quiet road. In addition, the West Highland Way, a popular walking route in Scotland, cuts through the field site. However, during the blue tit breeding season, disturbance of the habitat is low. A map showing location of nestboxes in these woods can be seen in Figure 1.3. In chapter 4, we utilized another nestbox population in woodland around Cashel farm, 2 miles south of SCENE (see Figure 1.4). In this location there are 81 nestboxes, all of the smaller cylindrical form

The woodland at both field sites is very similar and both comprise part of Loch Lomond & the Trossachs National Park. The majority of the 50,000 hectares of woodland and forest in the park consists of commercially planted coniferous species but the park also retains extensive semi-natural woodlands. The east shore of Loch Lomond where both study sites

are located, is an extensive area of oakwoods (*Quercus petraea* and *Quercus robur*) designated as a Site of Special Scientific Interest and a Special Area for Conservation (Bell, 2005). The woodland around SCENE are managed by the Forestry Commission. The woodlands around Cashel are managed by the Royal Scottish Forestry Society, and are part of a native woodland regeneration project "The forest for a thousand years".

The nestboxes at both sites are occupied by four species of breeding passerines. The predominant two species are blue tits (*Cyanistes caeruleus*) and great tits (*Parus major*). In addition, small numbers of pied flycatchers (*Ficedula hypoleuca*) and redstarts (*Phoenicurus phoenicurus*) also utilize the nestboxes. In all years since deployment in the woodland around SCENE, blue tits have been the most common nesting species, and numbers of breeding pairs have increased from 60 in the mid 1990's to 120 in 2005. The date of first egg laying has also varied from 29th of April in the 1990's to 9th April in 2005.

1.9 Ethical notes

All procedures in this thesis were carried out under Home Office licence. Each experiment was subject to an ethical review at WALTHAM® Centre for Pet Nutrition and the University of Glasgow before being carried out. Care was taken to minimize any potential stress or suffering, and none of the treatments or experiments resulted in injury or illness to a study animal.

1.10 Thesis content

In this thesis I aim to assess the roles of dietary antioxidants in mediating fitness-related traits in birds. I focussed on two themes of investigation: the effects of maternally derived α-tocopherol in mediating offspring phenotype, and fitness in blue tits, and the impacts of dietary antioxidant supplementation on oxidative stress, and flight behaviour in adult budgerigars

In **Chapter 2** I manipulate α-tocopherol availability during breeding in blue tits, to evaluate its impacts on reproductive output or offspring sex ratio. In **Chapter 3**, I then assess whether the chicks from α-tocopherol supplemented or control parents from Chapter 2, differ in development, oxidative stress or phenotype. Following this, in **Chapter 4** I dose blue tit chicks within the same brood with either a carotenoid, α-tocopherol, or control food supplement. I then investigate the different effects of the two dietary antioxidants on chick phenotype, with particular reference to plumage colouration and oxidative stress.

In **Chapter 5**, using correlational data from adult budgerigars I examine the links between oxidative stress, diet choice and unmanipulated behaviour. I then go on to experimentally manipulate exercise in the same domesticated budgerigars by initiating escape flights in **Chapter 6**. By assessing individual differences in flight performance and lipid peroxidation on an enhanced or reduced quality diet, the effects of dietary antioxidants on oxidative stress and physical performance will be revealed. In **Chapter 7** the influence of exercise training and dietary antioxidants on levels of oxidative stress will be examined in captive wild type budgerigars. Finally, I will bring together all of my results, and discuss the importance, problems and implications of all my experiments in **Chapter 8**.

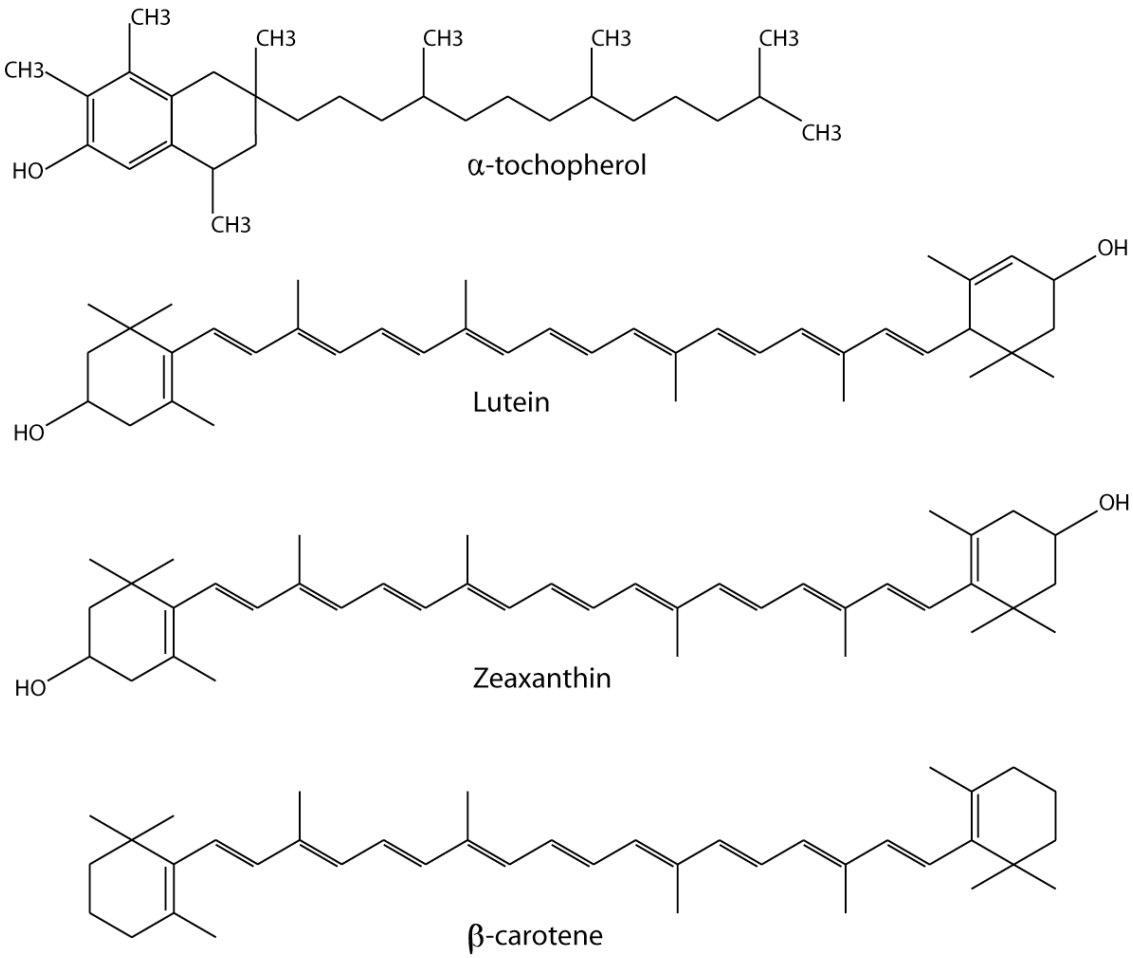

Figure 1.1 Structure of some dietary derived lipophillic antioxidants.

Figure 1.2 Location of Loch Lomond and SCENE in Scotland (from http://www.gla.ac.uk/departments/scene).

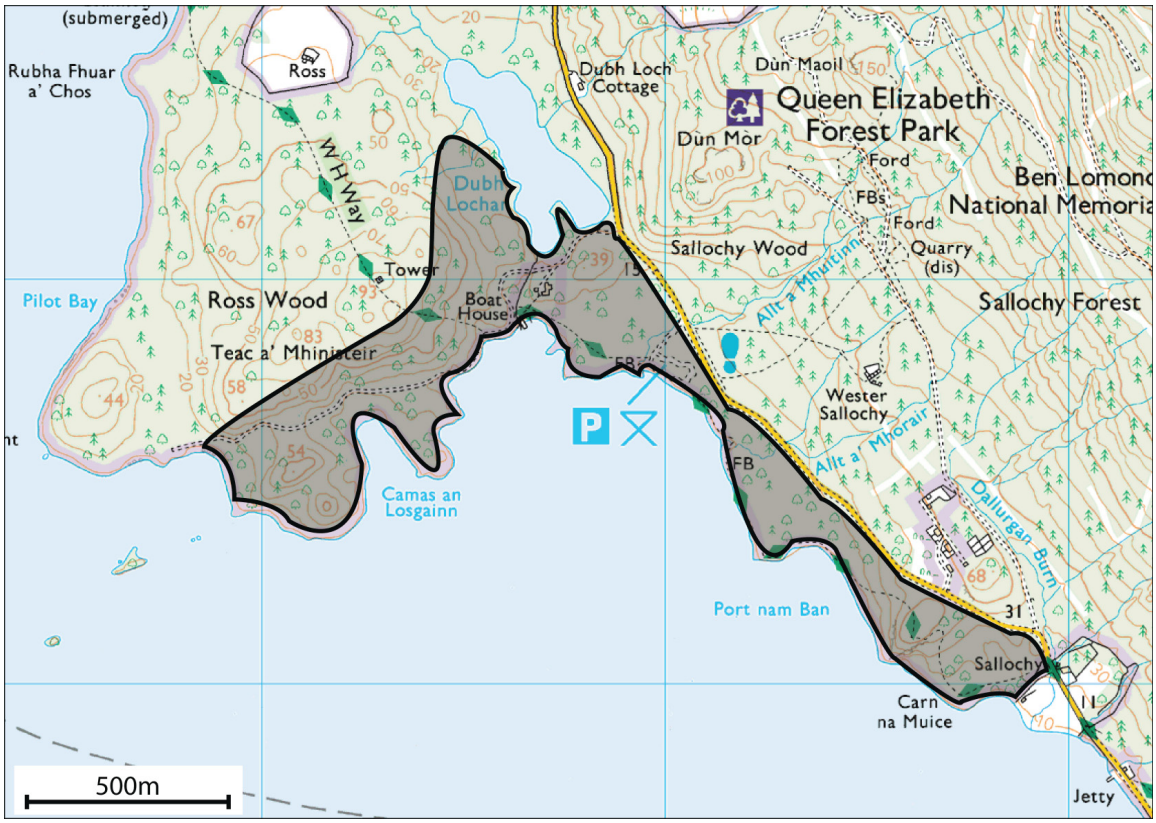

Figure 1.3 Map showing study site around SCENE on east coast of Loch Lomond, Scotland.

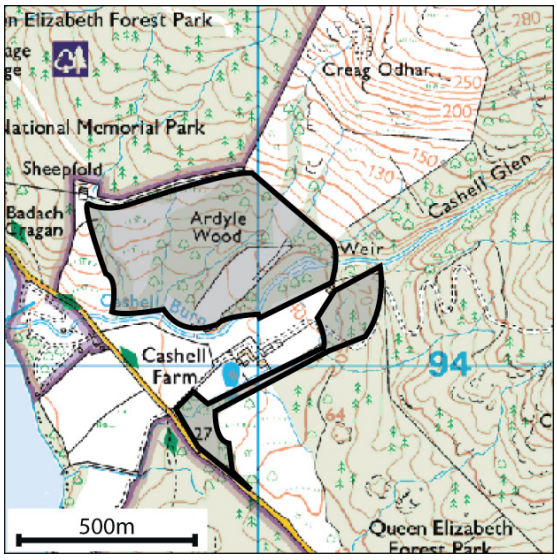

Figure 1.4 Map showing study site around Cashel Farm.

Chapter 2. The effects of α-tocopherol supplementation on maternal condition, reproductive effort, and brood sex ratio in blue tits *Cyanistes caeruleus*

2.1 Abstract

Breeding is a costly time for mothers, involving investment of energy and nutrients in offspring production and provisioning. Thus maternal condition is predicted to influence reproductive output. The metabolic costs of producing and caring for offspring are likely to involve an increase in the production of reactive oxygen species (ROS), which may cause lipid peroxidation, damaging tissue and cellular processes. Since dietary antioxidants may ameliorate this oxidative stress associated with breeding, mothers supplemented with additional dietary antioxidants may be in improved condition, and invest more resources in offspring. Providing more antioxidants to mothers, may also allow increased investment of antioxidants in eggs. These antioxidants would protect developing chicks from the damaging effects of ROS produced during growth. The major lipophillic antioxidant preventing lipid peroxidation is vitamin E, of which α-tocopherol is considered the most biologically active form. In this experiment I provided blue tit parents with either an α-tocopherol enriched, or a control food supplement during nest building and egg laying. I then examined measures of reproductive effort: average and total volume of eggs laid, hatching success, and incubation duration. I also removed the fifth laid egg from every nest to examine antioxidant deposition in yolks between mothers receiving control or α-tocopherol food. A further aspect of an ovum a mother can potentially manipulate is its sex. In line with theories of adaptive sex allocation I predicted that if α-tocopherol supplementation significantly improves maternal condition, she would benefit from overproduction of sons the more "expensive" sex. Males are faster growing and larger in blue tits, and may have increased nutritional demands during development. There were no detectable differences in reproductive effort between treatment groups, and no differences in yolk antioxidants. In contrast to my prediction, I found that mothers supplemented with α-tocopherol produced significantly more daughters than controls. I discuss potential reasons for the difference in sex ratio between treatment groups, and conclude that its adaptive significance cannot be addressed without considering chick development, phenotype and fitness. The true test of maternal allocation decisions is in assessing differences in phenotype and survival, of mothers and offspring alike.

2.2 Introduction

During breeding, the availability of nutritional resources will determine how an individual balances investment in the current reproductive attempt against investment in self maintenance, and future reproduction. Studies in which birds have been induced to lay larger clutches than "planned" have demonstrated that there is a cost to both mother and offspring of laying and rearing more young (e.g. Monaghan et al., 1998; Nager et al., 1999). Thus female condition is predicted to be an important determinant of reproductive output. In addition to the energetic costs of egg formation, females may deposit key substances into the yolk sac, albumen and shell which protect and nourish the developing chick. Hormones, immunoglobulins and antioxidants, are all reported yolk constituents, in addition to energy rich lipids (e.g. Royle et al., 1999; Grinstaff et al., 2003; Schwabl, 1993). By selectively incorporating more, or less, of a specific nutrient or hormone into eggs, a female may significantly alter the development and potentially the fitness of her offspring. Rapidly growing embryos produce high quantities of reactive oxygen species (ROS), as a by-product of high metabolism. Also, egg yolk is particularly high in polyunsaturated fatty acids (PUFAs), which are highly susceptible to attack by ROS (Cherian and Sim, 1997). Adequate antioxidant deposition into yolk is therefore vital to ensure normal development of chicks, particularly since antioxidant levels cannot be adjusted until after hatching. Many studies on avian taxa of commercial importance have demonstrated that levels of antioxidants in egg yolk have a significant bearing on levels of antioxidants in chick tissues, such as blood, brain and livers (e.g. Surai and Speake, 1998; Surai et al., 1998). By allocating extra antioxidants into yolk a female may improve the growth rate, health or condition of her chicks. In one study, carotenoid supplementation of adult blue tits during egg laying caused an increase in carotenoid levels in yolk (Biard et al., 2005). Chicks hatching from carotenoid supplemented eggs were larger, more brightly coloured and had enhanced immunity (Biard et al., 2005). As carotenoids have multiple roles within the body, in addition to their antioxidant function, it is difficult to assess the mechanism of observed effects. In order to tease out the role of antioxidants *per se* in neonatal development, I need to use a substance with proven antioxidant properties, and no direct role in pigmentation or cell signalling. Here I investigate the role of the antioxidant α-tocopherol, a form of vitamin E, in maternal allocation decisions and embryonic development. Vitamin E is the major lipophillic antioxidant involved in membrane defence (Tappel, 1962), and deficiencies in vitamin E are associated with a range of illnesses and disorders in many taxa (Zingg, 2007). Data from poultry science suggest widespread

beneficial effects of supplementary vitamin E for birds (Surai, 2002), though data for non-commercial species are scarce. I predict that if dietary antioxidant availability limits egg quality to a major extent, then individuals supplemented with α-tocopherol may lay larger clutches than non supplemented birds, and/or deposit more α-tocopherol into each yolk.

Another characteristic of an avian ovum, potentially manipulable by avian mothers, is its sex. The ability of birds to adjust the sex of their offspring is becoming increasingly clear (Pike and Petrie, 2003). Such sex ratio manipulation has attracted much attention, and several models for adaptive sex ratio manipulation have been suggested (for review see Cockburn et al., 2002). Trivers and Willard (1973) hypothesized that deviation from even sex ratios may occur in relation to resource availability, such as food. In the situation where the reproductive value of one sex benefits more from parental investment than the other, higher quality females should overproduce the more responsive sex (Trivers and Willard, 1973). Thus, the nutritional status of mothers may affect the sex ratio of their broods: females in better nutritional condition may produce a greater proportion of the more environmentally sensitive sex. α-tocopherol has never been implicated in sex ratio manipulation, but I predicted that mothers fed supplementary α-tocopherol should produce an excess of sons. Firstly, α-tocopherol may improve condition of mothers, allowing greater investment in parental care. Since male blue tits grow faster than females, and are larger, they are likely to have higher nutritional demands during development than females. Mothers in better condition will be better able to meet these demands than mothers in poor condition. Secondly, mothers receiving α-tocopherol may have increased availability of α-tocopherol for deposition in yolks. Sons are faster growing than daughters, and have higher levels of testosterone, both of which are likely to increase their exposure to ROS (Alonso Alvarez et al., 2007a). Thus male neonates may benefit more from increased yolk antioxidants than females.

There are several mechanisms by which maternal condition during breeding may be improved by dietary antioxidants. Reproduction is associated with increased metabolism, and a possible increase in oxidative stress at almost every stage, and oxidative stress may represent a major cost of reproduction (Alonso-Alvarez et al., 2004). Reproduction may increase levels of physiological stress suffered by mothers (Romero et al., 1997), and the links between stress response and oxidative stress are well documented (e.g. Şahın and Gümüşlü, 2007). Glucocorticoid release in response to stress has been documented in birds (e.g. Romero et al., 1997). One associated action of corticosterone, the most common avian

glucocorticoid, is in mobilization of energy substrates i.e. glucose. Increases in plasma glucose concentrations have been demonstrated in response to some stressors in birds (Scope et al., 2002; Remage-Healey and Romero, 2002), though in general data for wild birds are scarce (Ruiz et al., 2002). I predict that mothers supplemented with α-tocopherol, may eliminate some of the oxidative costs of reproduction and have improved condition Thus, I predict α-tocopherol supplemented mothers should have reduced levels of blood glucose, compared with controls. Additionally, oxidative stress has been implicated in a variety of pathways leading to immune suppression (e.g. Otsuji et al. 1996) and many studies have demonstrated a link between increased reproductive effort and immune suppression (Deerenberg et al. 1997; Nordling et al. 1998; Stjernman et al. 2004). The ratio between heterophils and lymphocytes (H/L ratio) is an indicator of immune suppression, and is known to increase in response to various stressors in wild bird populations (Hõrak et al. 1998; Kilgas et al. 2006). Leukocytes form the basis of the immune system and two of the main types of leukocytes are lymphocytes and heterophils. Heterophils are non-specific immune cells and their lysis during inflammatory response can be harmful to host tissue, whilst lymphocytes are always highly specific and cause no damage to host tissue (Ots and Hõrak, 1998). If α-tocopherol is capable of significantly improving female condition during breeding, we may expect birds with greater access to dietary α-tocopherol to be more capable of resisting a depression in immune function. I predicted that following egg laying and hatching, females supplemented with α-tocopherol would have lower H/L ratios, and lower concentrations of glucose in plasma, than birds receiving a control supplement.

In this study I wished to assess the effects of α-tocopherol supplementation of parents during nest building and egg laying, on maternal condition, reproductive effort and offspring sex ratio in a wild population of blue tits, *Cyanistes caeruleus*. α-tocopherol is the reference vitamin E molecule, and has the greatest biological activity (Machlin, 1991). For a population in Scotland, I provided feeders containing either lard enriched with α-tocopherol, or a lard only control, from halfway through nest building until clutch completion. When the eggs had hatched and chicks were 5 days old, I caught, measured and blood sampled each breeding female in order to test for treatment effects on female condition, H/L ratio, and blood glucose levels. Specifically I asked whether compared with a control, α-tocopherol supplementation: 1) improves female body condition or indices of stress and immune suppression; 2) increases reproductive effort; 3) alters yolk antioxidant deposition; and 4) adjusts the primary or secondary sex ratio of offspring?

2.3 Methods

This study was carried out in spring 2006 using nestboxes in Ross Woods around SCENE, Rowardennan, Loch Lomond. Full details of this study site can be found in the general introduction. Nest boxes were monitored at least every two days during nest building, and egg laying to record the stage of nest building, resident species, dates of egg laying, start of incubation and dates of hatching.

Supplemental diets

A total of 94 blue tit pairs (47 control and 47 α-tocopherol) were randomly assigned to the feeding trial, although some were excluded later, due to failure. Pairs were provided with either control lard or α-tocopherol enriched lard from the time that their nest was half built, until the start of incubation. Nests were defined as half built when nest cup construction had started, though prior to the addition of lining. Each nest was provided with approximately 125 grams of lard and the lard was changed every two days, to ensure that lard remained fresh and that the α-tocopherol did not oxidize. All food supplements were prepared the night before use. The control treatment was prepared by heating blocks of fresh lard and pouring the melted lard into foil lined moulds. These were left to cool and then stored in a -20 freezer overnight. The α-tocopherol treatment was prepared by melting the lard and first leaving it to cool to a thick liquid consistency, before adding α-tocopherol acid succinate (Sigma, Poole, UK) at a concentration 250 milligrams per kilo of lard. The lard was cooled before addition of the α-tocopherol to avoid the possibility of the α-tocopherol being denatured. I worked on an assumption that birds would eat approximately 1g of lard a day, and therefore α-tocopherol supplemented birds would receive ~ 0.25mg of α-tocopherol, within a biologically relevant range for this species (Arnold and Ramsay, unpublished data). This was thoroughly mixed using a hand held blender before being poured into moulds and stored quickly at -20ºC, to ensure the α-tocopherol did not drop out of the lard mix, and to store lard prior to use.

Lard was chosen as a vehicle owing to its palatability to blue tits and its low nutritional content compared to other hard fats (www.Nutrition.gov, US National Agricultural Library), it was also heated thoroughly and re-set to denature any antioxidants present prior to use (see Table 1 for information on the nutritional content of lard). I also followed

8 unmanipulated nests, where an empty feeder was placed next to the nest, but no food was provided throughout the course of the experiment.

The day after a nest was found to be quarter built, defined as a ring of moss in the bottom of the nestbox but with the floor centre still visible and uncovered, an empty feeder was placed on a branch, sapling or on the trunk of the tree within three meters (but usually less than 1.5m) of the nest box on the following day in order to habituate the birds to the feeders. Blue tits are territorial and within this area, will exclude conspecifics from the feeder. All feeders were identical 130x130x50mm green mesh suet feeders (Haiths, Cleethorpes, UK). When the nests were half built, the nest box was allocated at random to either control or α-tocopherol treatment. The appropriate food supplement was placed into the feeder the following day. Thereafter, the food was changed every two days during egg nest building and egg laying. When incubation commenced, and no new eggs had been laid for two days, the feeder was removed and the nest was not visited for ten days during incubation. After ten days the nests were checked daily for hatching. I classified Day 1 as when at least half of the eggs in the clutch had hatched. At the end of egg laying a subset of feeders were observed for two hours, to check that the target birds were consuming the food supplements.

Egg measurement and collection

When laying commenced, eggs were numbered with non-toxic, permanent ink to allow egg identification. I removed the fifth laid from each nest, on the day it was laid. This egg was then replaced with a dummy egg so females were not induced to lay an extra egg. The egg was kept chilled and taken immediately to a freezer where it was stored at -20ºC until analysis. The lengths and widths of all eggs were measured using vernier callipers to within 0.05mm. Egg volume was calculated using the equation $V = 0.51 \cdot LB^2$ (Carey, 1996).

Adult measurements

Adult birds were caught, blood sampled and measured when their chicks were five days old (or occasionally six). With chicks this age, adults are unlikely to desert the nest. Birds were caught in the nestbox when feeding or brooding the young: when the bird entered the nestbox, the hole was plugged and the bird was retrieved from within. Females were

distinguished from males by the presence of a brood patch. Within three minutes of capture, a small volume of blood was taken by venipuncture from a wing vein. The first drop of blood was used for glucose testing and to make two air-dried blood smears. We used a "blood glucose monitoring system" (OneTouch Ultra, Lifescan UK, Bucks, UK), whereby the end of a test strip connected to the glucose monitor was held against the puncture to collect a small drop of blood. Such glucose monitoring systems are used by human diabetics, but have also been used in studies of avian stress (e.g. Ruiz et al., 2002). All glucose readings and blood smears were taken within three minutes of capture, since indices of stress can change very quickly in response to handling (Le Maho et al., 1992). The repeatability of glucose readings within individuals was poor (Pearson correlation; 0.332, n = 5, p = 0.585) from blood taken a few minutes apart, although the sample size was very small. A small drop of blood was taken in ethanol for subsequent DNA extraction. Following blood sampling, the birds had their wings and tarsus measured, and using a field balance they were weighed to within 0.1 gram. Total handling time was minimized and most birds were released within ten minutes. For each bird, condition was calculated as the residuals from the regression of Ln(mass) on 3*Ln (tarsus).

Assessing H/L ratio

Before examination, the blood smears were stained with a Giemsa stain to allow differentiation and identification of different blood cells. The Heterophil to Lymphocyte (H/L) ratio was determined by examining the air dry blood smears under a light microscope at 1000× oil immersion. A total of at least 200 leucocytes were counted systematically (to avoid counting the same cell twice) including lymphocytes, heterophils, monocytes, basophils and eosinophils. The number of heterophils counted was then divided by the number of lymphocyte counted to obtain the H/L ratio. For each bird the better quality of the two blood smears was analysed, and eleven slides were examined twice in order to assess repeatability. H/L ratio was highly repeatable within individuals (Pearson correlation; 0.754, n = 11, p = 0.007). Edges of the smear were avoided for this examination, since this tended to include a larger concentration of lymphocytes compared to the rest of the smear.

Egg yolk antioxidant analysis

To analyse any difference in the antioxidant content of yolks from eggs laid by birds receiving different treatments, I used the fifth egg from each clutch for HPLC analysis. I used the fifth laid egg as most birds will lay at least five eggs, and this would allow birds time to access the feeder and assimilate antioxidants for deposition in yolks. Eggs were frozen at -40ºC until extraction took place. Eggs were removed from the freezer and their shells were removed with tweezers. The egg was then left to thaw until the albumen around the yolk had melted, leaving a frozen yolk. A dissecting needle was used to impale the yolk which was then rubbed over tissue paper until all albumen was removed. The yolk was then weighed to the nearest 0.001g, for us to test for an effect of treatment on yolk size. The yolk was then placed in an eppendorf and an equal volume distilled water was added to each (i.e. 0.222g yolk = 0.222ml water). The yolks were then homogenised.

Next, antioxidants were extracted from the yolk: 0.7ml of 5% NaCl was added to a 15ml plastic centrifuge tube. 200µl of yolk water solution was then added, before vortexing for 30 sec. 1ml of ethanol was then added, followed by 100µl of 4µg/ml astaxanthin solution, to act as an internal standard and allow identification of problems during extractions. Astaxanthin is not present in the yolks of tits. The tube was again vortexed for 30 sec. 2 ml hexane was added to each tube before vortexing again, then tubes were centrifuged for 10 min at 5000rpm. Using a glass Pasteur pipette, the top layer of hexane extract was added to another 15ml centrifuge tube. This tube was then stored in a fridge. 2ml hexane was then added to the original tube and this was homogenised for 30 sec, washing the homogeniser in distilled water between samples. The tube centrifuged as before, and the top layer of hexane was pipetted into the appropriate refrigerated tube. The hexane extract from each tube was then divided equally into three eppendorf tubes which were placed in a SpeedVac for 20 mins. After this the final extraction was dissolved in 300µl DCM and 300 µl methanol. These final extracts were then stored at -80C until processed by HPLC.

A Spectra Model 8800 HPLC pump system with a Phenomenex 250mm x 2mm id column was employed to determine antioxidant composition of each sample. I used HPLC at a flow rate of 0.2ml/min with a mobile phase of water/acetonitrile (2.5:97.5), and water/ethyl acetate (2.5:97.5) in a gradient elution. Using a Diode array absorbance detector type Thermo model UV6000, I detected carotenoids by absorbance at 445nm. α-tocopherol was detected and quantified using a Waters 474 Scanning Fluorescence Detector. Peaks were

identified by comparison with chromatography and retention times of several standards (Sigma, Poole, UK; Fluka, Gillingham, UK, Carotenature, Lupsingen, Switzerland). Owing to problems during HPLC analysis our final sample of eggs was reduced to 12 control and 12 α-tocopherol.

Sexing chicks

When the chicks were 14 days old they were brought into the SCENE field laboratory for blood sampling and measuring. Half of each brood was taken from the nestbox in a heated bag. Each bird was removed from the bag and blood sampled immediately, a small volume of blood was taken by venipuncture from a wing vein. One drop of blood was put in ethanol for subsequent molecular sexing (Griffiths et al., 1998; Arnold et al., 2007).

Statistics

Measures of female condition, reproductive output and yolk antioxidant concentrations were analysed using general linear models in SPSS v14 (SPSS Inc, Chicago, IL, USA). Treatment was entered as a fixed factor model, and hatching date as a covariate in every model. Since some birds took less time to complete nest building and begin egg laying following the treatment, the number of days of treatment before egg laying commenced was also ranked, and entered as a covariate in models. In the same way I entered the total duration of treatment into all models except explaining clutch size (feeders were removed when egg laying was complete, and so clutch size is inextricably linked with treatment duration). Initially, the interactions treatment*hatching date and treatment*treatment duration were included to account for treatment differences at different points in the breeding season, or after receiving the treatment for longer. However these terms were never significant and therefore were not included in final models. Each final model included:

Fixed factor: treatment
Covariates: hatching date, treatment duration,

To assess female body condition I used a univariate GLM with condition as a dependent variable. To assess female stress I used a multivariate GLM with H/L ratio and plasma glucose levels as dependent variables, with the additional covariates female body

condition, female body mass and total clutch volume. To assess reproductive effort I used a multivariate GLM with total clutch volume and egg number as dependent variables, with female body mass and female body condition as additional covariates. To assess egg quality firstly I used a multivariate GLM with average egg volume and yolk volume (5^{th} egg) as dependent variables, with additional covariates female body mass and female body condition. I analyzed yolk antioxidants using a multivariate GLM with total yolk carotenoid and total yolk tocopherol concentration as dependent variables, with additional covariates female body mass, female body condition, and total clutch volume. Incubation duration was assessed using a univariate GLM with additional covariates total clutch volume, female body mass and female body condition. Non-significant terms were excluded from the model by backwards-stepwise elimination, starting with non-significant interactions. Only significant interaction terms are reported.

I analysed the sex ratio of each nest using the number of males/number of offspring in a generalized linear model with a binomial error distribution, using SAS version 8 (SAS Institute Inc., Cary, NC, USA). In the same way I analysed hatching success as hatched eggs/total eggs (minus the collected 5^{th} egg), and fledging success as fledged chicks/hatched chicks. Within some nests, broken eggs or dead chicks were removed, or in some cases no embryonic tissue could be retrieved from unhatched eggs, meaning there was incomplete data to assess sex ratio (20 complete/56 total). To analyse primary sex ratio I used data only from nests where more than 89% of eggs laid were successfully sexed (minimum of 8/9 eggs). This meant a final sample of just 12 α-tocopherol supplemented nests and 7 control supplemented nests. I analysed secondary sex ratio in two ways. I used secondary sex ratio from nests with complete primary sex ratio information. I also used data from nests where all chicks that successfully fledged were sexed, excluding sex data from dead chicks or eggs. This resulted in a larger sample size of 28 α-tocopherol supplemented nests and 24 control supplemented nests Unfortunately I could not sex the 5^{th} laid egg in any nest, as it had not been incubated, thus this egg was excluded from estimates of clutch size in calculations involving proportions of sexed or hatched chicks (Arnold et al., 2003). Models were constructed in the same way as before.

2.4 Results

We did not have complete data for every female captured owing to problems with blood sampling and measuring, therefore degrees of freedom sometimes differ between tests.

At each nest, the target birds were observed to use the experimental feeders for both feeding treatments. Number of pecks per hour ranged from 0-7 on both treatments. Birds differed in the total duration of treatment (no. of days of feeder access) and in the duration of treatment before egg laying commenced. Data concerning duration of treatment can be seen in Table 1. I found no evidence of a difference in either total treatment length, or treatment days prior to laying between the treatment groups (GLM. Total treatment duration, $F_{1,69} = 0.55$ $p = 0.855$, Treatment duration before 1st egg: $F_{1,69} = 0.259$ $p = 0.613$). Duration of treatment, and the interaction between treatment and treatment duration did not significantly affect any aspect of reproductive output or female condition. The interaction between treatment and hatching date did not affect any aspect of reproductive output or female condition.

Female body condition

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

Female measures of stress

There was no significant difference in measures of stress (heterophil/lymphocyte ratio and glucose concentration) between α-tocopherol and control fed birds, when chicks were 5 days old (multivariate GLM, $F_{2,20} = 1.218$ $p = 0.317$). There was a marginally non-significant effect of hatching date on female stress (multivariate GLM, $F_{2,22} = 2.99$, $p = 0.070$), with birds breeding later in the season having a higher H/L ratio (univariate GLM $F_{1,25} = 4.882$, $p = 0.037$ see Fig 2.1a) and lower glucose concentrations (univariate GLM $F_{1,25} = 4.128$, $p = 0.053$ see Fig 2.1b). There was no effect of total clutch volume ($p=0.135$), or female mass ($p=0.744$) on stress.

Female reproductive effort

There were no differences in the number of eggs laid, nor the total clutch volume between control or α-tocopherol supplemented birds (multivariate GLM, $F_{2,28} = 0.151$, $p = 0.861$). Number of eggs and total clutch volume were positively correlated with female body mass (multivariate GLM, $F_{2,31} = 3.531$, $p = 0.041$). There was no effect of hatching date or female condition on number or volume of eggs laid ($p>0.404$). There were no differences in the average egg volume or yolk volume between control or α-tocopherol supplemented birds (multivariate GLM, $F_{2,23} = 0.218$, $p = 0.806$). There was no effect of female mass, or condition on egg volume or yolk volume ($p>0.203$ in all cases).

Yolk antioxidants

In fifth eggs, there were no differences in the concentrations of α-tocopherol and total carotenoids (multivariate GLM $F_{2,9} = 0.238$, $p = 0.793$) between treatments. Because some birds received no full days of treatment before the first egg was laid these were then excluded from the analysis. There were still no differences in the concentrations of α-tocopherol and total carotenoids ($p = 0.448$) between treatments. Mean concentrations of antioxidants in the yolks of all eggs (including those with just 5 days of treatment before the fifth egg was laid) were; α-tocopherol: control treatment (n=12) 232.88 ± 21.97 μg/ml, α-tocopherol treatment (n=14) 224.37 ± 26.42 μg/ml, total carotenoids: control treatment (n=12) 76.94 ± 10.24 μg/ml, α-tocopherol treatment (n=14) 82.59 ± 11.69 μg/ml). There was no effect of female mass or total clutch volume on concentrations of yolk antioxidants ($p>0.19$ in both cases), however there was a significant relationship between yolk antioxidants and female body condition (multivariate GLM $F_{2,12} = 9.396$, $p = 0.002$). Interestingly there appears to be a negative relationship between female body condition and yolk α-tocopherol (univariate GLM $F_{1,13} = 6.398$, $p = 0.026$, see Fig ????) and yolk carotenoid concentrations (univariate GLM $F_{1,13} = 9.613$, $p = 0.009$, see Fig ????).

Incubation duration

Feeding treatment did not affect incubation duration (means: control treatment 14.96 ± 0.33 days, α-tocopherol treatment 14.84 ± 0.31 days, univariate GLM $F_{1,30} = 0.001$, $p = 0.97$). There was no effect of total clutch volume, female condition, or date on duration of incubation (GLM $p > 0.3$ in all cases).

Reproductive success

There was no difference in hatching success between treatment groups, and no effect of female condition, H/L ratio or date on hatching success or fledging success (GLM, $p > 0.345$).

Sex ratio

I found a significant difference in primary sex ratio between the treatments (GLM, $F_{1,17} = 5.36$, $p = 0.033$) analyzing data only from nests where at least 89% of the clutch (both hatched chicks and unhatched eggs) was successfully sexed. Figure 2.3a shows that birds in the control group had male biased sex ratios whereas birds in the α-tocopherol supplemented group had female biased clutches. There was no effect of laying date, treatment length, or brood or clutch size on sex ratio ($p>0.35$ in all cases). It appears that both treatments caused skewed sex ratios, in different directions, though assessing which of the treatment groups is more skewed from an the population norm is difficult. I had 8 unmanipulated nests during the course of the experiment, and of these I had a sufficient amount of sex data for only 2 broods, the sex ratio (proportion males) for unmanipulated broods was 0.47 ± 0.05. It appears that the control fed birds deviated more from an even sex ratio than α-tocopherol birds (see Figure 2.3a). Next I analysed the secondary sex ratio of the treatment nests (chicks surviving to fledging). Analyzing secondary sex ratio (sex ratio of fledged chicks) sex ratio from the same subset of data I used for primary sex ratio (nests with 89% of clutch sexed) analysis yielded similar results (GLM, $F_{1,17} = 6.51$, $p = 0.021$, Figure 2.3b). Analyzing the primary and secondary sex ratio from the same subset of data should allow an assessment of whether differences in sex ratio are driven by selective embryo mortality, or by genuine sex-allocation. However, it appears that the sample of broods where I had near complete information on primary sex ratio, were probably those broods with less chick mortality, as these nests avoided the problem of sexing unhatched eggs. In addition to this limited sample I analysed the secondary sex ratio sex ratio from all nests where every fledged chick was sexed, i.e. all nests with complete secondary sex ratio. In this subset of data there was a trend for more male skewed sex ratios in control broods than α-tocopherol broods (GLM, $F_{1,50} = 2.42$, $p=0.0939$, Figure 2.4). In this case it appears that α-tocopherol supplemented broods differed further from an even sex ratio than controls. These results together do not allow me to determine whether the mechanism for a difference in sex ratio since, I do not know

the primary sex ratio for all of these broods. Of the unhatched eggs I had sex information for, there were 4 unhatched male eggs and 5 females from control nests, and 7 unhatched males and 2 females in the α-tocopherol nest. However, 5 out of 6 of these α-tocopherol nests were missing sexing data for at least 23 % of the clutch, and so we cannot treat these results with confidence. The other unhatched eggs that were collected showed no signs of development, of 82 eggs collected, 51 only contained blastocytes and so could not be sexed (Arnold et al., 2003)

2.5 Discussion

Although my treatment did not appear to affect any measure of reproductive effort, or female condition, I found evidence for a significant difference in sex ratio between the two treatment groups. Since male blue tits are also larger and faster growing than females, I predicted that α-tocopherol supplemented parents would produce more male-biased clutches and broods than controls. In fact, I found the opposite effect: α-tocopherol supplemented birds produced more female-biased sex ratios than control supplemented birds. Data from both primary and secondary sex ratios showed the same pattern in the difference between the two treatments. Thus I conclude that mothers adjusted the sex of their offspring in response to my nutritional manipulation. It should be noted that it is hard to determine whether both treatments, or just one treatment group, actually had a sex ratio that deviated from the population norm. The sex ratio data from unmanipulated broods was incomplete, so unfortunately direct comparisons between the sex ratios of both treatment groups, and controls are difficult. Since they appeared to deviate further from a 50/50 sex ratio, at least in primary sex ratio, control birds may have had a male-skewed primary sex ratio. A male bias in control broods may be understandable, since they also received a lard supplement, and therefore an energetic boost. If indeed male blue tits are more costly to produce, or benefit more from extra parental investment, then food provisioning during egg laying may cause an increase in males. Such condition dependent sex ratio biases have been shown with food supplementation studies in other species (Nager et al., 1999; Clout et al., 2002). Of course, this does not explain why α-tocopherol supplemented birds had more female-biased sex ratios than control birds. From feeder observations and measures of maternal condition, there did not seem to be a difference in total energy consumption between the treatments (Ducacroir, 2005 MRes thesis). There are several possible reasons for this unexpected difference between the treatments, and I shall discuss each in turn below.

Initially I predicted that male blue tits would have higher demands for antioxidants during development, and α-tocopherol availability during development would have greater fitness consequences for males than females, especially during within-egg embryonic growth where maternal antioxidant deposition cannot be adjusted after laying. Therefore α-tocopherol supplemented mothers would benefit from investing more in males than females. This would be adaptive, as the sons of α-tocopherol supplemented mothers should be more equipped to deal with the higher oxidative stress associated with faster growth and

the acquisition of large body size, compared with controls. Instead I found a more female biased primary and secondary sex ratio in the α-tocopherol group. It is possible that the difference in sex ratio does not relate to an adaptive strategy on the part of α-tocopherol females, rather a maladaptive effect of treatment. It is unlikely that the result reflects a toxic action of the α-tocopherol supplementation. Extremely high doses of α-tocopherol may have toxic side effects (Bendich and Machlin, 1988), but this requires doses far in excess of those provided here. Indeed, if the result reflected α-tocopherol toxicity I would expect some evidence of this in terms of reproductive effort or female condition. That I found no such effect leads me to suggest this can be discounted. Could this bias be the results of some unexpected interaction between α-tocopherol and the female reproductive system? Certainly none of the proposed mechanisms for sex manipulations (Pike and Petrie, 2003) appear obviously dependent on antioxidant levels. Interestingly, α-tocopherol was discovered as food factor X, a food derived vitamin which prevented foetal resorption in rats (Evans and Bishop, 1922). α-tocopherol may therefore have an important role during gestation, in determining viability and resorption of ova. Hormones such as corticosterone and testosterone have been implicated in mechanisms for sex allocation (Pike and Petrie, 2005; Bonier et al., 2007), and α-tocopherol has been implicated in the control of hormones in other taxa (e.g. Karanth, 2003). However, there is scant information on the interactions between hormones and α-tocopherol in birds. Indeed, it is unclear from this study whether the difference in sex ratio was due to sex allocation, or selective embryo mortality. Primary sex ratio was significantly different between treatments in broods where every chick and unhatched egg was sexed, suggesting that the sexes were indeed allocated pre-laying. It must also be noted that the sample size for sex ratio analyses was small, especially for primary sex ratio analyses (<20 samples), and small sample sizes may result in exaggerated significant sex ratio differences (Ewen et al., 2004). However, analysing sex ratio with larger samples, as in secondary sex ratio analyses, still suggested more male-biased broods in control treated nests, than in α-tocopherol broods. I cannot rule the possibility that state-dependent sex allocation occurred in this study (e.g. Cameron and Linklater, 2007), and it is possible that birds for whom I have complete sex ratio, may represent the best quality birds. This would explain why the male bias was strongest in control birds with complete sex ratio data, and the result was weaker when enlarging the sample: including more birds, may have inadvertently included birds of more variable quality, clouding the difference in sex ratio between treatment groups.

If, as our primary sex ratio analyses suggests, the difference in sex ratio between the treatment groups reflects a true sex ratio adjustment, then it may indicate potentially adaptive investment in daughters, mediated by α-tocopherol. Although sex ratio manipulation is now well documented in birds, the adaptive significance of a skewed sex ratio is less easy to demonstrate. The complexity of different life histories may influence sex allocation decisions in different ways at one time. The factors that may affect sex ratio, and the benefits to parents or offspring of sex biases are multifaceted. Moreover the mechanism for sex ratio adjustment is unknown (Pike and Petrie, 2003). My prediction that α-tocopherol supplemented mothers should overproduce sons had two potential reasons. Firstly, males are larger and faster growing, thus more sensitive to environmental perturbation. Mothers in better condition, should therefore overproduce sons. Evidence from long term data sets in another population of blue tits suggests that male quality is more important than female in determining reproductive success, though quality of territory influences reproductive success more than quality of parents (Przybylo et al., 2001). However, Råberg et al., (2005) recently suggested that females may be the more environmentally sensitive sex in blue tits. Interpreting which sex is more costly and/or environmentally sensitive can therefore be difficult. Indeed, in a recent study McGraw et al. (2005) reported zebra finch *Taeniopygia guttata* mothers fed a high carotenoid diet had male biased sex ratios compared to controls. However, other studies in this species have suggested females are the costlier, more environmentally sensitive sex (Kilner 1998). I also suggested the faster growth and likely increased ROS in male blue tits may lead to higher antioxidant demands. It is possible that female neonates may benefit from α-tocopherol supplementation in ways I did not previously consider. In this study, I found a correlation between maternal mass and both average egg volume and total egg volume, evidence that female reproductive output is partially dependent on body size. Access to increased α-tocopherol during development may infer a growth advantage, and increased condition of daughters. Therefore it is possible that α-tocopherol supplemented mothers may increase their fitness by overproducing females. In the following chapter, growth rate and phenotype of offspring of both sex will be assessed in relation to the supplementary feeding treatment of their parents.

A further consideration is that the treatments will have affected parents of both sexes as well as chicks. The implications of these effects for offspring sex ratio are unclear. A non-mutually exclusive alternative to Trivers and Willard's (1973) hypotheses for adaptive sex ratio manipulation suggests the fitness of parents may be reduced if producing one sex,

reduces their future chance of reproductive success more than the other sex (Gomendio et al., 1990). Here the impact is on the reproductive value of a mother, rather than her offspring. In blue tits, the faster growth and larger size of males may make them costlier to produce and rear than females. If α-tocopherol supplementation significantly altered the survival possibilities of adult females, this may impact on sex allocation decisions. The many health benefits of antioxidants in reducing disease and ageing may improve survival possibilities of adults, moreover, increased oxidative stress has been discussed as a proximate cost of reproduction (Alonso-Alvarez et al., 2004). In blue tits, impaired flight ability has been demonstrated during breeding conditions in females (Kullberg et al., 2002), an effect increasing with egg laying, and independent of body mass. It is possible that oxidative damage accumulating in muscle during breeding and flying (see Chapter 5-7) is responsible for this impairment. Increasing α-tocopherol levels in females during breeding may protect against oxidative stress and reduce locomotive impairment, thus increasing survival chances. In the following chapter I will examine maternal survival, and assess whether it was impacted by treatment or brood sex ratio.

Despite the difference in sex ratio between the treatment groups, apparently mediated by α-tocopherol availability, I did not detect a difference in concentrations of any yolk antioxidant. That I did not find a treatment difference in the fifth egg does not necessarily mean the treatment was unsuccessful: it is conceivable that α-tocopherol levels were different later in the laying sequence. Indeed, in the closely related great tit, a 40% decrease in α-tocopherol was reported, between first and last eggs (Hõrak et al., 2004). I collected the fifth egg to allow time for supplementary α-tocopherol to be incorporated into eggs (e.g. Salvante and Williams, 2002), but also because most birds will lay at least five eggs in our population. In Biard et al.'s study (2005) yolk carotenoid levels were measured in the last egg laid not in the fifth, although studies of carotenoid supplementation in great tits have also demonstrated increased yolk carotenoid concentrations in fifth laid eggs (Berthouly et al., 2007). Though both are important constituents of egg yolk, α-tocopherol and carotenoids are different molecules, so their patterns of metabolism and deposition in eggs may be very different. This is still to be explored. Therefore, expecting that α-tocopherol deposition should follow the same pattern as carotenoids may be erroneous. Similarly, levels of other yolk constituents may have been altered in response to female exposure to α-tocopherol that I did not measured. For example, maternal nutrition in gulls has been shown to affect the patterns of androgen allocation to eggs in different position in the laying sequence (Verboven et al., 2003). There is a possibility that something

associated with the α-tocopherol supplementation, aside from receiving extra α-tocopherol, may have led to the observed patterns. For example, if α-tocopherol feeders were more attractive to sympatric species, disturbance may have been higher around α-tocopherol supplemented nests. Unfortunately I was unable to look at internal antioxidant levels in mothers during egg laying. However, I believe that the treatment was successful insofar as providing α-tocopherol to adult birds. The birds used both α-tocopherol and control feeders with equal frequency (Ducacroir, 2005) and using a lipid vehicle ensures that α-tocopherol should be absorbed. In addition, the dose of α-tocopherol in lard was kept at biologically relevant levels, not higher than blue tits would encounter in natural prey items (Arnold and Ramsay, unpub). The fact that there was an effect of treatment on sex ratio, suggests that α-tocopherol levels in mothers were elevated by the treatment. It is interesting that both yolk carotenoids and tocopherol levels were lower in the 5th eggs of females in better condition. It could be predicted that birds in better condition would have increased availability of resources to invest in eggs. Condition in this sense refers to a greater mass per skeletal size, which may not reflect availability of specific nutrients i.e antioxidants. Of course, here I did not find an absence of relationship between condition and yolk antioxidants, rather a significant negative relationship between them. In this study because eggs were laid before female condition was measured (when chicks were 5 days old), this result may reflect decreased condition in birds that invested more in yolk antioxidants. However, it should be noted that the final sample for antioxidant analysis in yolks was small (12 control and 14 α-tocopherol), and further work is necessary to elucidate the effects of female condition and diet on yolk nutrient status.

There was no difference between treatments in any measure of reproductive effort, nor female condition. I also found no effect of feeding treatment on indicators of stress, however, there was a significant positive relationship between blood glucose and hatching date, and a significant negative relationship between heterophil to lymphocyte (H/L) ratio and hatching date. This finding is contrary to one other study, where stressed birds with a higher H/L ratio, had a higher concentration of blood glucose (Ruiz et al., 2002), as the difference in H/L ratio and glucose levels over the breeding season mean the two scores were negatively correlated with one another. High glucose levels may be a sign of acute stress, since glucose is mobilized by corticosterone as part of the stress response. However, blood glucose concentration is known to decrease during fasting or starvation (Savory and Smith, 1987) and the heterophil to lymphocyte ratio is known to increase during starvation (Maxwell, 1993). In this study, both measures may reveal the feeding status of a bird, as

opposed to an indicator of acute stress. This explains why the two measures were not positively correlated: as the breeding season progresses and food becomes more scarce, breeding females are under a greater starvation threat, reflected by higher H/L ratio and lower glucose levels. Finding an effect of treatment on any measures of reproductive output is confounded by natural variation in male and female condition, male attractiveness, habitat quality, the natural diet of birds, and by breeding date (Przybylo et al., 2001). Interactions between these factors are likely to determine breeding output to a large degree, though I did not have data for all of these variables to control for them in my models. A further complication with this supplemental feeding study is that the duration of treatment varied between birds. There was no significant treatment difference in the duration of supplementary feeding. However, length of treatment was highly variable, and in some cases feeding treatment may only have been accessible for a short time before commencement of egg laying. In addition, though feeders were watched to ensure both treatments were eaten I had no reliable method for assessing feeder use at every nest: the amount of supplement eaten will depend on other bird species. Thus my manipulation is less precise than would be achieved in a captive study. Though I found no effect of feeding treatment on measures of reproductive effort here, the costs of reproduction to females are likely to increase as chick development proceeds. The real test of treatments is in impacts on offspring fitness and phenotype. By assessing differences in the development of chicks laid by mothers of either treatment I may uncover treatment differences in reproductive traits not measured in this study.

In conclusion, I demonstrated that dietary α-tocopherol availability led to a significant difference in sex ratio in this population. I found no evidence for an increase in breeding effort or success associated with the α-tocopherol treatment. However, in this study I analysed only results pertaining to egg and clutch size. By assessing differences in chick development and condition, I would attain a more accurate insight into differences in reproductive performance, mediated by maternally derived α-tocopherol. In light of the difference in sex ratio between the treatment groups, assessing sex specific differences in chick development is of particular interest. This, and other aspects of offspring performance, will be considered in the following chapter.

Table 2.1 Nutritional content of lard per 125 grams, the amount provided in each feeder every two days (www.nutrition.gov)

Nutrient	**Unit**	**Value per 125 grams**
Energy	kj	4718
Protein	g	0.00
Total lipid	g	125.00
Zinc, Zn	mg	0.14
Selenium, Se	mcg	0.2
α-tocopherol	mg	0.75

Table 2.2 Duration of treatment in days for α-tocopherol and control supplemented birds. Range of durations (minimum and maximum), and means ± standard error, are given for total treatment duration, and duration of treatment prior to egg laying.

	Total Duration of Treatment			**Treatment before 1st Egg**		
	Mean (± SE)	*Min*	*Max*	*Mean (± SE)*	*Min*	*Max*
Control	15.09 ± 0.57	9	23	4.91 ± 0.61	0	17
α-tocopherol	15.31 ± 0.72	9	28	4.47 ± 0.61	0	15

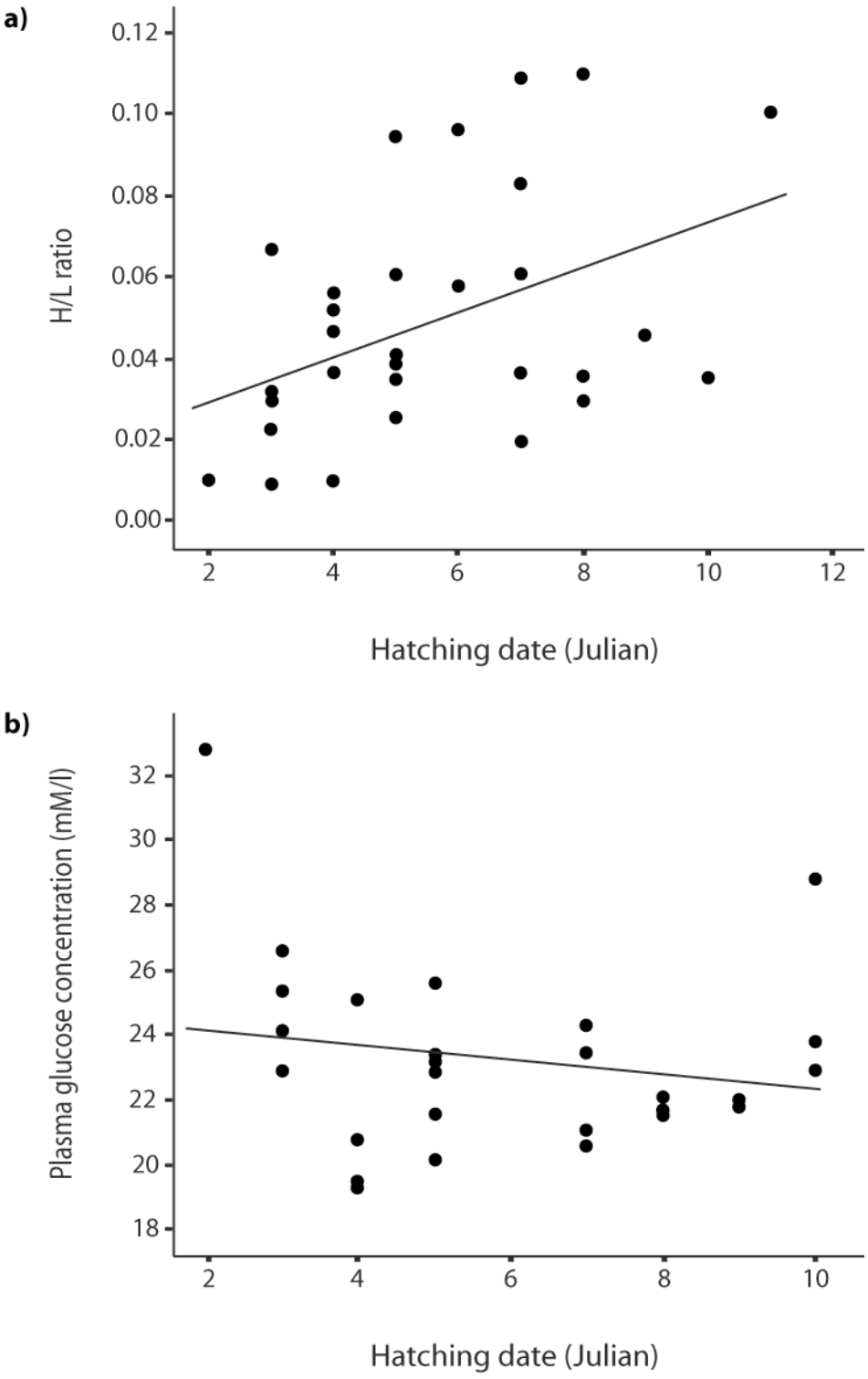

Figure 2.1 a) Heterophil to lymphocyte ratio increased with brood hatching date in breeding females, and **b)** glucose concentration decreased with brood hatching date when chicks were 5 days old. Day 1 = 19th May.

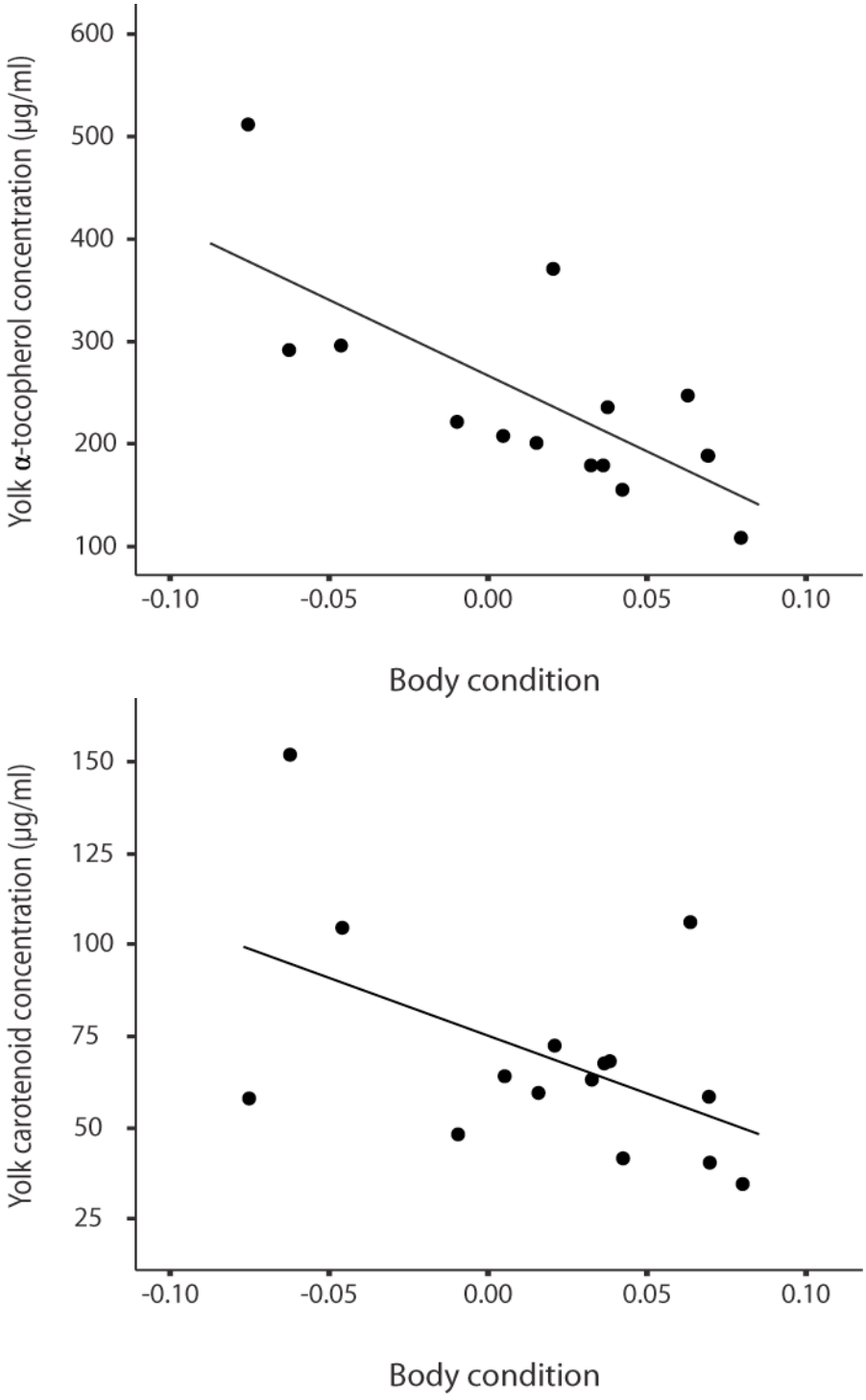

Figure 2.2 a) Concentration of yolk α-tocopherol and b) total carotenoid decreased with female body condition (residuals of ln (mass) on 3*ln (tarsus)

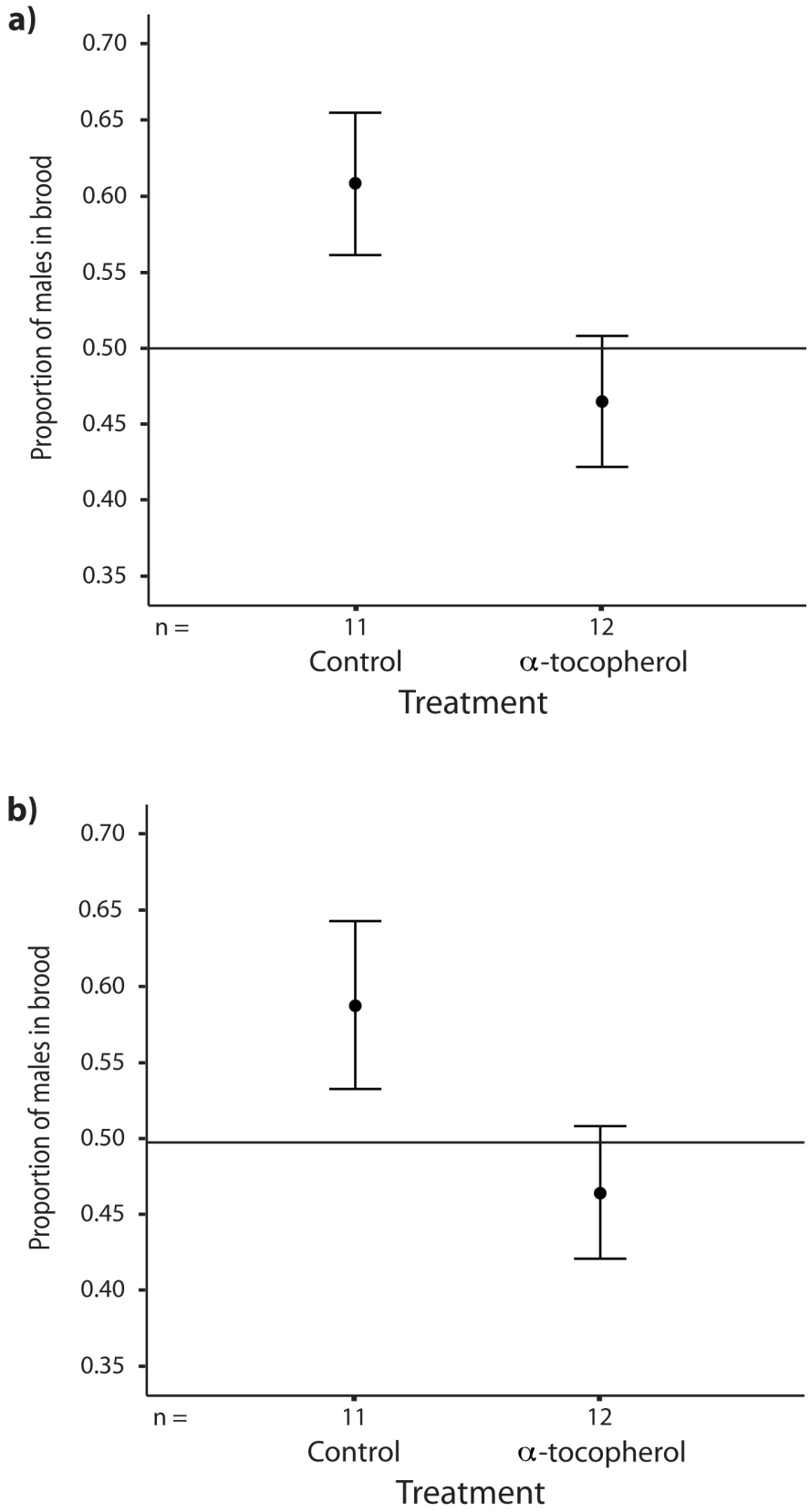

Figure 2.3 a) Primary sex ratio in α-tocopherol and control broods. Data only from broods with >89% eggs sexed (GLM, $d.f._{1,17}$ F=5.36, p=0.033). **b)** Secondary sex ratio in α-tocopherol and control broods. Data only from broods with >89% eggs sexed (GLM, $d.f._{1,17}$ F=6.51, p=0.021). The lines represent an even sex ratio.

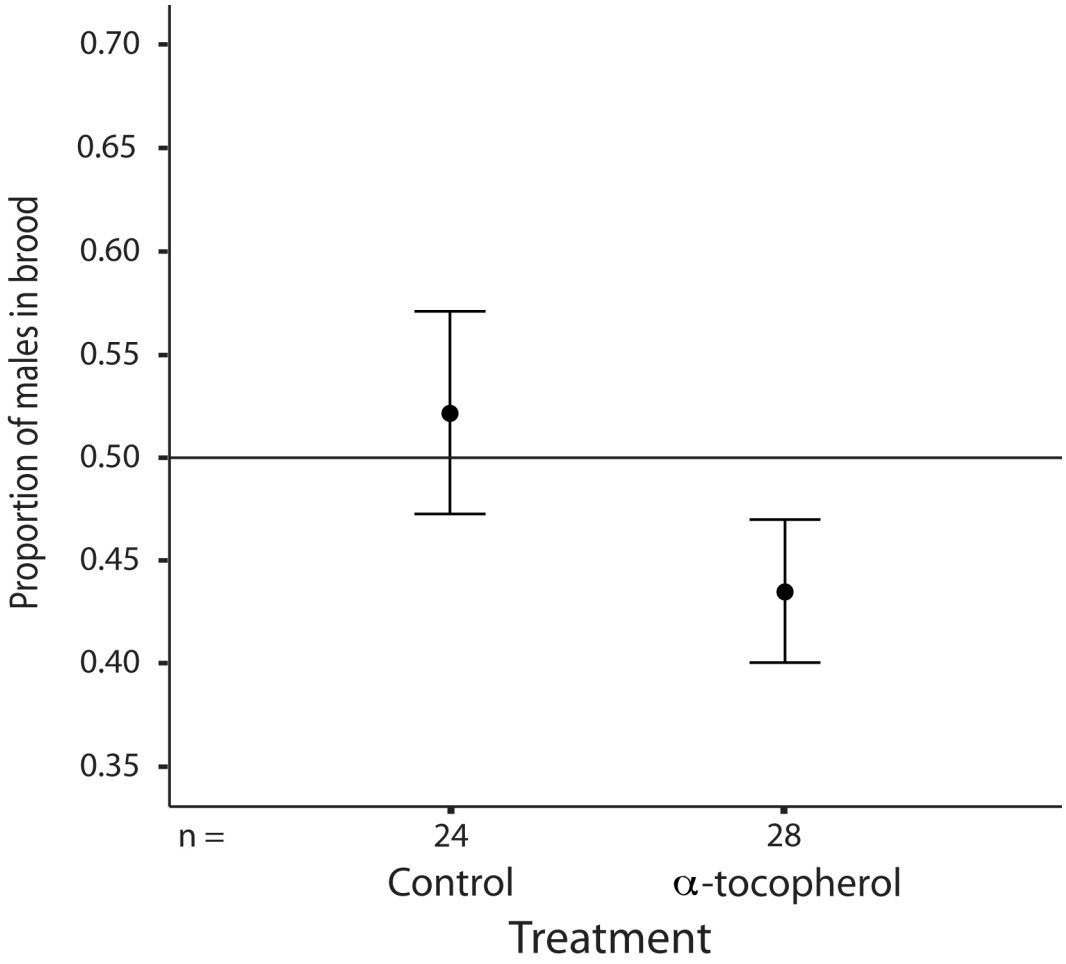

Figure 2.4 Secondary sex ratio in α-tocopherol and control broods (data from broods where all fledged chicks were sexed, GLM, $d.f._{1,50}$, F= 2.42, p=0.0939). The line represents an even sex ratio.

Chapter 3. Experimental evidence for the impacts of maternally derived α-tocopherol on offspring growth, plumage colouration and lipid peroxidation

3.1 Abstract

Maternally derived antioxidants are capable of altering offspring phenotype in different ways. Firstly, deposition of antioxidants in yolk may have benefits in terms of reducing oxidative stress and improving growth of neonates. Also, mothers supplemented with additional antioxidants may also be able to invest more in chick feeding; feeding more often, or better quality food. However, to date very few studies have addressed the role of antioxidants specifically in altering phenotype. Following from Chapter 2, I cross fostered split broods of blue tit *Cyanistes caeruleus* chicks between breeding pairs that had been treated with either α-tocopherol, or control food supplement before and during egg laying. In this way I could assess the relative contribution of pre and post hatching effects on offspring growth rate, oxidative stress, body condition and plumage colour. In accord with other studies, I found effects of egg parents (i.e. genetic, egg quality and incubation effects) were more important in determining body size and growth rate, than effects of rearing parent. In contrast, effects of rearing parent were more important in determining plumage colouration and levels of MDA, a measure of lipid peroxidation. My α-tocopherol supplementation affected the growth rate of chicks, with chicks from α-tocopherol supplemented mothers growing faster, regardless of rearing environment. They did not pay a cost of increased lipid peroxidation despite this increased growth. However, because chicks from α-tocopherol eggs were also smaller than controls post-hatching, there was no difference in mass at day 14 between them. Finally, I showed some evidence that more α-tocopherol mothers returned to breed the following year, with higher reproductive success, than control mothers. This study has implications for our understanding of how antioxidants may alleviate the costs of reproduction.

3.2 Introduction

Maternal effects are defined as the impact of parental phenotype on the offspring's phenotype, holding genetic sources of variation constant (Kirkpatrick and Lande, 1989). Such maternal effects have garnered much interest, since there is growing understanding of the pervasive effects of early development on long term fitness (Lindström, 1999). In birds, maternal nutritional status has been shown to effect egg size (Nager et al., 2000), as well as the deposition of substances within the egg such as antibodies, lipids, proteins and hormones (e.g. Schwabl, 1996; Grindstaff et al., 2003). These in turn can influence offspring phenotype (e.g. Navara et al., 2006a). Another important group of nutrients known as antioxidants are also deposited into eggs (Blount et al., 2000) where they have multifaceted roles including protecting against damage caused by reactive oxygen species (ROS), such as free radicals. Most studies investigating the roles of antioxidants in avian eggs have focussed on carotenoids, a class of lipophillic antioxidants. Experimental manipulations of carotenoid levels in eggs, either indirectly via the mother (e.g. Surai and Speake, 1998) or by direct injection into the yolk (Saino et al., 2003), have shown that carotenoids can reduce oxidative susceptibility (Surai and Speake, 1998; Blount et al., 2002) as well as improving offspring immunity (Saino et al., 2003) and body size (Biard et al., 2005, but see Remeš et al., 2007). Except for some poultry studies (Surai and Kuklenko, 2000; Surai, 2000), there are few data on the roles other antioxidants derived from the egg can have in influencing embryonic and neonatal development in birds. This is surprising given that other nutrients present in eggs may have more powerful antioxidant actions than carotenoids (Hartley and Kennedy, 2004).

α-tocopherol (vitamin E) is the major membrane bound lipophillic antioxidant, and is of vital importance in resisting lipid peroxidation (Burton and Ingold, 1981). Since growing tissue is a site of high ROS production, α-tocopherol availability during development may allow faster growth, or limit oxidative damage associated with faster growth. ROS may damage proteins, lipids and DNA, and oxidative stress is linked with virtually all diseases (Cui et al., 2004). Conditions in early development are likely to have a significant bearing on long term phenotype (reviewed by Lindström, 1999), thus oxidative stress in neonates may have important consequences for fitness. The effects of α-tocopherol on growth rate and oxidative stress in wild birds are unclear. Data from other taxa (e.g. Kahn-Thomas and Enesco, 1982; Cowey et al., 1984) suggests that α-tocopherol supplementation can result in faster embryonic or neonatal growth. Also, chickens from faster growing "lines" have

higher requirements for α-tocopherol, than those from slower growing lines (Surai et al., 2002). Faster growth in altricial species may allow earlier fledging, which can decrease the risk of predation and parasitism of parents and offspring in some species (Berggren, 2005).

Although manipulation of egg quality is one mechanism for the action of maternal effects, nutrient supplementation of parents may also affect adult reproductive behaviours post-hatching. In birds, the nutrient and energy requirements to provision chicks are considered important factors limiting reproductive success (Stearns, 1992). Providing supplementary food to adults during chick provisioning may alleviate some of the energetic and nutritional costs involved in self-maintenance. Data from one study of blue tits *Cyanistes caeruleus* showed that food supplemented adults brought chicks larger food items after a lull in begging by chicks compared to control adults (Grieco, 2001). This may indicate that supplemented parents were using time to find high quality food for offspring in begging gaps, while control birds invested more time feeding themselves. Food supplemented adults were also quicker to respond to begging by returning with food (Grieco, 2001). Thus the nutritional state of parents influences the quality of care they provide. During chick rearing increased metabolic rate in adult birds (e.g. Hodum et al., 1998; Weimerskirch et al., 2003) will increase production of ROS. Access to antioxidants may alleviate this oxidative cost, and allow greater investment in chick rearing. Breeding is also associated with a decline in flight ability for females (Kullberg et al., 2002). If antioxidant supplementation can improve flight performance (Chapters 5-7), then parental provisioning may be increased by antioxidant availability. These represent two potential mechanisms by which dietary antioxidants may alter parental provisioning behaviours. To my knowledge, only one study has assessed the effects of specific nutrient availability on chick provisioning. In adult great tits *Parus major*, females receiving a high carotenoid diet, provisioned their chicks well regardless of brood size, whereas control birds provisioned small broods less than large (Helfenstein et al., 2008). However, this is a weak example, and the reason for this effect is unclear, and cannot be attributed specifically to antioxidant function. In this study I will assess the effects of α-tocopherol supplementation during egg laying on chick provisioning behaviour.

α-tocopherol is a potentially limiting nutrient during reproduction, since breeding involves a shifting of energetic and nutritional resources from self maintenance and potential survival, to egg formation, incubation and chick rearing. This relocation of resources will be managed such that offspring survival can be achieved, but also survival and future

reproduction of parents (Stearns, 1992). In Chapter 2, I demonstrated no difference in female condition mediated by α-tocopherol, during early chick development. However, the costs of reproduction are likely to accumulate as chick development proceeds. By assessing survival of adults, and recruitment of chicks, a more effective measure of the effects of dietary treatment on fitness may be attained, but assessing fitness in wild populations can be difficult (Irschick, 2003). The year following from this experiment, any ringed female observed nesting and breeding in the nestbox population was caught. In this way I hoped to assess the effect of α-tocopherol supplementation on survival for both adults and their chicks.

As discussed, there is potential for parental α-tocopherol availability to influence offspring phenotype through effects of egg quality and incubation, and also through post hatching provisioning. Following hatching of clutches produced by mothers receiving either a α-tocopherol enriched, or control food supplement, I paired nests of opposing treatments, matching for brood size and hatching date. Growth rate, as well as plumage reflectance and condition at fledging were measured. Plasma concentrations of Malonidialdehyde (MDA), a by-product of lipid peroxidation were assayed. Since the major function of membrane bound α-tocopherol is in protection against lipid peroxidation (Burton and Ingold, 1981), this measure allows an assessment of the affect of pre-laying α-tocopherol treatment on oxidative damage (see also Chapter 5). The covariance of variation in offspring traits owing to either egg effects, or rearing environment were thus assessed. In addition, I previously demonstrated that α-tocopherol supplemented females, produced more female biased sex ratios, than control females (Chapter 2). I predicted that this may be an adaptive response, if daughters of α-tocopherol supplemented mothers were of improved quality at fledging, compared to daughters of control birds. Here, I can test whether nestlings of different sex from broods of either treatment, differed in development or phenotype. Specifically I aimed to investigate: 1) Controlling for egg laying and rearing environment, does prelaying α-tocopherol supplementation affect offspring phenotype and/or levels of oxidative damage? 2) Does prelaying α-tocopherol supplementation affect parental food provisioning behaviour? 3) Are there sex-specific differences in offspring phenotype related to parental treatment? 4) Does prelaying α-tocopherol supplementation affect survival and probability of breeding in the following season?

3.3 Methods

This study follows on from the project outlined in Chapter 2. The experimental manipulation was carried out in spring 2006 and recruitment was assessed from spring 2007 to spring 2008. A nestbox breeding population of blue tits was studied in Ross Woods around SCENE, Rowardennan, Loch Lomond (see Chapter 1.7). Information about supplemental diets, and nestbox monitoring until hatching can be found in Chapter 2. In brief, adult blue tits were provided with either a control or α-tocopherol enriched lard supplement during nest building and egg laying, the fifth egg from each brood was collected, and all eggs were measured.

Cross fostering and chick measurements

After hatching (day one was when more than half of eggs within a clutch had hatched), broods were left alone until the chicks were three days old. During this time appropriate cross fostering pairs were devised. I aimed to cross foster half broods of chicks from α-tocopherol with half broods from control parents in order to disentangle the egg effects (i.e. genes, egg quality and incubation environment), from the effects of rearing environment. Nests were paired according to feeding treatment, brood size (± 1 chick) and exact hatching date. On day 3, pairs of nests were checked at the same time, to ensure that brood sizes matched prior to cross fostering. Where an appropriate pair was found, every chick within each nest was individually marked with a unique colour combination on the three patches of down on their heads using non-toxic ink. The chicks were weighed to within 0.1g using a field balance and then randomly selected for fostering. At each nest a coin was tossed to determine whether the first bird selected would be fostered or not. Following this, chicks were randomly removed from their nest and every second bird was fostered. The birds to be cross fostered were carried to their new home in a heated box. Nestlings that were not cross-fostered were also kept out of the nest in a heated box to control for the disturbance involved in cross-fostering. Chicks were returned to the appropriate nestbox within 30 minutes. Where broods were unsuitable for cross fostering, all chicks were marked and measured at the nest site, and returned to their own nest. All nests were then revisited every second day until day 14, when chicks were identified and weighed. At day 9, chicks were re-marked, using a unique combination of toenail clipping. No nestlings suffered any ill effects from handling or marking. There was a final total of 24 cross fostered broods.

Provisioning behaviour

Adult provisioning behaviour was recorded when their chicks were 4 days old. Small black and white video cameras, approximately 50x50x20mm, were attached to the inside of the nest box back wall. The camera was positioned facing the entrance hole to the nest box, to capture parent's entrances and allow identification of beak contents. The cameras were connected to a videocassette recorder (VCR) powered by a deep cycle battery. All equipment was contained in a waterproof box, sealed with waterproof tape and secured to the base of the tree which the nest box was located, by padlocks and chains. The boxes were finally covered with forest litter, moss and ferns to help camouflage the box and prevent any further disturbance around the nest area. The video recording equipment was installed the day before filming to allow adults to habituate, and the nest boxes were not visited on this day to ensure normal behaviour. The VCR was set to record from 0600 to 1200 hours the following day, which corresponds to the peak chick provisioning times.

Videos were replayed on television monitors using a VCR with a timer facility and were paused and rewound to record the time when a visit or exit was made from each nest. Where possible, the contents of the adult's beak when a food item was brought to the nest were identified. The following categories were used. 1. caterpillar – judged on size and appearance being similar to that of a caterpillar, 2. non-caterpillar – if object in beak did not resemble that of a caterpillar nor a spider, but was definitely a prey item 3. spider – if object had a body structure similar to that of a spider with distinct thin appendages which resembled legs, but with no visible wings, 4. unknown – if object did not resemble a typical prey item, 5. not-visible – if the bird's beak could not be seen or was obscured. Several videos were watched as a group to ensure consensus in the definitions of different prey types between observers. Several tapes were analysed independently by two different observers to calculate repeatability in identification of prey types. Repeatability was high. All data was recorded in tabulated form and any unusual nest activity was recorded along with each individual's entry and exit time to and from the studied nest.

Chick blood sampling, morphometrics, and colour analysis

When the chicks were 14 days old they were brought into the SCENE field laboratory for blood sampling and measuring. Half of each brood was taken from the nestbox in a heated bag. Each bird was removed from the bag and blood sampled immediately, a small volume

of blood was taken by venipuncture from a wing vein. One drop of blood was put in ethanol for subsequent molecular sexing (Griffiths et al., 1998; Arnold et al., 2007). The remaining blood was collected in 75µl heparinised capillary tubes. The capillary tubes of blood for MDA analysis were centrifuged and haematocrit readings were taken from each, before these were stored at -20°C. After blood sampling each bird had its wing and tarsus measured. Using a spectrophotometer (Ocean Optics S2000) two reflectance readings were taken from each bird's chest plumage, and two from its crown plumage. Where feathers were in pin, or underdeveloped, this was noted and data from that patch were excluded from analyses. After each half brood was completed, it was returned to the nestbox and swapped for the remaining half brood. Birds were removed from their nests for no longer than one hour. After 25 days every nest was checked for fledging. Any unfledged chicks were identified and noted. Growth rate was calculated for each bird between days 3 and 13 as: (mass day 13 – mass day 3) / 10, giving a rate of daily body mass gain in g/day.

Plumage colouration

In order to assess whether chicks from parents of either treatment differed in plumage colouration, plumage reflectance was analyzed using the SPEC package (http://www.bio.ic.ac.uk/research/iowens/spec). I used data on the spectral sensitivities of the four single cones in a blue tit's retina (Hart et al., 2000), to analyse the spectral data. The benefits of analyzing the colour in this way are twofold: First, I was able to separate colour into several components and analyse these individually. Since I used two distinct regions of plumage, of different colours, this allowed an expansive assessment of differences in plumage colour between chicks. Second, by utilizing visual sensitivity data I also ensured that any differences in colour between chicks would be identifiable by adult birds. This is important where chicks have been cross fostered, and any difference in colour may lead to differences in food provisioning etc. The SPEC program multiplies cone sensitivities by the reflectance spectrum from the plumage patch (Hadfield and Owens, 2006). This is done for every wavelength to which the cones are sensitive, and these values are then summed for each cone type, to give four quantal cone catches; UVS (Ultraviolet sensitive), SWS (Short wavelength sensitive), MWS (Medium wavelength sensitive) and LWS (Long wavelength sensitive) (Vorobyev et al., 1998). A mean quantal cone catch was calculated from the two readings taken for each plumage area.

In this experiment I use this quantal cone catch from the MWS and LWS cones to analyse carotenoid mediated colour of yellow chest plumage and the UV portion of both chest and crown feathers, since there is considerable interest in how the UV portion of the spectra affects social signalling in blue tits (e.g. Korsten et al., 2006). From the chest plumage I analysed the LWS chromatic signal and the UVS chromatic signal. From the head feathers, I analysed the UVS chromatic signal. Thus, I analysed spectral data from the LWS, MWS, SWS and UVS cones for the crown plumage and the UVS, and SWS cones for the head plumage. Analyses of chromatic cues permit the distinction of stimuli of different spectral composition regardless of intensity, typically achieved by chromatic opponency of signals from photoreceptors (Osorio et al., 1999). I calculated chromatic signals with the following formulae using the quantal cone catches calculated by SPEC (Osorio et al., 1999): 1) Chest chromatic signals: a) LWS chromatic signal = (LWS - MWS) / (LWS + MWS) b) UVS chromatic signal = (UVS – SWS)/(UVS + SWS; Head feather chromatic signals a) UVS chromatic signal = (UVS – SWS)/(UVS + SWS); b) SWS chromatic signal = (SWS – MWS)/(SWS + MWS).

MDA analysis

In order to assess the effect of supplemental feeding treatment on oxidative damage, malonidialdehyde, a by-product of lipid peroxidation, was quantified in the plasma of a subsample of chicks. Owing to the relatively large volume of plasma required for these analyses, I could not analyse all birds, instead I analysed plasma samples from at least one chick of each sex, per treatment per brood. This meant a final sample size of 90 samples. MDA analysis was performed as in Young and Trimble (1991) with some modifications.

Thiobarbituric acid (0.044M,100µl) and phosphoric acid (1.22M, 100µl) were mixed together and added to 50µl of plasma (per bird) in a test tube. An inert atmosphere was created by applying a nitrogen blanket, and the test tubes were sealed and vortexed prior to heating (60 min, 70-75^{o}C). Samples were cooled in water, then 200µl was transferred to a centrifuge tube containing sodium hydroxide (1M, 100µl). Methanol (500µl) was added and mixed. Samples were centrifuged (10 min, 12000 g) and the supernatant analysed on a Summit HPLC system (Dionex, Idstein, Germany) using Chromeleon software (Dionex). An Acclaim 120 C18 5µ 4.6 x 250 mm column (Dionex) and guard were used with fluorescence detection (excitation 532nm and emission 553nm). The mobile phase was isocratic, 40:60 methanol:phosphate buffer (40mM, pH 6.5), with a flow rate of 1ml/min,

and a run time of 7 min. Samples were assayed against a standard of malonidialdehyde bis (dimethyl acetal), (Sigma Aldrich, Poole, UK) that was simultaneously taken through the same procedure.

Offspring recruitment and parent survival

In order to assess parental survival and F1 recruitment, adults were trapped on the nest during the 2007 breeding season and also mistnetted at feeding sites throughout the study site over winter 2007/8. From April 2007, nestboxes were checked weekly for signs of nest building. When nests were fully built they were checked every 3 days for egg laying and when no new eggs were laid for two consecutive days, the final clutch size was noted. During incubation females were inspected for existing leg rings by observation either on or off the nest. Adults were caught when their chicks were 5 days old, focussing on nests where at least one of the parents was observed to be ringed. Investigation was focussed on females, since they were easier to catch at the nest than males. In contrast, males were more likely to be caught at feeders over the winter (Herborn and Arnold, unpublished data). Birds were sexed by the presence or absence of a brood patch. A small volume of blood was taken by venipuncture from a wing vein within three minutes of first trapping the bird in the nestbox for another project. A small drop of blood was stored in ethanol for subsequent molecular sexing, to confirm visual sexing of adults (Griffiths et al., 1998; Arnold et al., 2007). Following blood sampling, the birds had their wings and tarsus measured, and using a field balance they were weighed to within 0.1 gram. The ring number of any ringed birds was noted, and any unringed birds were ringed. Total handling time was minimized and most birds were released within ten minutes. All ringed or unknown females within the study site were caught to assess female recruitment from previous years within our population. I assessed the reproductive success of all captured female bird ringed a previous year. I noted clutch size, and hatching success and at 25 days nests were checked for fledging, and any unfledged birds were noted. In winter 2007/2008 feeding stations were erected throughout the field site for several weeks to encourage birds to feed in particular areas. Mist nets were deployed at these feeding sites throughout the winter, and any birds caught were identified and blood sampled. As before, a small drop of blood was stored in ethanol for subsequent molecular sexing (Griffiths et al., 1998; Arnold et al., 2007).

Statistics

Throughout the results and discussion, for clarity I will refer to chicks laid by α-tocopherol treated parents as chicks from α-tocopherol eggs, and chicks laid by control treated parents as chicks from control eggs. Differences in female condition, and differences in reproductive output prior to cross fostering, in only cross fostered nests, were analysed using general linear models in SPSS v 14. For assessment of differences in chick provisioning I analysed data from all nests, including nests that were not cross fostered, as I assumed that the cross fostering process would not affect parental behaviour. Where data from both are used, this will be described in the results section. Data on chick growth, size, plumage colouration, and oxidative stress were analysed using general linear mixed models (GLMM) in SAS v8 (SAS Institute Inc., Cary, NC, USA). Identity (ID) of egg parent's nest, and identity (ID) of rearing parents nest were added as random factors in each model, to control for non-independence of nestlings of the same origin and hatching environment, or rearing environment respectively. I entered sex, parental treatment, rearing treatment and all possible two-way interactions into each model. Models were simplified by dropping non-significant terms from the model, starting with non-significant interactions, until only factors significantly contributing to the model remained. In the results below non-significant values are provided at the point the term was omitted from the model, only significant interaction terms are reported. For these analyses I used only data from cross fostered nests. Means ± 1 standard error are reported throughout the results.

3.4 Results

Comparing just cross fostered nests, I found no significant differences in total clutch volume (α-tocopherol: n=12, 15953.15 mm^3 ± 828.73, Control: n=12, 15630.15 mm^3 ± 901.72, GLM $F_{1,23} = 0.09$, p= 0.79) or average egg volume (α-tocopherol: n = 12, 1445.85 mm^3 ± 22.47, control n= 12, 1463.20 mm^3 ± 27.79, GLM $F_{1,23} = 0.278$, p= 0.62) between the treatment groups, prior to cross fostering. When chicks were 3 days old, prior to cross fostering, chicks from α-tocopherol treated parents weighed significantly less than birds from control treated parents (GLMM, $F_{1,188} = 24.28$, $p<0.0001$, Figure 3.1a). There was no significant sex difference in body mass day 3 (GLMM, $F_{1,192} = 0.019$, p = 0.66). Half-broods of chicks were then cross-fostered between treatments.

Full statistical data from models (including non-significant terms at the point they were dropped from the model), explaining chick phenotype can be found in Tables 3.1- 3.4. The main findings are described below.

Growth rate (Table 3.1)

Growth rate was faster for chicks whose egg parents received α-tocopherol treatment, than chicks whose parents received control treatment (see Figure 3.1b). Feeding treatment of rearing parent had no effect on growth rate (GLMM $F_{1, 15.1} = 0.48$, p=0.50), and males gained more mass than females between the ages of 3 and 14 days. Both random factors appeared to affect mass gain between days 3-13.

Mass (Table 3.2)

Chicks raised by control fed adults were of greater mass at day 14 than those raised by α-tocopherol fed adults (Figure 3.2a). At day 14 there was no longer a significant effect of egg parents' feeding treatment on mass (GLMM $F_{1, 38.1} = 0.69$, p = 0.41). Males were also significantly heavier than females There was no significant interaction between sex, and either treatment of egg ($F_{1, 174} = 1.71$, p = 0.193) or rearing parents (GLMM $F_{1, 174} = 1.81$, p = 0.179), though plotting the data does suggest that males raised by control treated birds were heavier than males raised by α-tocopherol treated birds (Figure 3.2a). This effect is less clear in females. Mass at age 14 was affected by identity of egg parents, but not by identity of rearing parents (Table 3.2).

Tarsus length (Table 3.3)

At 14 days of age, chicks from α-tocopherol supplemented egg parents had smaller tarsi than chicks from control eggs (Table 3.3). There was also a significant interaction between treatment of rearing parents and sex on tarsus length (Table 3.3). It appears that although in general males had longer tarsi than females (means: males 17.14 ± 0.05 mm, females 16.57 ± 0.06 mm), male chicks raised by control treated adults had longer tarsi than male chicks raised by tocopherol treated adults (Figure 3.2b). Tarsus length was significantly affected by identity of egg parent, though not by identity of rearing parent.

Body condition (Table 3.4)

There was a non-significant trend for birds from α-tocopherol fed egg parents to be in better condition (greater mass for skeletal size) than birds from control fed egg parents ($p = 0.071$, Figure 3.1c). There was no significant effect of treatment of rearing adults (GLMM $F_{1, 20.9} = 0.97$, $p=0.34$) or sex (GLMM $F_{1, 184} = 2.61$, $p=0.11$) on condition (Table 3.4). As with most morphometric measures, there was a non-significant trend ($p = 0.06$) for variance in offspring condition attributable to identity of egg parents, but not to identity of rearing parents.

Oxidative damage

In spite of the differences in chick mass and growth between treatment groups, I found that neither genetic nor foster parent treatment had a significant effect on plasma levels of MDA (GLMM: parents treatment, $F_{1, 79.7} = 0.35$, $p = 0.55$ see Figure 3.4, rearing treatment, $F_{1, 19.4} = 0.19$, $p = 0.67$). There were no sex differences in MDA (GLMM $F_{1, 80.5} = 0.29$, $p = 0.59$). In contrast to morphometric measures, MDA was affected by both identity of rearing parent ID and by egg parent ID (random factors: egg parent $Z = - 3.58$, $p = 0.0003$, rearing parent $Z = 2.21$, $p = 0.0274$).

Plumage colouration

Males had a greater LWS chromatic signal (relating to yellow chest plumage) than females (GLMM $F_{1, 191} = 19.38$, $p < 0.0001$, Figure 3.3), however, there were no effects of either

treatment on chest LWS chromatic signal. There was no effect of sex (GLMM $F_{1, 173}$ = 0.12, p = 0.729), rearing treatment (GLMM $F_{1, 17.9}$ = 0.27, p = 0.61), or egg treatment (GLMM $F_{1, 58.8}$ = 0.41, p = 0.527) on chest UVS chromatic signal. Interestingly, variation in MWS chromatic signal of chest plumage was significantly affected by ID of rearing parents, but not by ID of egg parents (random factors: egg parent, Z = 0.32, p = 0.374; rearing parent Z =1.85 p = 0.0321), whereas UVS chromatic signal of chest plumage was affected by ID of egg parents, but not by ID of rearing parent (random factors: egg parent Z = 2.85, p = 0.0022, rearing parent Z =0.23, p = 0.410).

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

Parental provisioning behaviour

Because there was no difference between feeding rates in first and second hour watched, chick feeding data were pooled. I found no effect of treatment on any aspect of chick provisioning. To enhance the sample size I added non-cross fostered nests to the model. There was no difference between in either number of feeds (Feeds per 2 hours. α-tocopherol: n = 12, mean 56.14 ±9.28; control: n = 17, mean 46.67 ± 1.33, GLM, $F_{1, 28}$ = 0.103, p = 0.751), or proportion of caterpillars fed by parents receiving either treatment when chicks were 4 days old (GLM $F_{1,28}$ = 0.005, p = 0.94, proportion caterpillar α-tocopherol: n = 12, mean 0.87 ± 0.03; control: n = 17, mean 0.87 ± 0.04). Using data only from cross fostered broods also showed no difference in number of feeds per brood (GLM, $F_{1, 15}$ = 0.719, p = 0.411) or number of feeds per chick (GLM, $F_{1, 15}$ = 1.68, p = 0.215). There was a non-significant trend for proportion of caterpillars provided to decline with

date (GLM $F_{1,28} = 3.35$ $p = 0.07$). Thus parents from different treatments did not vary in the amount or type of prey provided to chicks, that I could detect. Nor were there any treatment differences in number of feeds per chick (feeds per chick per 2 hours: α-tocopherol: n = 12, mean 7.03 ± 1.16; control: n = 17, mean 5.76 ± 0.25, GLM, $F_{1, 28} = 0.39$, $p = 0.54$) in the two hour period they were observed.

Chick fledging and recruitment and parental survival

During the course of the experiment only 5 chicks out of 203, from fostered nests died post-hatching, and there was no effect of genetic or foster parent treatment. I attempted to catch all breeding females, in spring 2007 to assess recruitment of F1 females, and females breeding in both 2006 and 2007. Breeding data from retrapped mothers, and female F1s in 2007 can be seen in Tables 3.5 and 3.6 respectively. Although the sample size for both chicks and adults is too small for statistical analysis, it appears that there were more α-tocopherol fed adult females reproducing again in 2007, than control birds. They also appeared to successfully fledge more chicks. There are no apparent differences in either recruitment or breeding success of chicks from either control or α-tocopherol fed parents in 2007. In winter of 2007/2008 mistnetting was used to catch as overwintering birds in our population. This data concerns male and female chicks from this study, and can be seen in Table 3.7. In this case there were more chicks from α-tocopherol supplemented egg parents, than controls. Only one adult from 2006 was recaught in winter; a control female.

3.5 Discussion

Supplementing parents with α-tocopherol during egg laying had significant impacts on the growth and body condition of resultant offspring compared with controls. Utilising a cross-fostering design, I could separate variation in offspring phenotype assignable to egg effects (i.e. genetic, egg composition and incubation environment effects) versus those of rearing environment. Egg effects explained some of the variance in all morphometric measures, however, effects of rearing environment did account for some of the variation in both MDA and yellow plumage colouration. Prior to cross fostering, 3 day old chicks from α-tocopherol eggs were significantly smaller than chicks from control eggs, despite both total and mean egg volume being unaffected by feeding treatment. Chicks from α-tocopherol eggs grew faster than chicks from control eggs, and by day 14 there was no significant difference in mass mediated by parent's treatment.

Previously, I predicted that chicks hatching from eggs with enhanced levels of α-tocopherol, should grow faster than those from control eggs, if α-tocopherol protects against ROS produced during embryonic and neonatal growth. In this study, chicks from α-tocopherol eggs gained more mass between days 3 and 13 than chicks from control eggs. Interestingly, in one of few previous studies in a wild bird, tocopherol availability during nestling growth was shown to weakly, though significantly, affect growth rate (de Ayala et al., 2006. In chickens, it has been demonstrated that faster growing breed lines, have a higher demand for vitamin E than slower growing lines (Surai et al., 2002). Also, α-tocopherol appears capable of preventing oxidative stress induced growth retardation in chicken embryos (Satiroglu-Tufan and Tufan, 2004), and growth rate increases with supplementation of tocopherol have been demonstrated other taxa (e.g. Lee and Dabrowski, 2004). In the closely related great tit, nestlings from carotenoid fed mothers gained more mass between days 9-14 than chicks from control parents (Berthouly et al., 2008). I hypothesized that faster growth would offer chicks a selective advantage, if increased growth allowed earlier fledging. In blue tits, earlier fledging may allow chicks to avoid nest predation and parasitism. The exact dates of fledging for the broods in this study are unknown, though fledging times may have been altered by the split-brood experimental design. The prediction of a benefit for increased growth rate, assumes that chicks would start at the same size, and thus faster growing birds would reach full grown size quicker. In Berthouly et al.'s study of great tits (2008), the difference in mass mediated by carotenoids only became visible at 14 days old. Here, chicks from α-tocopherol eggs weighed less on

day 3, than chicks from control eggs and I shall discuss this further below. Owing to their increased rate of growth this difference was absent by day 14. However, it should be noted that in this study "growth rate" is inferred from differences in mass at the start of nestling growth and mass prior to fledging. Although this means chicks must have differed in their mass gain per day, this is a crude approximation of actual growth rate. The exact rate of growth of each bird will depend on skeletal size and growth at different ages. Indeed, in this study chicks from control eggs had longer tarsi prior to fledging than chicks from α-tocopherol eggs. In addition, male chicks *raised* by control supplemented rearing parents had significantly longer tarsi than males raised by α-tocopherol birds.

It is worth considering the implications of the differences in tarsus length, condition and mass between chicks in this study. Some studies in blue tits have suggested that tarsus length, is a good measure of body condition and rearing conditions in blue tits (Senar et al., 2002). In this study, male chicks raised by α-tocopherol rearing parents had smaller tarsi in, than control rearing parents. There did not appear to be any difference in female tarsus length between those raised by α-tocopherol, and those raised by control parents. This result may indicate that rearing conditions were poorer, at least for males, in α-tocopherol nests. However, despite being skeletally smaller, on day 14, chicks laid by α-tocopherol supplemented parents were in better condition. Condition scores have also been used to assess rearing conditions and survival probability in a range of bird species (Merilä et al., 1999). Indeed, in blue tits, the survival probabilities of chicks in one population were shown to be dependent on body mass, and only indirectly by tarsus length (Råberg et al., 2005). The costs and benefits to chicks from eggs of either treatment are thus hard to determine from morphometric data at fledging. Certainly, my prediction of a demonstrable benefit for chicks from α-tocopherol eggs by increased growth was not observed. The fitness consequences for chicks from different treatment groups in terms of survival and recruitment will be considered below. A further interesting aspect of differences in tarsus length apparently attributable in part to rearing parents treatment is that chicks may have been provisioned differently in the different nests. I found no evidence of a difference in chick provisioning rate, or in proportion of caterpillars, when chicks were 4 days old, though prey size and quality may have differed.

Thus far, explanations for the observed difference in growth rate have been discussed, but not the fact that chicks were smaller on day three in α-tocopherol nests. Our experimental procedure involved cross fostering chicks aged 3 days, to account for asynchronous

hatching and ensure that fostered nests were matched for brood size. Therefore, it is possible that this difference was due to a difference in chick feeding by α-tocopherol and control fed parents prior to cross fostering. There was no difference in feeding rates at day 4, though this does not exclude the possibility that chicks were provisioned differently at an earlier time. The difference in size at day 3 could be due to differences in mass at hatching. I did not weigh chicks prior to day 3, but there were no treatments difference in egg yolk or clutch volume between treatments. If α-tocopherol chicks did hatch at a smaller mass and this was not related to the quantity or quality of the yolk, then other factors affecting embryonic growth pre-hatching must be considered. Incubation conditions are known to play a role in determining embryonic growth and subsequent hatching mass, especially in commercial species (Hulet, 2007). The role of incubation conditions in other species is less clear. Recent work has shown that incubation behaviour is a costly and much overlooked aspect of maternal care, capable of affecting offspring fitness (Gorman et al., 2005). Indeed, in herring gulls *Larus argentatus* high thermal constancy in early incubation was shown to result in greater body mass at fledging (Kim and Monaghan, 2006), although this also shortened total incubation time. I did not show a significant difference in the total duration of incubation between the treatment groups (Chapter 2), but incubation duration was not precisely measured, and specific incubation conditions in nests are unknown. Incubating less regularly may allow increased time in self maintenance for mothers, with the cost of hatching smaller chicks. However, if females provisioned with α-tocopherol invested more α-tocopherol in yolks, their chicks may be better able to resist the oxidative costs of faster growth post-hatching. Alternatively, mothers provisioned with dietary antioxidants may anticipate a similarly antioxidant rich environment for rearing chicks. In this case the oxidative costs of faster growth for their chicks could be ameliorated by quality food provisioning.

It is interesting that the effects of egg parents' treatment on growth persisted, despite differences in rearing environment. Indeed, egg effects explained some variance in all morphometric measures, where effects of rearing environment did not. This strongly suggests that some aspect of egg or chick development was 'programmed' prior to the cross fostering. In chickens it has been demonstrated that yolk carotenoid concentration significantly affects carotenoid levels of chick tissues post hatching. In yellow legged guul, chicks from high carotenoid eggs fed a control diet had higher levels of carotenoids than chicks from control eggs fed a high carotenoid diet (Karadas et al., 2005a). The effects of

early antioxidant levels on the ability to assimilate these later has also been demonstrated in zebra finches *Taeniopygia guttata* (Blount et al., 2003). Therefore, maternal allocation of antioxidants may be an adaptive strategy, improving the oxidative status of chicks, regardless of post hatching diet. In studies similar to this, females supplemented with carotenoids increased the concentrations of carotenoids in egg yolk, leading to a range of benefits for chicks (Biard et al., 2005; Berthouly et al., 2008). Levels of yolk α-tocopherol would be the most likely aspect of egg quality, manipulable by females in this experiment. In Chapter 2 I found no difference in levels of tocopherol, or carotenoids, in yolks of fifth laid eggs between parents of either treatment. As discussed in Chapter 2, yolk antioxidants may have been different in eggs other than the fifth laid, this may be an artefact of the sampling regime, as antioxidant levels in yolk decrease across the laying sequence in tit species (Hõrak et al., 2004; Biard et al., 2005). Thus, α-tocopherol may still have been responsible for the differences in growth rate and size observed in this experiment. In this study I found no effect of treatment on MDA levels even though α-tocopherol hatched birds appeared to grow faster. I would have expected MDA levels to be higher in the faster growing α-tocopherol birds, if antioxidant levels were the same in both. Therefore α-tocopherol, birds did not appear to pay an oxidative cost of faster growth, evidence perhaps of better antioxidant utilisation. This suggests at least some aspect of antioxidant status may have differed between chicks from different treatments.

Alternatively, other yolk constituents may have been modified by females in response to α-tocopherol supplementation. Lipids, peptides, hormones and antibodies have all been shown to have adaptive roles in egg yolk (e.g. Royle et al., 2001; Grindstaff et al., 2003). Testosterone in particular, has been demonstrated as being an important predictor of growth rate (Navara et al., 2006a), and also promotes ROS production and oxidative stress (Alonso-Alvarez et al., 2007a). Royle et al. (2001) demonstrated that testosterone concentrations increased across the laying sequence, while concentrations of antioxidants (including α-tocopherol) decreased across the laying sequence, in lesser black backed gulls *Larus fuscus*. This is interpreted as an adaptive response: in favourable environmental conditions females will be able to provide chicks from later eggs with a high antioxidant diet, reducing the oxidative stress associated with increased testosterone. In unfavourable conditions i.e. when dietary antioxidants are scarce, the oxidative costs associated with testosterone will ensure later hatched chicks are uncompetitive, reducing unnecessary parental investment. This demonstrates there may be a link between yolk testosterone and nutritional conditions. Though blue tits have a different reproductive strategy, it is not

inconceivable that females with enhanced access to dietary antioxidants, may detect this and upregulate, or downregulate, levels of testosterone in accordance (e.g. Verboven et al., 2003). More work is required on mechanistic links between antioxidant and androgen functions during development.

The links between specific nutrient availability, and reproductive investment are probably more complicated than I initially predicted. During any breeding season, many species must trade-off the energetic and nutritional demands of current reproduction, versus chances of survival and future reproduction (Stearns, 1992). Although I found evidence of enhanced condition in chicks laid by mothers receiving additional dietary α-tocopherol, the benefits of this to chicks is unclear, particularly since chicks from eggs laid by α-tocopherol mothers were skeletally smaller. It should be noted that the benefits to the parents, and in particular the mother, of the treatment were largely untested here (but see Chapter 2). It is possible that by having smaller chicks (though in better condition) that were faster growing, mothers were able to invest more time in self maintenance, and subsequently increase their chances of survival. To test the effects of treatment on survival and recruitment in both adult birds and their chicks, the vast majority of ringed females nesting at our field site during the year following this experiment were retrapped. I found no evidence for a treatment specific difference in F1 recruitment or breeding success, but I found a tendency for more mothers supplemented with α-tocopherol during this experiment to breed in the following season than controls. These birds also seemed to have a greater breeding success. Unfortunately, due to the small sample size, this result cannot be considered statistically robust. Following this, birds were recaptured during a programme of mist netting in the winter of 2007/2008. Again the number of birds recaught was small, though on this occasion there were more F1 chicks, of both sexes, from egg parents supplemented with α-tocopherol, than controls.

My finding that egg effects are more often important than rearing effects in determining condition, size and growth is in agreement with other studies (e.g. Biard et al., 2006). Egg effects may indicate genetic effects, and/or differences between broods in egg quality and incubation. In passerines, the amount of genetic variation in body size and condition is known to depend on environmental conditions, with more genetic variation in good years than bad, in blue tits (Merilä et al., 1999). This is the first study to assess the reasons for variation in oxidative damage in birds. I found that there was an effect of both rearing parents' ID and egg parents' ID on plasma MDA concentration. This suggests that

oxidative damage in chicks near fledging, may be determined through a combination of antioxidant levels acquired by deposition in yolk, and also by parental provisioning. This may explain why α-tocopherol treatment had no effect on MDA levels: I removed food supplements after egg laying, prior to chicks hatching. Thus quality of provisioning is likely to have been similar in parents of both treatments. In tit species, several studies have shown an effect of common rearing environment on carotenoid mediated colour, as my result for yellow component of chest plumage shows here (Biard et al., 2006; Fitze et al., 2003a). This further supports the idea that carotenoid based plumage colouration is determined sometime during early post hatching development (Fitze et al., 2003b). Conversely, I found that the UV portion of chest plumage was affected more by rearing environment than egg effects. Although carotenoid plumage spectra typically have a peak in the UV, as well as long wavelengths, there is some evidence that the mechanism for the UV colour is not carotenoid pigmentation (Prum, 2006). The results here are in agreement with this suggestion. Interestingly, neither component of crown plumage I measured was significantly affected by any aspect of egg quality, rearing environment or feeding treatment.

In Chapter 2, I showed that there was a significant sex ratio difference in broods laid by parents of either treatment. Contrary to my initial predictions, α-tocopherol supplemented females produced more female biased sex ratios than control broods. In the previous chapter, I suggested that the sex ratio difference in α-tocopherol broods may be adaptive, if daughters of α-tocopherol supplemented mothers fared better than daughters from control parents. In this chapter I found no evidence to suggest this was the case. In fact, the only sex specific treatment difference I found suggested that male birds raised by α-tocopherol rearing parents had significantly smaller tarsi than male birds raised by control rearing parents. In contrast, females did not appear to differ in tarsus length with respect to rearing parent's treatment. This may suggest that males in α-tocopherol supplemented nests may have fared worse than males in control nests. If this is true then investment in females by α-tocopherol supplemented females may be adaptive, although as discussed above the costs or benefits of increased tarsus length are difficult to demonstrate. I also suggested that the potentially lower costs of raising daughters may have benefited α-tocopherol mothers via increased chances of survival and future reproduction. There was insufficient data here to test this. Though there was some evidence of an increase in survival of α-tocopherol supplemented mothers, this seemed unrelated to brood sex ratio. While interesting, I can conclude very little about the adaptiveness of the sex ratio difference

between α-tocopherol and control birds. Future experiments in more controlled circumstances are required to investigate whether α-tocopherol is capable of influencing sex allocation decisions, or whether these results represent an artefact.

In conclusion, I found evidence that manipulation of access to α-tocopherol during nest building and egg laying was capable of altering offspring phenotype. Chicks from α-tocopherol eggs were smaller early in development, though gained mass faster than their control foster-siblings. This faster growth was not associated with an increase in oxidative stress, or decrease in plumage colouration in chicks from α-tocopherol supplemented parents. However, further work is necessary to elucidate the benefits, or indeed costs, of this difference in offspring phenotype. The results from retrapping adults during the following breeding season indicated the α-tocopherol supplement during egg formation may have ameliorated the costs to mothers of breeding. There can be little doubt that dietary antioxidants are important nutrients, vital for reproduction, and normal development. Future experiments are required to elucidate the benefits of dietary α-tocopherol to both adult birds, and their offspring.

Table 3.1 Output from GLMM testing effects of feeding treatments and sex on growth rate (mass gain per day) of chicks between days 3-13. Non-significant interactions shown below were removed from the model in stepwise fashion and values are given at point of removal. Significant main effects are marked *.

Random factor	Estimate	Wald's Z	P
Egg parent ID	$1.13x10^{-3} \pm 6.2\ x10^{-4}$	1.85	0.033
Rearing parent ID	$6.9\ x10^{-4} \pm 4.3\ x10^{-4}$	1.61	0.054
Residual	$3.4\ x10^{-3} \pm 4.1\ x10^{-4}$	8.44	<0.0001
Main Effects		$F_{d.f.}$	*P*
Egg treatment		$10.33_{1, 25.2}$	0.0036*
Rearing treatment		$0.48_{1,15.1}$	0.499
Sex		$23.56_{1,160}$	<0.0001*
Egg treatment x Rearing treatment		$0.70_{1,154}$	0.403
Sex x Egg treatment		$2.78_{1,160}$	0.0976
Sex x Rearing treatment		$1.28_{1,152}$	0.260

Table 3.2 Output from GLMM testing effects of feeding treatments and sex on mass in chicks aged 14 days. Non-significant interactions shown below were removed from the model in stepwise fashion and values are given at point of removal. Significant main effects are marked *.

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

Table 3.3 Output from GLMM testing effects of feeding treatments and sex on tarsus length in chicks aged 14 days. Non-significant interactions shown below were removed from the model in stepwise fashion and values are given at point of removal. Significant main effects are marked *.

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

Table 3.4 Output from GLMM testing effects of feeding treatments and sex on body condition (mass/tarsus) in chicks aged 14 days. Non-significant interactions shown below were removed from the model in stepwise fashion and values are given at point of removal. Significant main effects are marked *.

Random factor	Estimate	Wald's Z	P
Egg parent ID	$2.9x10^{-4} \pm 1.9\ x10^{-4}$	1.57	0.058
Rearing parent ID	$8.1\ x10^{-5} \pm 1.1\ x10^{-5}$	0.76	0.224
Residual	$1.1\ x10^{-3} \pm 1.2\ x10^{-5}$	8.92	<0.0001
Main Effects		$F_{d.f.}$	*P*
Egg treatment		$3.69_{1,17.3}$	0.071
Rearing treatment		$0.97_{1,20.9}$	0.335
Sex		$2.61_{1,184}$	0.108
Egg treatment x Rearing treatment		$0.27_{1,168}$	0.601
Sex x Egg treatment		$1.79_{1,181}$	0.183
Sex x Rearing treatment		$0.013_{1,179}$	0.721

Table 3.5 2006 feeding treatments of adult females retrapped in breeding season of 2007. Table includes secondary brood sex ratio from breeding season of 2006, and number of chicks successfully fledged in breeding season of 2007. I did not have information on sex ratio from unmanipulated broods.

Ring number	Treatment	Secondary Sex Ratio(2006)	No chicks fledged (2007)
V205208	Control	0.28	10
V205007	Control	0.57	9
V205012	Control	0.28	0
V205013	α-tocopherol	0.45	7
V205014	α-tocopherol	n/a	9
V205021	α-tocopherol	0.5	8
V205015	α-tocopherol	0.55	11
V205020	α-tocopherol	0.2	13
V205054	unmanipulated	n/a	10
V205209	unmanipulated	n/a	8

Table 3.6 F1 females from 2006 retrapped in 2007. Table shows treatments of both egg parents, and rearing parents, and number of chicks successfully fledged in 2007.

Ring number	Egg Parents' Treatment	Rearing Parents' Treatment	No chicks fledged
V205201	Control	Control	9
V205351	Control	Control	5
V205357	Control	Control	11
V205411	Control	Control	7
V205475	Control	α-tocopherol	11
V205450	α-tocopherol	α-tocopherol	5
V205427	α-tocopherol	α-tocopherol	10
V205326	α-tocopherol	Control	8
V205732	α-tocopherol	α-tocopherol	11
V205383	α-tocopherol	Control	9
V205442	α-tocopherol	α-tocopherol	8
V205739	Unmanipulated	Unmanipulated	12
V205738	Unmanipulated	Unmanipulated	9

Table 3.7 Treatments of egg parents and rearing parents of F1 chicks from 2006 recaptured in winter 2007/2008.

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

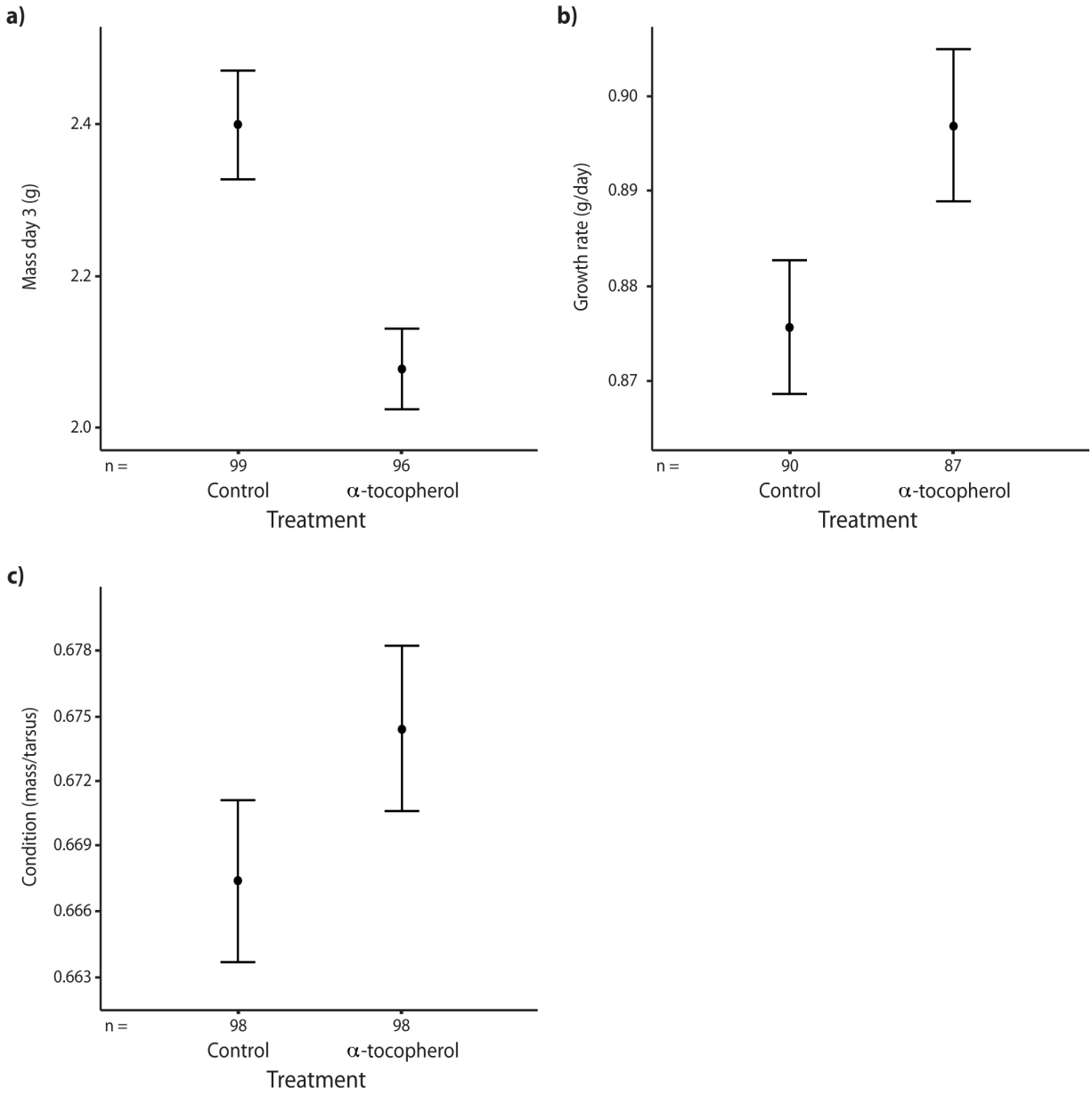

Figure 3.1 Mean (± 1 S.E.) differences between chicks from egg parents that had received either α-tocopherol, or control diet in: **a)** Mass of chicks age 3 days; **b)** Mass gain per day between days 3-13; **c)** Body condition of chicks aged 14 days

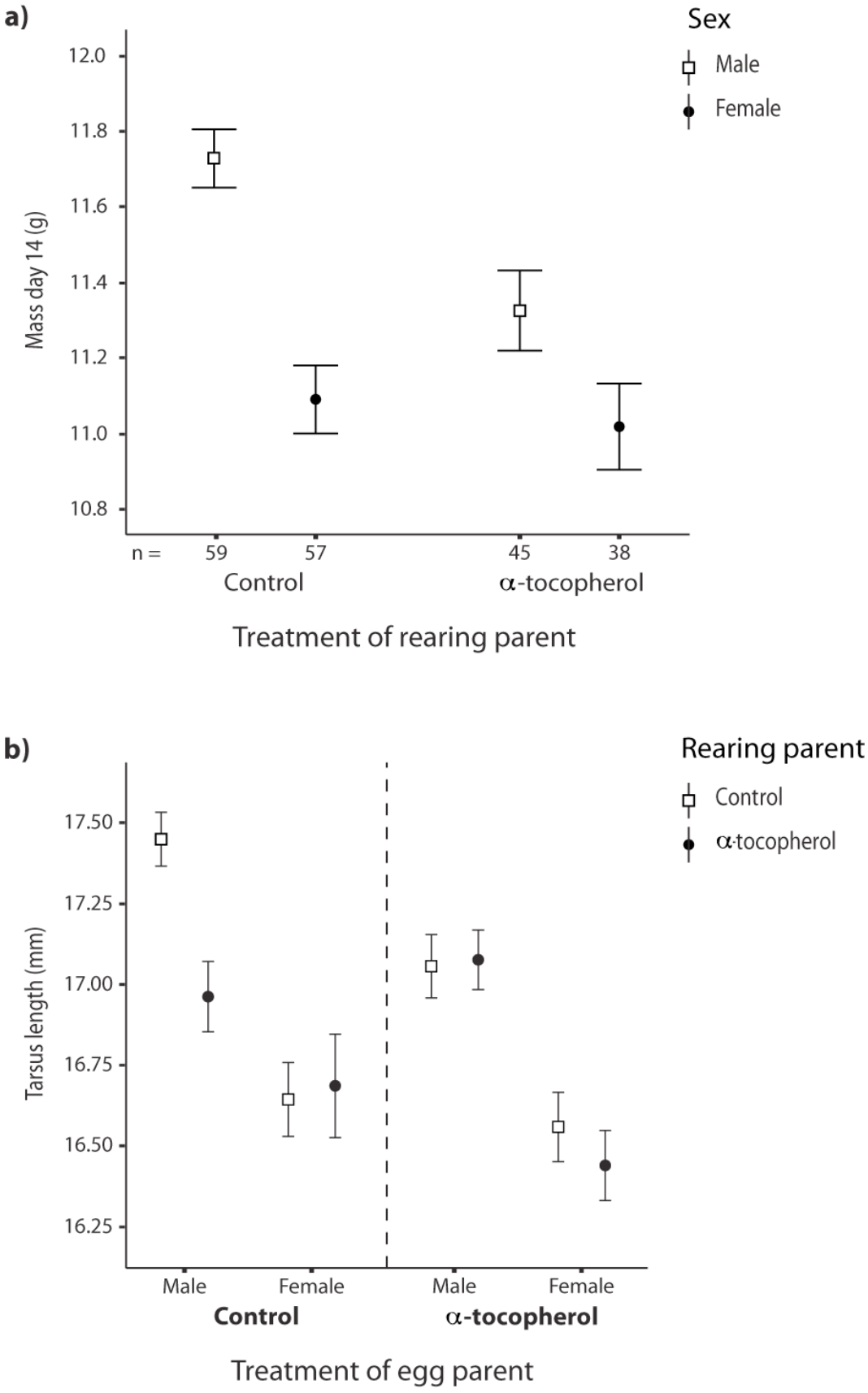

Figure 3.2 Differences (Mean ± 1 S.E.) between chicks a). Mass aged 14 days of male and female chicks, reared by different parents b) Tarsus length chicks, laid by either α-tocopherol, or control treated parents, and raised by α-tocopherol, or control treated parents

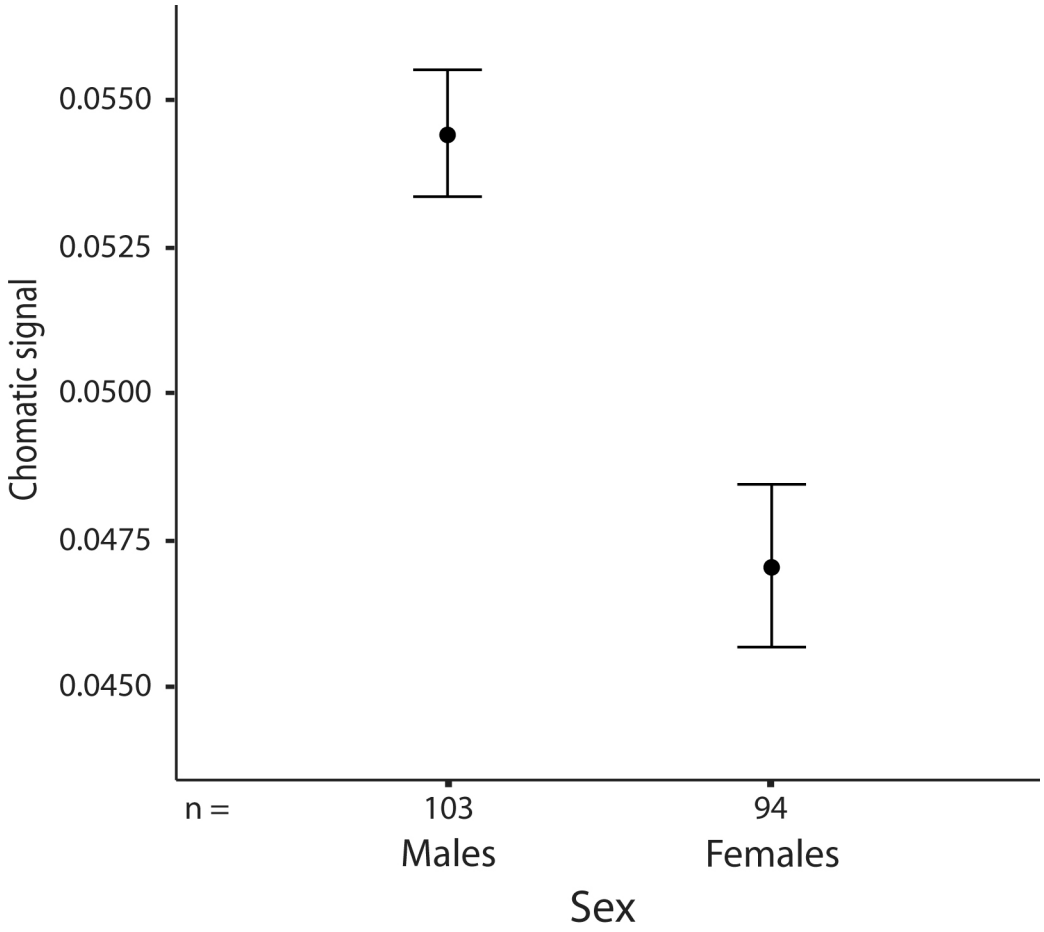

Figure 3.3 Mean (± S.E.) chromatic signal from yellow chest plumage in male and female chicks aged 14 days

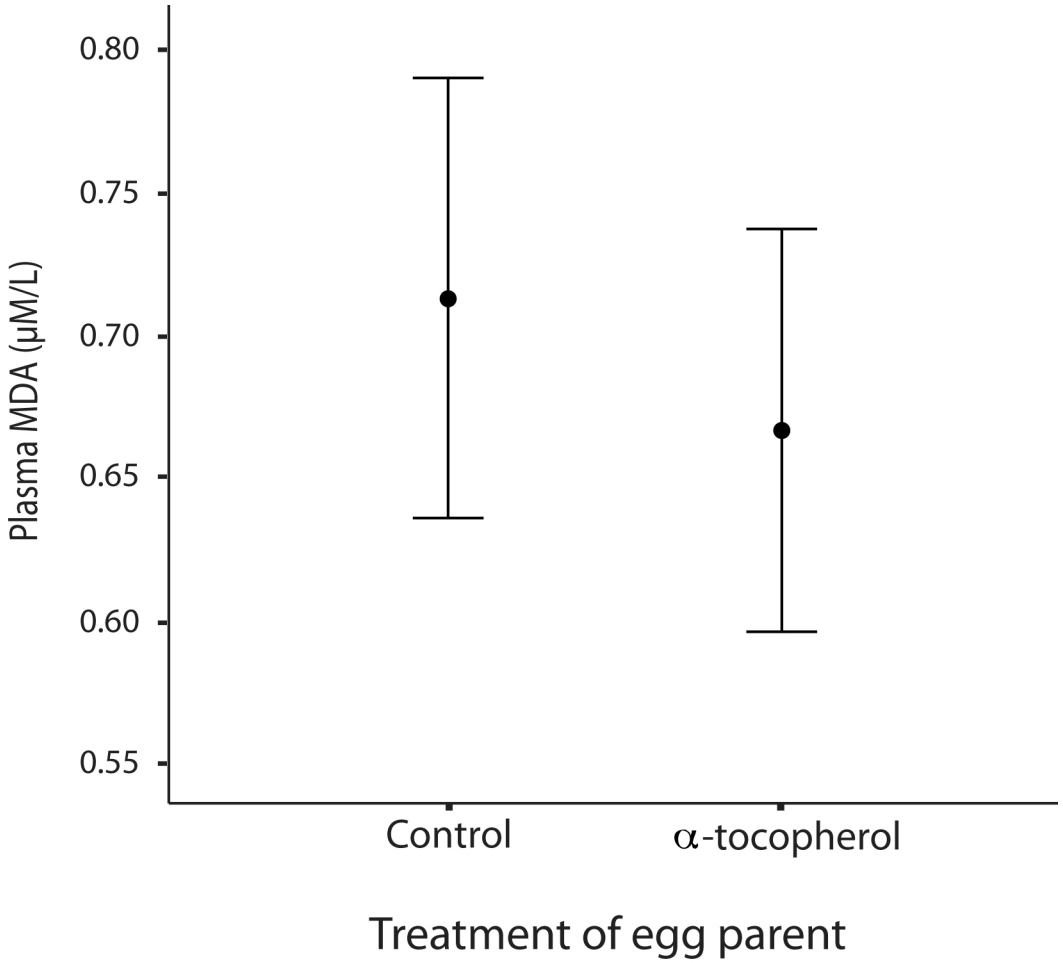

Figure 3.4 Mean (± S.E.) MDA concentration in chicks aged 14 days, from either control or α-tocopherol eggs.

Chapter 4. The roles of α-tocopherol and carotenoids in determining plumage colouration, oxidative damage and growth rate in nestling blue tits *Cyanistes caeruleus*

4.1 Abstract

Carotenoid pigments are responsible for many of the red, yellow and orange plumage and integument traits seen in birds. One idea suggests that as carotenoids act as antioxidants, carotenoid-mediated colouration may reveal an individual's ability to resist oxidative stress. Conversely, it has been argued that carotenoid-mediated colouration may act as a "Red Herring" in indicating antioxidant levels. Since antioxidants can prevent carotenoid pigments from bleaching, higher levels of non-pigmentary antioxidants may be predicted to increase plumage colouration through protection of carotenoid-mediated colour. Here, I tested this hypothesis by supplementing neonatal blue-tits *Cyanistes caeruleus* with one of three diets; control, carotenoid treatment, or α-tocopherol treatment. After 14 days I measured the plumage colouration of each bird, and plasma levels of malonidialdehyde (MDA), a by-product of lipid peroxidation. I predicted that α-tocopherol supplemented birds should have enhanced plumage colour compared to controls if non-pigmentary antioxidants protect carotenoids against oxidative discolouration. It was also predicted that α-tocopherol supplemented birds should have lower levels of oxidative stress (and hence MDA) than carotenoid supplemented and control groups. I found that only the carotenoid supplemented birds tended to increase in chromatic signal of the chest, and α-tocopherol had no effect on plumage colouration. There were no differences in oxidative stress between treatment groups. However, I found that the chromatic signal of the chest was positively correlated with levels of MDA, suggesting that increased carotenoid-mediated plumage may reveal higher levels of oxidative stress. Interestingly, faster growing birds had a higher chromatic contrast, and higher circulating levels of zeaxanthin, but suffered more oxidative stress. Although I found no support of the Red Herring hypothesis, neither was carotenoid-mediated plumage an honest signal of resistance to oxidative damage. Further integrative studies are necessary to elucidate the roles of dietary derived antioxidants in physiological trade-offs.

4.2 Introduction

Carotenoid pigments are the basis for many of the red, yellow and orange colours seen across nature (Olson and Owens, 1998). Since carotenoids are also considered important in internal processes, including the immune and antioxidant systems, they have long been the focus for studies of trade-offs between competing physiological systems. Central to many studies is the idea that carotenoids act as antioxidants; scavenging reactive oxygen species (ROS) which cause damage to proteins, lipids, and DNA, vital for normal cell function. Thus, yellow or red colouration of feathers or integument is suggested to reflect the antioxidant status of an individual (von Schantz et al., 1999). However, the extent to which carotenoids are genuinely useful antioxidants has recently been questioned (Hartley and Kennedy, 2004; Costantini and Møller, 2008; Isaksson and Andersson, 2008). Carotenoids have a lower antioxidant capacity compared to many non-pigmentary antioxidants, and they cannot be recycled. For example the efficiency of α-tocopherol (vitamin E), the major lipid-soluble antioxidant used in membrane defence, in inhibiting formation of malonidialdehyde (MDA), one of the major products of lipid peroxidation, has been shown to be 40-50x greater than β-carotene (Palozza and Krinsky, 1991). Moreover, carotenoids are precursor molecules for the metabolism of vitamin A and other retinoid compounds. These molecules play a major role in immunity against viral, protozoan and bacterial infection (Semba, 1999). Hartley and Kennedy (2004) suggest that it is unlikely that the primary role of dietary carotenoids will be as antioxidants, since their function in the immune system is extremely important, and in this role carotenoid molecules are short lived.

Recently, Hartley and Kennedy outlined the "Red Herring hypothesis" which states that although carotenoids are unlikely to be important biological antioxidants, since non-pigmentary antioxidants like α-tocopherol are suggested to protect carotenoid pigments from oxidative bleaching, carotenoid signals may reveal the levels of these other antioxidants (Hartley and Kennedy, 2004). The red herring hypothesis offers two testable predictions: Firstly, increasing levels of non-pigmentary antioxidants should increase expression of carotenoid-based signals. Secondly, that antioxidant function is unlikely to be the primary use of carotenoids *in vivo*.

Non-pigmentary antioxidants have been shown to significantly increase expression of integumentary carotenoid-mediated colouration in both the red nuptial patch of

sticklebacks (Pike et al., 2007), and the red bill colouration of zebra finches (Bertrand et al., 2006). The patterns for plumage colouration may be different because feather colouration is determined by carotenoid deposition during the time of feather growth, and cannot be modified until the following moult. As such it represents a less dynamic system than bill or skin colouration, where carotenoids can be redeployed in response to an immune challenge (Faivre et al., 2003). Unlike in feathers, integumentary carotenoids are often esterified (Czeczuga, 1979), and carotenoids are found in higher concentrations in feathers than other tissues (McGraw et al., 2003). Thus, there is reason to suspect that feather colouration may react differently to antioxidant supplementation than integumentary colouration. In one recent study, Karu et al. (2008) found that vitamin E had no effect on plumage colouration in greenfinches *Carduelis chloris*, where carotenoid supplementation increased colouration. Interpretation of this study is tricky because carotenoid supplementation was provided within a vitamin E rich substrate, which may have masked the effects of vitamin E. The yellow chest of blue tits, *Cyanistes caeruleus*, is a patch of carotenoid-mediated plumage present in both adult and chicks. It has been shown that supplementing nestling blue tits with carotenoids significantly increases plumage colouration (Hadfield and Owens, 2006), though the effect of non-pigmentary antioxidants has not been tested. Also, the function of carotenoid colouration in blue tit nestlings is unknown. In the closely related great tit (*Parus major*) carotenoid colouration is not related to begging or provisioning rate (Fitze et al., 2003a; Tschirren et al*.,* 2005), or fledging success (Fitze and Tschirren, 2006). In adult blue tits, males with more yellow plumage raise offspring in better condition with enhanced immunity (Hidalgo-Garcia, 2006; Senar et al., 2002). Although the function of nestling colouration is unclear, supplementing nestling birds during early development allows manipulation of dietary antioxidant levels throughout the entirety of feather growth. According to the Red Herring hypothesis, if increasing levels of non-pigmentary antioxidants increases the expression of carotenoid-based plumage colouration, then nestlings fed α-tocopherol and nestlings fed carotenoids should both increase in colouration, compared to controls.

The second prediction of the Red Herring hypothesis is that antioxidant function is unlikely to be the primary use of carotenoids *in vivo*. To test this, an appropriate measure of oxidative stress is vital. When suggesting carotenoid signals are an indicator of oxidative status (von Schantz et al., 1999), levels of antioxidants are often referred to as antioxidant status or wealth, and many studies of the biological actions of carotenoids have measured antioxidant capacity of plasma (i.e. Tummeleht et al., 2006; Hõrak et al., 2006;

Pike et al., 2007). However, it is important to draw a distinction between antioxidant levels or capacity, and oxidative status. The potential of carotenoids to act as antioxidants does not imply that this is their primary function, or that they actually prevent oxidative damage *in vivo*. Malonidialdehyde (MDA) is a by-product of lipid peroxidation and therefore is directly representative of levels of cell damage caused by oxidative stress (Young and Trimble, 1991). In Chapter 3, I showed that variation in MDA in the same population of blue tits was partly determined by effects of rearing environment. Thus, I expect that chicks fed α-tocopherol should have reduced levels of MDA compared to chicks fed either carotenoids or the control, if carotenoids are ineffective biological antioxidants in birds.

Blue tits provide a good model for testing the Red Herring hypothesis for a number of reasons. In addition to being well studied and experimentally malleable, both sexes have yellow carotenoid mediated chest plumage, present in nestlings as well as adults. In order to test the effects of non-pigmentary antioxidants on plumage colouration and oxidative damage, I supplemented neonatal blue-tits with one of three diets; control, carotenoid or α-tocopherol, in a within-brood design. Supplementation was kept within biologically relevant limits, and plumage colouration, plasma antioxidant levels and plasma MDA concentrations were measured shortly before fledging. To my knowledge, this is the first test of relationships between MDA and dietary antioxidants between birds, in an ecological context. Specifically, I addressed the following questions: Does α-tocopherol or carotenoid supplementation enhance plumage colouration compared with a control? Does α-tocopherol or carotenoid supplementation decrease oxidative stress compared with a control?

4.3 Methods

This study was carried out in spring 2005 using nestboxes at two adjacent field sites in Scotland (see *study sites*, general introduction). The first site was Ross Woods, the mixed oak woodland around Scottish Centre for Ecology and the Natural Environment (SCENE), near Rowardennan on the east side of Loch Lomond. At this location I utilized 11 of approximately 300 nestboxes, the rest were used in another project (Arnold et al., 2007). The second site was the woodland around Cashel Farm, two miles south of Ross Woods, where 16 of approximately 90 nestboxes were occupied by blue tits, and used in this study. Nest boxes were monitored at least every two days between nest building and egg laying, to record the stage of nest building, resident species, dates of egg laying, start of incubation and dates of hatching. All work was conducted under Licence from the UK Home Office, and no chicks died as a result of the experiment.

Feeding treatment

Due to differences between nests in territory quality and parental provisioning, I performed a within-brood manipulation. Each chick in a nest was assigned to one of three treatments: control, carotenoid or α-tocopherol. Gelatine, which contains protein and some lipid, was chosen as a vehicle rather than water since I did not want to satiate a chick without providing nutrition. Based on known rates of prey consumption (Arnold et al., 2007) and antioxidant content of prey (Ramsay and Arnold, unpublished data) I estimated the daily intake of carotenoids and α-tocopherol for blue tit chicks in order to calculate dosage. I prepared fresh food every night for use the following day. Leaf gelatine was dissolved in boiled water and allowed to cool before adding the appropriate supplement. For the carotenoid treatment I used oro glo powder (Kemin Ltd, Lincoln, UK) which consists of lutein and zeaxanthin (20:1). For α-tocopherol I used α-tocopherol acid succinate (Sigma, Poole, UK). When chicks were between 3-7 days old each was provided with 0.05ml of gelatine. Between days 7-14, 0.1ml was provided. The effective dosage per bird was 0.11 mg carotenoids, and 0.079 mg α-tocopherol between days 3-7; and 0.22 mg carotenoids, and 0.159 mg α-tocopherol between days 7-14. The carotenoid doses are similar to doses used in other studies (Biard et al., 2006; Hadfield and Owens, 2006), although I am unaware of any other supplementation of α-tocopherol to blue tits.

When the chicks were three days old they were assigned to a feeding treatment. The brood was removed from the nestbox and placed temporarily in a heated box. A coin was tossed to determine the treatment of the first chick selected randomly from the box, and each following chick was allocated to a treatment in the order; control, carotenoids, α-tocopherol etc. Every chick within each nest was individually marked with a unique colour combination on the three patches of down on their heads using non-toxic ink, allowing easy identification for food supplementation. After marking, the chicks were weighed to within 0.1g using a field balance, and then fed the appropriate food. Using a 1ml syringe without a needle, the appropriate volume (see above) of each gelatine treatment was squeezed directly into the chick's mouth, ensuring it was swallowed. Whole broods were returned to their nestbox within 30 minutes. Nests were then revisited every other day and chicks were identified, and fed. At day 9, chicks were re-marked, using a unique combination of claw clipping since after this age the down patches on the head recede.

Morphometric and growth measurements

When the chicks were 14 days old they were processed in half broods, to avoid parental desertion, at SCENE field laboratory. Half of each brood was taken from the nestbox to the laboratory in a heated bag. Each bird was removed from the bag and blood sampled immediately: a small volume of blood was taken by venipuncture from a wing vein. One drop of blood was put in ethanol for subsequent DNA extraction and molecular sexing using a standard technique, previously used for blue tits (Griffiths et al., 1998; Arnold et al., 2007) The remaining blood was collected in 75µl capillary tubes. The capillary tubes of blood for MDA and antioxidant analysis were centrifuged and haematocrit readings were taken from each, before these were stored at -20ºC, and later at -80ºC. After blood sampling each bird was weighed and had its wing and tarsus measured and a BTO leg ring was applied, before they were analysed for colour measurements. Using a spectrophotometer (Ocean Optics S2000), two readings were taken from both side of the yellow portion of every bird's chest. The spectrophotometer was standardised against both a black and white standard before each reading. Where feathers were in pin, or underdeveloped, this was noted and the colour data was excluded from further analysis. After each half brood was completed, it was returned to the nestbox and swapped for the remaining half brood. Birds were removed from their nests for no longer than 60 minutes. Particularly hungry chicks were fed after weighing and sampling, before being returned. Calculating true growth rate is difficult for nestling birds, since avian growth follows a

sigmoidal curve, with a slow start, a rapid linear increase to an inflection point, then a slow down in rate until adult size is assumed (e.g. Brown et al., 2007). In this study I wished to assess growth rate of nestlings fed different antioxidant treatments, since rapid growth and cell proliferation will cause increased ROS production and oxidative damage (Surai, 1999). As such, the best indication of rate of growth would include skeletal growth and cell genesis, as well as mass gain by fat or muscle deposition. Unfortunately, in this study I did not measure tarsus length throughout nestling growth, but birds were weighed daily between the ages of 4-13 days. However, in line with my prediction that the rapid linear phase of growth should correspond with an increase in oxidative stress, I calculated the rate of mass gain during linear growth, as a surrogate for growth rate. To deal with differences in starting mass between different birds, I calculated the natural logarithm (ln) of daily mass data. I then plotted ln(mass) vs age for every bird. Plotting this data allowed me to determine the phase of linear growth in these nestlings as between days 4 and 10. Finally, for each bird I calculated growth rate as (ln (mass day 10) - ln (mass day 4))/ 6. Any unfledged chicks were identified and noted. Only 7 chicks died out of 239 and this did not differ between treatments. This mortality level was similar to unmanipulated broods (Arnold, unpublished data). There was no evidence that either handling or diet manipulation had a negative impact on welfare.

Analyses of plumage colouration.

In this study, I wished to investigate whether a non-pigmentary antioxidant was capable of enhancing the carotenoid-mediated component of chest plumage. I calculated colouration in two ways. Firstly, I calculated the "carotenoid chroma" of feathers. From the reflectance spectrometry data carotenoid chroma was calculated as $(R_{700} - R_{450})/ R_{700.}$ The wavelength of maximum absorption of carotenoid pigments is 450nm and maximum reflectance is 700nm. Thus, this measure provides an assessment of the relative difference between maximum and minimum carotenoid absorbance, and provide a measure of carotenoid mediated colour (Isaksson et al., 2005)

Since the hypothesis tested in this experiment ultimately relates to the signal content of carotenoid plumage, I also analysed spectral data with reference to the spectral sensitivities of the visual system of blue tits. I used the SPEC package (www.bio.ic.ac.uk/research/iowens/spec) for R1.6.1 (www.R-Project.org) to reduce the spectral data into four quantal cone catches, relative to the sensitivities of blue tit retinal

cones (Hadfield and Owens, 2006). These cones were ultraviolet sensitive, short wavelength sensitive, medium wavelength sensitive, and long wavelength sensitive (UVS, SWS, MWS and LWS respectively) (Hadfield and Owens, 2006). Since this experiment was investigating the component of plumage colouration specifically attributable to carotenoids, I used spectral data from only the LWS and MWS cones in the analyses. These cones relate to the region of the spectrum mediated by carotenoids. The wavelength of maximum sensitivity (λ_{max}) of these cones (± one standard deviation) are: MWS = 502.9 ± 1.6; and LWS = 563.1 ± 2.1 (Hart et al., 2000). Using the spectral readings from the chest plumage of each bird, I calculated a mean value per cone per bird. Chromatic cues permit the distinction of stimuli of different spectral composition regardless of intensity, typically achieved by chromatic opponency of signals from photoreceptors (Osorio et al., 1999). I calculated the "chromatic signal" of the LWS and MWS cones with the following formula, from Osorio et al. (1999):

chromatic signal = (LWS-MWS)/(LWS+MWS)

Since feathers with greater carotenoid content have been shown to have higher values of chroma (Saks et al., 2003), a greater chromatic signal should indicate increased carotenoid-mediated colour. Analyzing colour in both these ways has the advantage of allowing variation in different aspects of colour to be analyzed, and also permits an assessment of the relationship between both colour measures. I found that both measures were strongly positively correlated with one another (Pearson's rho= 0.406, $p < 0.0001$).

Blood plasma antioxidant analysis

In order to assess the effect of feeding treatment on plasma levels of the manipulated antioxidants, I analysed plasma concentrations of lutein, zeaxanthin, and α-tocopherol. I also measured plasma retinol concentration. In order to extract the antioxidants, 4µl ethanol was added to 20µl of plasma and vortexed thoroughly. 50µl of hexane was then added and vortexed before the hexane layer, containing the antioxidants was drawn off. This was repeated with 40µl of hexane, before the hexane extract was placed in a SpeedVac for 20 minutes. The final antioxidant extract was then dissolved in 20ml of methanol.

I used a Spectra Model 8800 HPLC pump system with a Phenomenex 250mm x 2mm id column to determine antioxidant composition of each sample. I used HPLC at a flow rate of 0.2ml/min with a mobile phase of water/acetonitrile (2.5:97.5), and water/ethyl acetate (2.5:97.5) in a gradient elution. Using a Diode array absorbance detector type Thermo model UV6000, carotenoids were detected by absorbance at 445nm, α-tocopherol at 295nm, and retinol at 325nm. Peaks were identified by comparison with chromatography and retention times of several standards (Sigma, Poole, UK; Fluka, Gillingham, UK; Carotenature, Lupsingen, Switzerland).

Oxidative damage assay

In order to assess the effect of supplemental feeding treatment on oxidative stress, I measured malonidialdehyde, a by-product of lipid peroxidation, in the plasma of a subsample of chicks. Owing to the relatively large volume of plasma required for these analyses, I analysed plasma samples from one chick per treatment per brood. This meant a final sample size of 85 samples. MDA analysis was performed as in Young and Trimble (1991) with some modifications.

Thiobarbituric acid (0.044M,100µl) and phosphoric acid (1.22M, 100µl) were mixed together and added to 50µl of plasma (per bird) in a test tube. An inert atmosphere was created by applying a nitrogen blanket, and the test tubes were sealed and vortexed prior to heating (60 min, 70-75^{o}C). Samples were cooled in water, then 200µl was transferred to a centrifuge tube containing sodium hydroxide (1M, 100µl). Methanol (500µl) was added and mixed. Samples were centrifuged (10 min, 12000 g) and the supernatant analysed on a Summit HPLC system (Dionex, Idstein, Germany) using Chromeleon software (Dionex). An Acclaim 120 C18 5µ 4.6 x 250 mm column (Dionex) and guard were used with fluorescence detection (excitation 532nm and emission 553nm). The mobile phase was isocratic, 40:60 methanol:phosphate buffer (40mM, pH 6.5), with a flow rate of 1ml/min, and a run time of 7 min. Samples were assayed against a standard of malonidialdehyde bis (dimethyl acetal), (Sigma Aldrich, Poole, UK) that was simultaneously taken through the same procedure.

Statistics

Effects of feeding treatment on nestling characteristics was tested using a General Linear Mixed Model (GLMM, SAS v8, SAS Institute Inc., Cary, NC, USA). In all models I used nest identity (ID) as a random effect, in order to control for the non-independence of data from siblings. I tested several aspects of nestling phenotype. Firstly to test the effect of treatment on plasma antioxidants and oxidative damage I used lutein, tocopherol, retinol and MDA as dependent variables, with treatment and sex as fixed factors and mass, growth rate and hatching date as covariates. I tested for treatment differences in growth rate with treatment and sex as fixed factors and hatching date as a covariate. To test the effect of treatment on colour I used chromatic signal and carotenoid chroma as dependent variables with treatment and sex as fixed factors and mass, growth rate, hatching date, and MDA as covariates. In all cases models were simplified by dropping non-significant terms until only significant factors remained. All significant terms are reported below, and data are presented as means ± 1 standard error throughout. Sample sizes vary between analyses because only a subset of blood samples were used for MDA or antioxidant analysis.

4.4 Results

The final sample size was 232 chicks in 26 nests. All models below provided a better fit including the random factor, nest ID, than without it (Wald's $Z > 1.33$, $p < 0.09$ in all cases).

Effect of feeding treatment on manipulated antioxidants

Lutein concentration was significantly higher in the plasma of carotenoid fed nestlings, than those in both other treatment groups (Treatment: $F_{2,92.4} = 6.14$, $p = 0.003$, Figure 4.1a). However, there was no significant difference in plasma α-tocopherol between treatment groups (Treatment: $F_{2,78.3} = 0.14$, $p = 0.866$, Figure 4.1b). There was a negative relationship between body mass and plasma α-tocopherol ($F_{1,79.5} = 7.14$, $p = 0.0091$). There was no significant difference in plasma retinol between treatment groups, and plasma retinol concentration declined across the breeding season ($F_{1,18.6} = 7.18$, $p = 0.015$).

Plumage colouration

Chromatic signal (calculated as perception of yellow chest plumage by blue tit visual system) was positively correlated with plasma concentration of MDA ($F_{1,60} = 7.92$, $p = 0.0066$, Figure 4.2a), and male chicks had a significantly greater chromatic signal than females ($F_{1,53.6} = 17.35$, $p = 0.0001$, males = $0.055 \pm 9.8 \times 10^{-4}$; females = $0.048 \pm 97.4 \times 10^{-4}$). There was a marginal trend for carotenoid supplemented birds to have a greater chromatic signal than α-tocopherol and control fed birds ($F_{2,42.9} = 2.62$, $p = 0.0843$, Figure 4.2). There were no significant sex or treatment differences (see Fig 4.2b) in carotenoid chroma (calculated as the amount of carotenoid-mediated colour in chest plumage), however there was a non-significant positive relationship between carotenoid chroma and MDA ($F_{1,59} = 2.86$, $p = 0.0961$) .

Oxidative damage and growth rate

MDA was unaffected by feeding treatment, but plasma MDA was positively correlated with growth rate ($F_{1,47.1} = 6.56$, $p = 0.0137$, Figure 4.4) and negatively correlated with hatching date. ($F_{1,17.8} = 5.27$, $p = 0.0341$). There was no sex difference in MDA. There was no treatment difference in growth rate though there was a marginally non-significant sex

difference in growth rate, with males tending to grow faster than females ($F_{1,172} = 3.34$, $p = 0.069$) . Since I did not have both plasma antioxidant and MDA values from the same individual, I could not assess the relationship between plasma MDA and antioxidant levels.

4.5 Discussion

Experimentally supplementing nestlings with either α-tocopherol or carotenoids permitted an analysis of the different effects of these nutrients in determining plumage colouration, and oxidative stress. Supplementation with carotenoid treatment significantly increased plasma concentration of lutein, the predominant carotenoid in the treatment. I did not detect increased plasma α-tocopherol levels in α-tocopherol supplemented birds, compared with the other treatment groups. However, the α-tocopherol was provided using the same supplementation technique, and α-tocopherol supplemented nestlings did not appear to show any detrimental effects of the treatment. The lack of an effect of treatment on plasma α-tocopherol levels may simply reflect the much lower plasma concentrations of this molecule in general, since α-tocopherol is most effective when utilised as a membrane bound antioxidant. Therefore, as in other studies (e.g. Biard et al., 2006), I suggest that both treatments were successful in raising internal levels of the intended lipophillic antioxidants, though in the case of α-tocopherol this was not reflected in plasma levels.

In this experiment, I found a trend for carotenoid supplemented birds to have greater chromatic signal of yellow chest plumage, than both control and α-tocopherol birds. In contrast to my predictions, there was no difference in plumage colouration between the control and α-tocopherol birds. There was no significant treatment difference in carotenoid chroma between the treatment groups, though again a trend for a difference between carotenoid supplemented birds, and controls was found. Thus neither measure of colour provides evidence that α-tocopherol significantly affects colouration. Interestingly while there was a significant sex difference in chromatic signal, that is the signal of yellow chest plumage as perceived by other blue tits, there was no difference in carotenoid chroma, the amount of specifically carotenoid mediated colour in plumage. This suggests that though some aspect of colour differed between the sexes, this was not related to carotenoid pigmentation. Further work is required to elucidate the exact mechanism behind this difference. Contrary to these results, the prediction that integumentary carotenoid colouration reveals the abundance of non-pigmentary antioxidants has been supported

recently for both sticklebacks (Pike et al., 2007) and zebra finches (Bertrand et al., 2006). In this study I supplemented α-tocopherol, a potent antioxidant prevalent in the diet of blue tits (Arnold and Ramsay, unpublished data) which has a high potential to protect plumage pigments from bleaching. In the only other study of the effects of α-tocopherol on plumage colour, Karu et al. (2008) also found no effect of vitamin E on plumage colouration. One possible reason for the lack of an effect of α-tocopherol on colouration is that dietary carotenoids may not be limiting in the diet of nestling blue tits and thus α-tocopherol makes no difference to the expression of the carotenoid-mediated trait. The "red herring hypothesis" depends on non-pigmentary antioxidants protecting relatively rare carotenoid pigments. However, the carotenoids appear to cause some change in plumage colouration, particularly with respect to chromatic signal. It is possible that because carotenoids are so prevalent in the diet they are "dumped" rather than invested in feathers, since carotenoids in high doses may exhibit pro-oxidant activities in the body (Burton and Ingold, 1984). Alternatively, problems in calculating dosage may result in the lack of any effect of α-tocopherol on colouration. The highly specialist caterpillar based diet of blue tit chicks makes assessment of levels of α-tocopherol ingested by chicks relatively precise. Nevertheless, there is some evidence that α-tocopherol absorption may be impaired by carotenoids, through competition (Woodall et al., 1996; Parker, 1996), and the bioavailability of α-tocopherol for chicks is unclear. In this study I provided α-tocopherol to one set of chicks, and carotenoids to another, though never the two in concert. However, the diet of blue tits is already rich in carotenoids and α-tocopherol (Ramsay and Arnold, unpublished data). Feeding chicks shortly after a feeding by their parents may have interfered with absorption of the supplements, and I did find small differences in plasma levels of lutein, but not α-tocopherol between treatments. More work is needed to determine whether α-tocopherol may have dosage dependant impacts on colour, particularly in carotenoid deficient individuals.

Plumage colouration changes in response to carotenoid supplementation have been demonstrated before in nestling great tits (Tshirren et al., 2003; Fitze et al., 2003b; Biard et al., 2006). In nestling blue tits Hadfield and Owens (2006) reported a change in blue tit colouration with carotenoid supplementation, whereas Biard et al. (2006) found no effect. So far, the function of nestling plumage colouration, and therefore the relevance of enhanced colouration, is unclear. As in other species, offspring plumage colouration may be used by parents to determine food provisioning (Lyon et al., 1994), although this was not true for one study of great tit nestlings (Tschirren et al., 2005). Moreover, nestling

colour was found to be unrelated to adult plumage colouration in great tits (Fitze et al., 2003a) and nestling colouration in at least in one population of great tits (Fitze and Tschirren, 2006), seems unrelated to fitness. In contrast, there is some evidence that the colouration of yellow chest plumage is related to fitness traits, e.g. parental abilities, in adult blue tits (Senar et al., 2002). Plumage colouration may therefore be an honest signal of quality in adulthood but not in nestlings. α-tocopherol can be stored in the liver during the nestling phase (Surai et al., 1998), and thus might have impacts later in life. For example, it could be utilised to protect pigments when carotenoids are relatively scarce in the diet, prior to moulting into breeding plumage, but this remains to be tested.

In line with the Red Herring hypothesis, I predicted that birds supplemented with α-tocopherol would have lower levels of MDA, and therefore oxidative stress than other nestlings. However, I found no difference in MDA levels between treatment groups. Unfortunately I could not perform both MDA and plasma antioxidant analysis for any individual bird. Nevertheless, I found a significant treatment difference in plasma lutein concentration, though not in oxidative stress. This suggests that for nestling blue tits, dietary carotenoids, or at least dietary lutein the most abundant carotenoid in the blue tit diet, are not the most important predictors of individual lipid peroxidation. In this experiment, nest ID, the random factor, contributed to the model (though was marginally non-significant), suggesting inter brood differences, were bigger than intra brood differences, in levels of MDA. Additionally, in Chapter 3, I showed an effect of rearing parents' ID and egg parents' ID on MDA levels in nestling blue tits. This suggests differences in MDA between chicks are at least partly determined after hatching. If this is unrelated to provisioning of antioxidant rich foods, then other inter brood differences must be considered. ROS are produced by the immune cells as part of the immune response, and therefore higher levels of MDA may indicate immune activation. For example, de Lope et al. (1998) demonstrated ectoparasites are capable of inducing an immune response in house martin *Delichon urbica* nestlings. Hen fleas *Ceratophyllus gallinae* are common ectoparasites of tit nestboxes (Fitze et al., 2004) present in our population, though the differences between nestboxes are unknown. Ectoparasite mediated immune activation is often associated with a decrease in growth rate, plumage colouration and body size (e.g. Tschirren et al. 2003a; Tschirren et al. 2003b). However, in this study MDA was actually positively correlated with growth rate and plumage colouration, the opposite of what would be predicted if oxidative stress was caused by a costly immune response. Although MDA analysis cannot be used as an assessment of total oxidative stress (Dotan et al., 2004;

see also Chapter 5), I suggest that measuring an index of lipid peroxidation is a more effective measure of oxidative status than measuring antioxidant capacity, since the potential of molecules to act as antioxidants does not necessitate that this will be their role. Since I found an increase in carotenoid levels caused by feeding treatment, simply using antioxidant capacity as a measure of oxidative status, as in other studies, may have led to misleading conclusions.

Interestingly, I found a positive correlation between MDA and chromatic signal, and a trend for a positive relationship between MDA and carotenoid chroma, the opposite of what may be expected if plumage colouration is a signal of oxidative status (von Schantz et al., 1999). Since carotenoid treatment did appear to increase concentrations of lutein and also colouration, at least for chromatic signal this suggests that while plumage colouration in nestling is partially mediated by carotenoid availability, increased plumage colour does not reveal lower levels of oxidative stress. In fact, quite the opposite trend was observed here. In addition, in captive great tits, newly fledged chicks did not differ in plumage colouration, after being given a specific oxidative challenge (Isaksson and Andersson, 2008). Also, antioxidant activity of plasma was shown to be unrelated to either plumage colouration, or plasma carotenoid concentrations in adult or nestling great tits (Isaksson et al., 2007a) This is further evidence that plumage colouration (particularly in nestlings), is unrelated to antioxidant, or oxidative status. As with nestling colouration, the biological relevance of nestling oxidative stress is unclear. Some factors influencing oxidative stress will not be encountered until after fledging such as the cost of flight exercise (see Chapters 5, 6 and 7). The negative effects of oxidative stress are likely to be cumulative, and it is unclear whether nestling oxidative stress will be translated into chronic long-term oxidative stress, or whether it will be ameliorated once growth is completed. Certainly, identifying trade offs is more difficult in developing birds, where the need to grow and compete for care may have more immediate fitness consequences than the costs associated with oxidative stress.

In this study, faster growing birds were found to have higher levels of MDA. Growing tissue is a site of high ROS production, so this may be expected. Since increased dietary antioxidants may prevent oxidative damage and allow faster growth, I may have predicted differences in growth rates between carotenoid/α-tocopherol and control treated birds. Indeed, in barn swallows *Hirundo rustica*, supplementing nestlings with additional vitamin E was found to increase body size and growth rate at some developmental stages, though

the effect size was small (de Ayala et al., 2006). In this study I found no significant effect of α-tocopherol or carotenoid supplementation on growth rate. It is worth considering here that calculating growth rate is a complicated procedure. My prediction that faster growth is associated with oxidative stress was based on the idea that cell proliferation and high metabolism during growth increase rates of ROS production (Surai, 1999). However, in this study, growth rate is calculated as a gain in mass that need not be reliant on skeletal or body growth. While it is true that birds gaining more mass between days 4 and 10 had higher levels of MDA, this ignores vital factors such as body size at hatching and fledging, and the pattern of growth between. However, the results here seem to support the assumption that more rapid growth will result in a higher oxidative cost, and care was taken to analyse only the fast linear phase of growth. Although skeletal growth may have stopped before day 10 (the final day used in my growth rate calculation), the days covered by my growth rate analyses should at least provide a reasonable indication of growth and mass gain. However, the fact that higher circulating lutein associated with the carotenoid treatment, was not also associated with a decrease in MDA, or increase in growth is further demonstration that high levels of carotenoids may not indicate a better oxidative status. Peters (2007) suggested that testosterone and carotenoids may be considered two sides of an integrated physiological mechanism involved in carotenoid ornamentation. Since increased testosterone may increase levels of carotenoids in circulation, testosterone may regulate expression of carotenoid ornamentation (McGraw et al., 2006). Testosterone can also act as an immunosuppressant (Roberts et al., 2004) and increase oxidative stress (Alonso-Alvarez et al., 2007). However, these costs to increased testosterone mediated colouration, may be alleviated by carotenoids through their roles in the immune system, and also potentially through antioxidant function. It is possible that levels of testosterone are responsible for increasing growth rate in nestling blue tits, as has been shown in other species (Navara et al., 2006a). In this study, males grew faster than females, and had increased chromatic signal of plumage. Interestingly, there was no difference in MDA between the sexes, perhaps suggesting sex differences in antioxidant defences. These sex differences, combined with the complex relationships between growth rate, MDA and plumage colour may imply a role for testosterone in mediating growth rate, oxidative stress, and colouration in nestlings.

In contrast to the red herring hypothesis, I did not find evidence of non-pigmentary antioxidants increasing carotenoid-mediated plumage colouration. By directly measuring MDA and carotenoid levels, I was able to separate the antioxidant effects of carotenoids

from their other functions. The results concerning the role for carotenoid-mediated plumage in signalling oxidative and/or antioxidant status were complex. Plumage colouration was actually positively correlated with MDA. Moreover, carotenoid supplementation tended to increase the chromatic signal of yellow plumage but did not affect levels of MDA. Therefore, I found evidence that carotenoids are not particularly effective antioxidants, as also suggested by Hartley and Kennedy (2004), and carotenoid-mediated plumage may actually reveal higher levels of oxidative stress, or at least the by-products of fast growth. In future, integrative studies are required to assess the relationships between hormones, dietary antioxidants and oxidative stress, and elucidate their impact on plumage colouration.

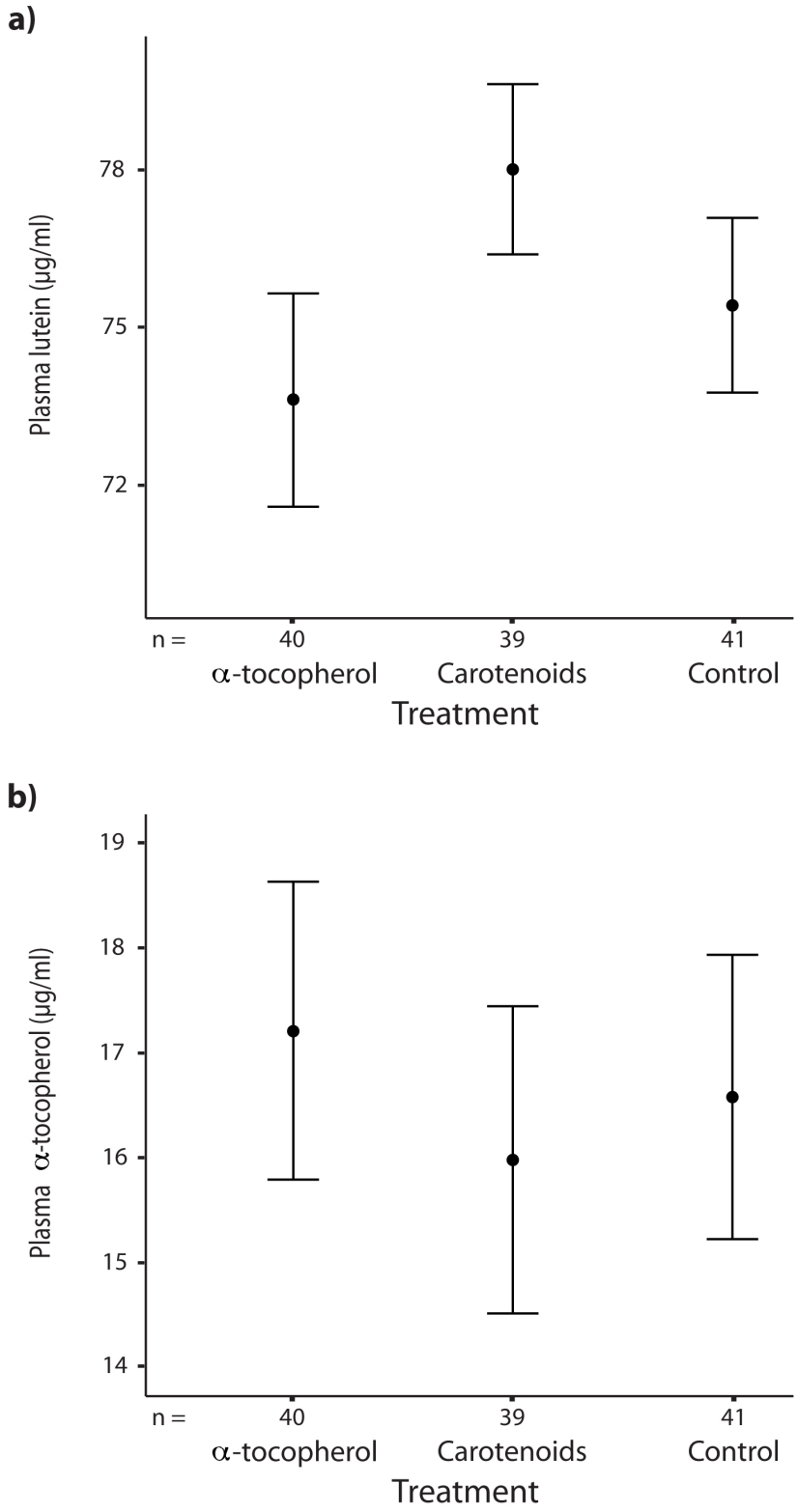

Figure 4.1 Mean (± S.E.) blood plasma concentrations of **a)** lutein and **b)** α-tocopherol in 14 day old nestlings following supplementation with the α-tocopherol (gelatine and α-tocopherol), Carotenoids (gelatine, lutein and zeaxanthin) or Control (gelatine only) treatments.

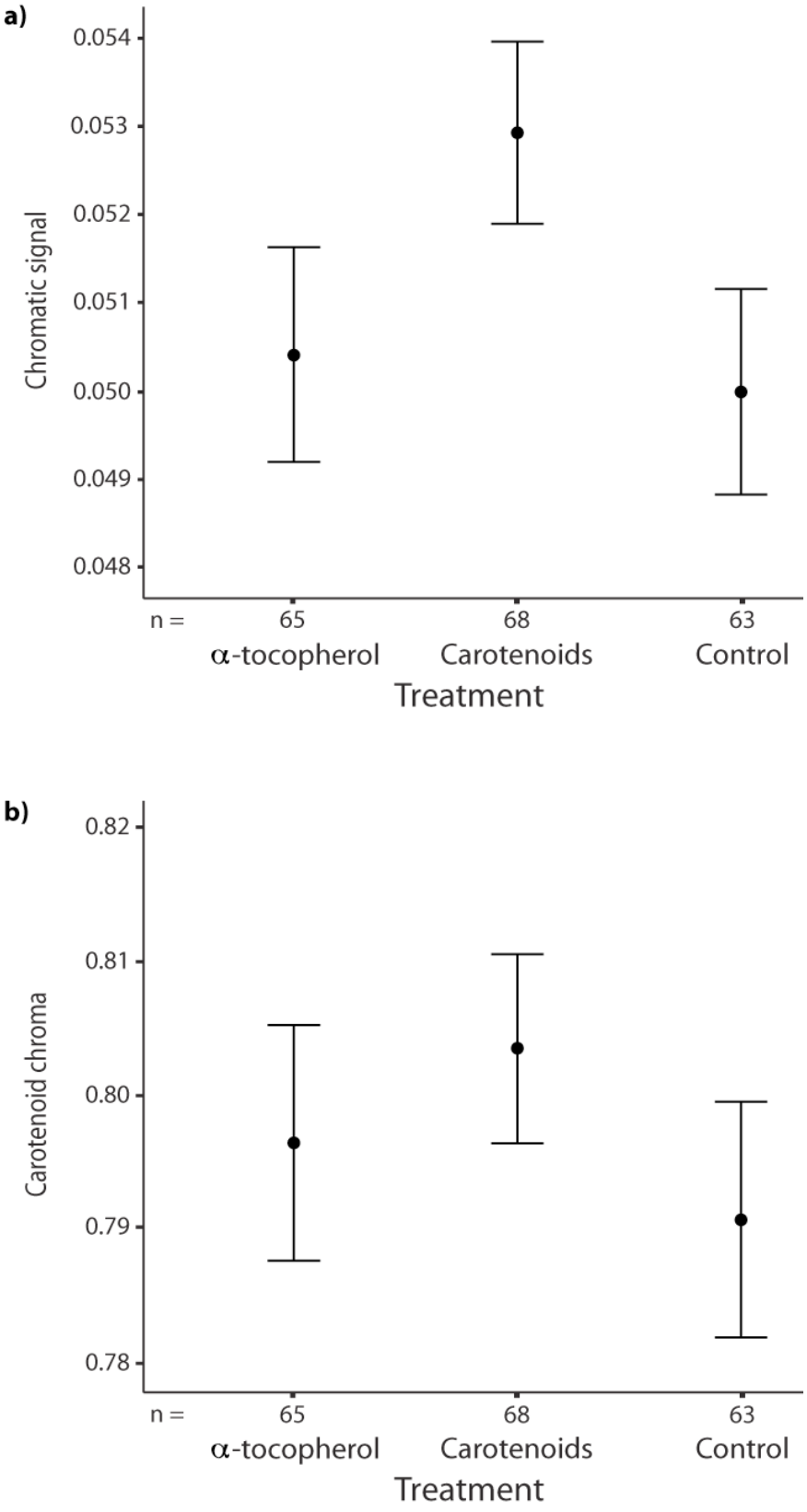

Figure 4.2 a) Mean chromatic signal (± S.E.) of the yellow chest plumage and b) carotenoid chroma of 14 day old nestlings following supplementation with the α-tocopherol (gelatine and α-tocopherol), Carotenoids (gelatine, lutein and zeaxanthin), or Control (gelatine only) treatments.

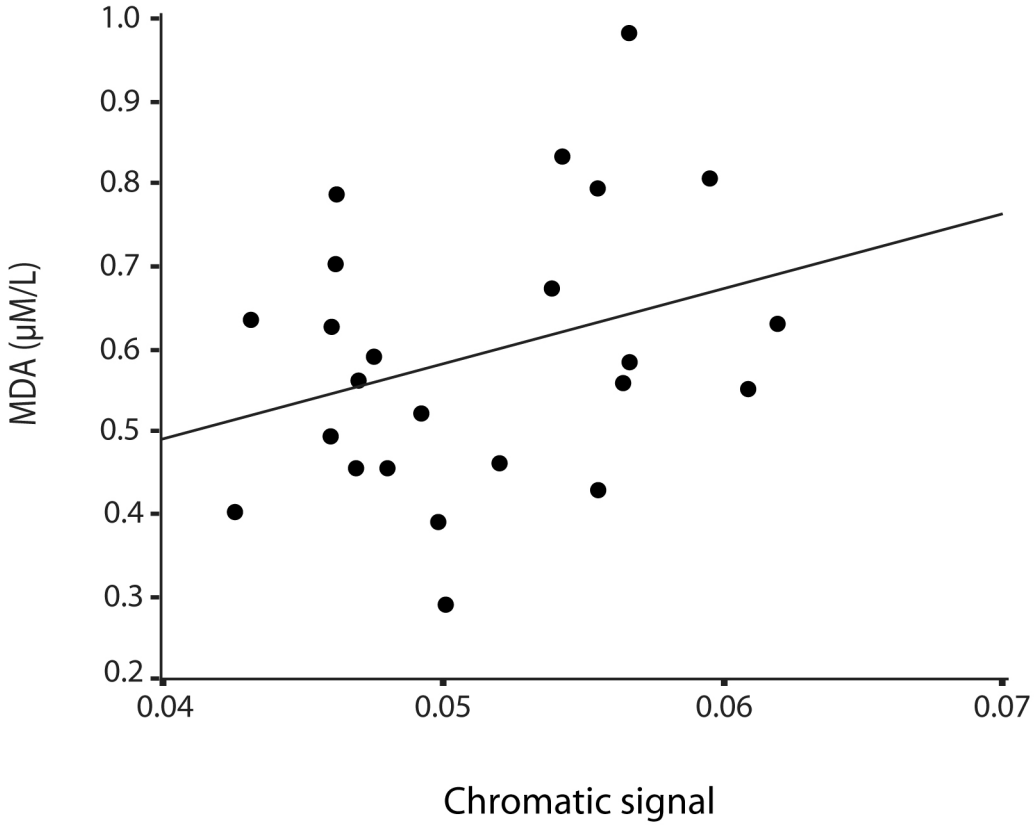

Figure 4.3 Relationship between the chromatic signal of yellow chest plumage of 14 day old nestlings and plasma MDA concentration at age 14 days. Since nest ID affected the model, data points represent brood means.

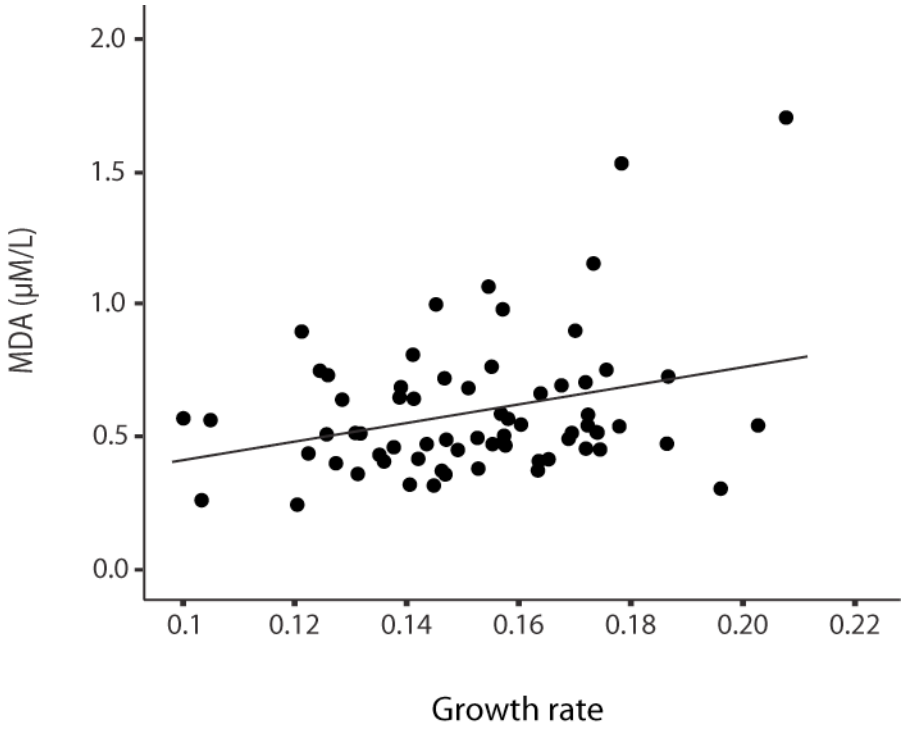

Figure 4.4 Relationship between growth rate between the ages of 4 to 10 days (transformed) and plasma MDA concentration at the age of 14 days. When the outlier to the far right was removed the relationship was marginally non significant (GLM $F_{1,55.9} = 3.51$, $p=0.0662$).

Chapter 5. Behavioural activity and oxidative damage measures in adult budgerigars *Melopsittacus undulatus*

5.1 Abstract

Oxidative stress, the imbalance between the production of reactive oxygen species (ROS) and the ability of the antioxidant defence system to ameliorate the damaging impacts of ROS, appears to be a key fitness determinant. An individual's behaviour is predicted to affect its levels of oxidative stress via a number of pathways: ROS production may be both a cause and consequence of an individual's physical activity rate. Conversely, the selection of antioxidant rich foods can enhance the body's defences against ROS. So far, the links between behaviour and oxidative stress in non-human animals are relatively unstudied, and are likely to be complex. Here, these links were explored here using a range of techniques. After 28 days receiving a standard diet, I assessed the dietary selection of an antioxidant rich nutritional supplement by adult budgerigars, *Melopsittacus undulatus*. I also filmed the birds in their home cages, and scored active, and maintenance behaviours, before blood sampling birds for antioxidant and oxidative damage analyses. I assessed three types of oxidative damage: single stranded DNA breaks, alkali-labile sites in DNA, and malonidialdehyde (MDA), a by-product of lipid peroxidation. Birds that were naturally more active had more single stranded DNA breaks, than less active birds. I suggest this is evidence that normal activity levels can affect oxidative stress. In addition, birds with more alkali-labile sites in their DNA, selected more pieces of the antioxidant-rich supplement. Thus, it appears that birds may be capable of detecting their current oxidative status, and can choose food items accordingly. Also of interest, is the lack of correlation between the three oxidative stress assays, which shows that they are measuring different aspects of oxidative damage. In the future, studies are required to determine the extent to which individual differences in behaviour may affect oxidative status, and also to assess the biological relevance of different measures of oxidative stress.

5.2 Introduction

Unstable reactive oxygen species (ROS), produced during metabolic processes can cause damage to lipids, proteins and DNA necessary for maintaining biological function (Knight, 1998). This naturally produced oxidative stress has led to the evolution of endogenous antioxidant defences within all animals (Surai, 2002). Since oxidative stress is implicated in cellular damage, almost all disease processes, and infertility and ageing it may represent a key fitness determinant (Alonso-Alvarez et al., 2004). The factors affecting an individual's susceptibility to oxidation are therefore of interest to behavioural ecologists, although currently these are poorly understood. Endogenous antioxidant systems are augmented by antioxidants acquired through the diet, and these dietary antioxidants may explain some variation in levels of oxidative stress (Surai, 2002). Indeed, dietary antioxidants have been shown to reduce oxidative stress in many taxa (e.g. Kolosova et al., 2006), including birds (e.g. Woodall et al., 1996).

Since ROS production is increased by metabolic processes, an individual's level of physical activity is likely to have an effect on its oxidative balance (Urso and Clarkson, 2003). Several studies have shown that exercise increases oxidative stress in humans and other animals (Hartmann et al., 1995; Aniagu et al., 2006), including birds (Costantini et al., 2008). These studies usually involve enforced strenuous exercise, and the extent to which an individual's "natural behaviour" will affect levels of oxidative stress is unclear. It is possible that more active individuals will suffer increased oxidative stress. Alternatively, only individuals with more efficient antioxidant systems, or greater access to dietary antioxidants, may be able to increase levels of activity. The extent to which dietary antioxidant uptake and levels of oxidative damage are related to exercise behaviour has been unexplored in birds. Birds, and particularly parrots, make an interesting model for studies of oxidative stress, since they live longer than would be expected for their size, in spite of having high metabolic rates (Speakman, 2005). Given that there is now a widely accepted link between ageing and oxidative stress, birds may have very efficient antioxidant defences compared with mammals.

To investigate the ecological and evolutionary implications of oxidative stress, a reliable assay is an important tool. One method is to measure oxidative damage caused by ROS by direct measurement of oxidation products of proteins, lipids and DNA. Measurement of malonidialdehyde, a product of lipid peroxidation, is one of the most commonly used

techniques (Young and Trimble, 1991). Another method for assessing oxidative damage is by measuring DNA damage. It is estimated that ROS are responsible for around 10,000 base modifications a day, and oxidation is one of the important factors affecting DNA damage (Diplock, 1994). The single cell gel electrophoresis (SCGE), or comet assay measures DNA fragmentation after electrophoresis to assess the level of DNA damage (Tice et al., 2000). Comet assay can be applied at different alkalinities in order to reveal different types of DNA damage. At a lower pH (0.03M NaOH) it is thought to reveal only DNA strand breaks, and at higher pH (0.3M NaOH) both DNA strand breaks and alkali-labile sites are revealed. This technique may be particularly useful in birds where nucleated blood cells can be easily and ethically obtained, though to my knowledge all previous studies using comet assay in birds have focussed on commercial poultry (e.g. Frankic et al., 2006).

In this study I investigated whether oxidative status in adult budgerigars, *Melopsittacus undulatus*, fed a standard diet, was related to age, sex, behaviour, or plasma antioxidant status. I created four categories of behaviour: activity (e.g. walking, climbing etc), flying, maintenance behaviours (e.g. preening, stretching) and feeding. I also assessed the links between behaviour, oxidative damage and selection of antioxidant rich food items. In order to measure oxidative damage I used different measures; MDA analysis and two variations of the comet assay. My specific aims were to answer: 1. Are active or maintenance behaviour rates related to levels of oxidative damage? 2. Are behavioural rates related to dietary antioxidant consumption? 3. Is dietary antioxidant consumption linked to levels of oxidative damage?

5.3 Methods

The experiment lasted one month from November 15th 2004 at the WALTHAM® Centre for Pet Nutrition. 12 male and 12 female (green & yellow) domesticated budgerigars were removed from large, stock aviaries. Each bird was weighed and health checked before being randomly housed with a member of the opposite sex. The birds did not breed during the experiment. Each pair was housed in a cage measuring 1002 x 545 x 410mm. Birds had *ad libitum* access to water and food throughout the experiment, except during food choice trials. Video-monitoring equipment was placed in front of each cage from the start of the study to habituate the birds to it prior to the behavioural trials. The cages were kept at a standard temperature and day length. Birds were given ~10g of seed each day, which was weighed in and out of the cage on a daily basis. Water and cage lining were also changed each day of the experiment.

This experiment lasted 28 days with all birds receiving the same diet of standard Trill, which consists of a seed mix with 3% inclusion of Nutrivit®. Nutrivit® is a vitamin supplement in the form of a small seed like grain that is mixed into the seed mix "Trill" produced by Mars® (Mars, Csongrad, Hungary). The main nutritional effect of is to provide a higher concentration of antioxidants than is present in seed alone. Concentrations of antioxidants in the seed diet are: α-tocopherol 0.75 IU/g; retinol 0 IU/g; vitamin C n/a, 1.35 µg/g β-carotene 0.06 µg/g; lutein 4.19 µg/g; zeaxanthin 1.35 µg/g. Concentrations of antioxidants in Nutrivit® are α-tocopherol 1668.4 IU/g; retinol 220000 IU/g; vitamin C 764.4 µg/g β-carotene 0.6 µg/g; lutein 2.8 µg/g; zeaxanthin 2.6 µg/g. From day 24-28, behavioural and diet choice trials were performed, and all birds were blood sampled.

Food choice and behavioural recording

On day 26 of the experiment at 8.00am, feeding dishes were removed from each cage for a period of 2 hours to standardise hunger, and cages were cleaned. Next, pairs were separated with the female on the left of the cage in all cases. Individual budgerigars were presented with a food bowl containing a prepared 10g food sample containing identical proportions of each seed and Nutrivit®. The video camera in front of each cage was turned on during the food choice trial to record behaviour without the confounding effects of social interactions, though the birds were not acoustically isolated. The dish and tray, along with any spilled seed, were removed after 2 hours and the camera was turned off. The

contents of the dish & cage floor were then sorted and weighed, and the number of Nutrivit® particles was counted. Food choice was defined as the number of Nutrivit® pieces eaten, and the total mass of food eaten in the two hour period

Analysis of behavioural recording

Initial analyses showed that the first hour of each observation period accurately reflected the behaviour of the birds over the entire filmed period. So, for each bird, from the video the frequency of different behaviours per ten minute interval during the first hour on the trial was analysed. I calculated "Maintenance" behaviour as frequency of preening, stretching and bill wiping. "Active" behaviour was scored as the frequency of walking, climbing, and hopping. Frequency of "Flying" was assessed as a different variable, since flight is the most energetically demanding aspect of exercise in birds, and may be associated with different forms of oxidative stress than non-flight activity. Finally "Feeding" was calculated as the combined frequency of eating andor drinking.

Blood and plasma analysis

All birds were blood sampled after 28 days of the experiment. Individual birds were weighed and tarsus, wing and mass measurements were taken. A small blood sample (~250µl) was taken from the jugular vein via a syringe. 50 µl of the whole blood was diluted in 1ml PBS immediately in a sodium citrate tube for comet assay. Capillary tubes of blood for antioxidant and MDA analysis were centrifuged and haematocrit readings were taken from each. Plasma was stored at -70^{o}C, prior to antioxidant and MDA analysis.

Antioxidant extraction and analysis

At the University of Glasgow, I analysed levels of alpha-tocopherol, lutein, zeaxanthin, and retinol in order to uncover any effect of feeding treatment or activity on plasma antioxidant profile. In order to extract the antioxidants, 40µl ethanol was added to 20µl of plasma and vortexed thoroughly. 50µl of hexane was then added and vortexed before the hexane layer, containing the antioxidants was drawn off. This was repeated with 40µl of hexane, before the hexane extract was placed in a SpeedVac for 20 minutes. The final antioxidant extract was then dissolved in 20 ml of methanol.

A Spectra Model 8800 HPLC pump system with a Phenomenex 250mm x 2mm id column was employed to determine antioxidant composition of each sample. I used HPLC at a flow rate of 0.2ml/min with a mobile phase of water/acetonitrile (2.5:97.5), and water/ethyl acetate (2.5:97.5) in a gradient elution. Using a Diode array absorbance detector type Thermo model UV6000, I detected carotenoids by absorbance at 445nm, α-tocopherol at 295nm, and retinol at 325nm. Peaks were identified by comparison with chromatography and retention times of several standards (Sigma, Poole, UK; Fluka, Gillingham, UK). Levels of α-tocopherol in budgerigar plasma were very low, in some samples too low to be detected (<0.3µg/ml) and in others so low that there was an unacceptably high error in calculating concentration. Plasma α-tocopherol concentrations were therefore excluded from the analyses.

MDA analysis

The MDA method was based on that of Young and Trimble (1991). Thiobarbituric acid (0.044M,100µl) and phosphoric acid (1.22M, 100µl) were mixed together and added to 50µl of plasma (per bird) in a test tube. An inert atmosphere was created by applying a nitrogen blanket, and the test tubes were sealed and vortexed prior to heating (60 min, 70-75^{o}C). Samples were cooled in water, then 200µl was transferred to a centrifuge tube containing sodium hydroxide (1M, 100µl). Methanol (500µl) was added and mixed. Samples were centrifuged (10 min, 12000 g) and the supernatant analysed on a Summit HPLC system (Dionex, Idstein, Germany) using Chromeleon software (Dionex). An Acclaim 120 C18 5 4.6 x 250 mm column (Dionex) and guard were used with fluorescence detection (excitation 532nm and emission 553nm). The mobile phase was isocratic, 40:60 methanol:phosphate buffer (40mM, pH 6.5), with a flow rate of 1ml/min, and a run time of 7 min. Samples were assayed against a standard of malonidialdehyde bis (dimethyl acetal), (Sigma Aldrich, Poole, UK) that was simultaneously taken through the same procedure.

Comet assay

The alkaline comet assay procedure was performed according to Tice et al. (2000) at two different pHs for each bird. Electrophoresis at Low pH (0.03M NaOH) to reveal DNA strand breaks and electrophoresis at high pH (0.3M NaOH) which also converts alkali-labile sites into single strand breaks. Slides were made and analysed on the same day as blood sampling. 50µl SYBR Gold was used in each gel for visualisation of comets. Slides

were viewed by epifluorescence microscopy using an Olympus BX-51(Olympus Optical Co., Tokyo, Japan) with a 460nm UV filter for SYBR Green. Komet software (v.6, Kinetics Imaging, Nottingham, UK) was used for image analysis on 100 randomly selected cells for each bird and treatment. Cells were scored according to % DNA in the comet head, as a measure of DNA intactness.

Statistics

Comet data were proportions and so were arcsine square root transformed. Count data were square root transformed prior to analysis. Correlations between the different measures of oxidative damage, and different levels of plasma antioxidants were assessed using Pearson's correlations in SPSS version 14. Since I was using multiple correlations on 6 different measures, we used a bonferroni transformation to calculate a new significance value of $p=0.0083$ for these tests. In order to explain variation in oxidative damage we used a general linear model for each measure: MDA, high pH comet and low pH comet. We included sex as a fixed factor in each model, and included behavioural scores, amount of seed and nutrivit eaten, age and body condition as covariates in every model. Non-significant terms were deleted from the model in backwards stepwise fashion, until only significant terms remained. All significant results are presented below. There are varying sample sizes reported throughout the results since in some cases we did not have sufficient blood to perform some of the tests of oxidative damage or antioxidant analyses.

5.4 Results

Figure 5.1 shows the mean activity profiles of birds during the one hour observation period. Birds exhibited a broad range of behaviours, most often engaging in Active behaviours (excluding climbing), and feeding. Birds were rarely seen at their water during the experiment.

Oxidative damage and plasma antioxidants

None of the measures of oxidative damage were significantly correlated with one another (see Figure 5.4). There were no significant relationships between plasma antioxidant concentrations and any measure of oxidative damage (see Table 5.1), and with the exception of lutein and zeaxanthin, levels of each antioxidants were not correlated with each other (Table 5.1). Mean values of antioxidant concentrations in budgerigar plasma in μg/ml were: lutein 33.78 ± 2.82, zeaxanthin 31.83 ± 3.22, retinol 0.69 ± 0.08, α-tocopherol 2.07 ± 0.83.

Oxidative damage and behaviour

Using low pH comet assay there was a marginally non-significant trend for a lower proportion of intact DNA in more active birds, than less active birds (GLM $F_{1,19,}$ $p = 0.053$, Figure 5.2). Active behaviour was not related to levels of MDA or high pH comet ($P > 0.74$ in both cases). There was no relationship between flying or maintenance behaviour and either comet assay. Using high pH comet, there was a negative relationship between intactness of DNA and number of Nutrivit® particles eaten (GLM $F_{1,21,}$ $p = 0.043$, Figure 5.3), though not with total amount of seed eaten ($p > 0.5$). Therefore, birds with more DNA damage ate more Nutrivit®. There were no significant relationships with any other measured variable. Concentration of MDA was not significantly related to any other variable.

5.5 Discussion

Here, relationships were found between activity, diet selection, and oxidative damage. One measure of DNA damage was marginally non-significantly related to active behaviour. Selection of a dietary antioxidant supplement was significantly related to another measure of DNA damage. Several studies have shown that exercise and activity are capable of increasing correlates of oxidative stress (e.g. Hartmann et al., 1995; Aniagu et al., 2006). However, many of the studies linking exercise and oxidative stress have enforced strenuous exercise on experimental subjects and the extent to which "natural" behaviour is linked to oxidative stress is currently unclear. By filming budgerigars in their home cages and scoring individual behaviours, I aimed to understand how behavioural activity was related to current oxidative condition. I found that birds that were more active, engaging in walking, climbing and hopping in their cages, had less intact DNA measured by low pH comet, than less active birds.

Interestingly, I also showed that birds with less intact DNA, measured by high pH comet assay, consumed significantly more Nutrivit® than birds with more intact DNA. This relationship was independent of the total amount of food eaten, and thus reflects specific uptake of the supplement. The effects of Nutrivit® supplementation on oxidative damage following enforced exercise will be addressed in Chapters 6 and 7. However, it seems unlikely that increased uptake of Nutrivit® could cause enhanced DNA damage, since the supplement consists mainly of antioxidant substances. Therefore I suggest that birds with higher levels of oxidative stress, select more Nutrivit® from their food mix, than birds suffering less oxidative stress. This result is interesting since it suggests that the birds were capable of detecting the nutrients contained within Nutrivit®. There are a multitude of reasons why birds should favour antioxidant rich food items. Proving that birds have selected food items based on antioxidant levels, or can detect such differences is more difficult. In a recent study, Schaefer et al. (2008) demonstrated that blackcaps could potentially detect antioxidants in different fruits based on fruit colour. Given that Nutrivit® is a non-colourful seed like pellet, if birds did preferentially select it, this would involve a considerable capacity for taste and learning as well colour vision.

If birds can assess their oxidative status, then this raises the question of why the active birds did not consume more Nutrivit® or reduce their levels of activity, if this was responsible for DNA damage. It is possible that the increased oxidative damage in more

active birds was not caused by activity *per se*, rather, activity and oxidative damage were both affected by another factor. The links between metabolic rate and oxidative stress are well documented (Speakman, 2005), and metabolic rate may also be related to certain active behaviours (Levine, 2002). The stress response also raises levels of oxidative stress (Şahın and Gümüşlü, 2007), and it is possible that the increased activity seen in some birds was representative of stressed behaviour. In this study, flying behaviour was unrelated to oxidative stress, in spite of being more energetically demanding than walking, climbing or hopping; the constituents of the activity score. Given that birds were food deprived before the experiment, and pairs were separated during filming, birds more prone to stress in these circumstances may have been considered more active using my score. Indeed, prior to commencement of this experiment, birds were kept in large, flight aviaries with a mix of birds of different ages and sex. Here, birds were paired in smaller cages, and although filming and analysis took place 4 weeks after being housed in the new cages, birds may not yet have acclimated to their new environment. Owing to the small sample size and limited behavioural measures I cannot conclude a definitive reason for the link between activity and oxidative stress. However, this pilot study does suggest that oxidative damage and activity are linked in some capacity in budgerigars.

For any study of the role of oxidative stress in physiological trade-offs, an effective measure of oxidative stress, or its effects, is necessary. Using several different measures of oxidative damage allowed us to assess the relationships between different aspects of oxidative damage and behaviour. Another interesting aspect of these results is that different measures of oxidative stress were not correlated with one another. Indeed, behavioural activity was related to DNA damage using the low pH comet assay, and Nutrivit® uptake was related to DNA damage using the high pH comet assay. Using a high pH to conduct the comet assay reveals the broadest range of DNA damage: single and double-stranded DNA breaks, single strand breaks where DNA excision repair is incomplete, and also converting alkali-labile sites to detectable DNA breaks. Low pH comet does not detect these alkali-labile sites. Alkali-labile sites represent specific types of DNA damage such as base and phosphate alkylation, or intermediates in base excision repair. It is not possible to determine exactly which kind of DNA damage was related to Nutrivit® uptake, though I can speculate that either birds were only capable of detecting specific types of alkylation, or perhaps that increased Nutrivit® consumption was related to ongoing DNA repair mechanisms. Although the long-term implications of DNA damage associated with behavioural activity is unclear from this study, increases in DNA damage

can eventually lead to apoptosis (Monti et al., 1992). Since avian lymphocytes, the cells used in comet assay, are only produced early in development, and circulate for lengthy periods (Glick, 1979), long-term DNA damage may induce a reduction in lymphocyte number and therefore immunocompromise an individual. It is noteworthy that in spite of the links between oxidative stress and ageing (Finkel and Holbrook, 2000), and testosterone (Alonso-Alvarez et al., 2007a), that I found no significant effects of sex or age on any measure of oxidative stress. However, with a small sample size and uneven distribution of age classes, this is by no means conclusive.

These results show that defining oxidative status is complex: absence of any effect on one measure of oxidative damage does not indicate a *de facto* absence of change in oxidative stress. MDA is directly representative of levels of lipid peroxidation, one of the major types of oxidative damage. It is interesting that lipid peroxidation, which has been reported as being affected by exercise (Vollaard et al., 2005), was unrelated to activity in this study. All of the results discussed above as oxidative damage, relate to DNA damage measured by comet assay. Comet assay uses only DNA from lymphocytes, while the origin of plasma by-products of lipid-peroxidation like MDA is unknown. Thus there may be tissue-specific effects of oxidative stress. Plasma levels of antioxidants were unrelated to either Nutrivit® uptake, or levels of oxidative stress. It is possible that antioxidants in plasma were used up countering oxidative stress prior to blood sampling or that important antioxidants are stored, rather than immediately used in plasma (Surai, 2002). Although I found no effect of Nutrivit® on plasma antioxidants, the fact that oxidative stress appears to influence Nutrivit® selection suggests that Nutrivit® is involved in antioxidant defence in some capacity. Of the antioxidants I was able to detect in plasma, only tocopherol and retinol are increased in Nutrivit® to a major extent. It is possible that other antioxidants may be important, or that different antioxidants act synergistically (Ewen et al., 2006), in limiting oxidative damage in this species.

In this study, I used correlational data from budgerigars to assess whether oxidative damage was linked with undisturbed behaviour. I found that the propensity to engage in active behaviour was linked with oxidative damage, in that more active birds had more single stranded breaks in their DNA, than less active birds. I also found that birds with increased alkali-labile sites in their DNA selected more antioxidant supplement. These results offer evidence that behaviour may be capable of modifying levels of oxidative stress, though also that birds can potentially detect oxidative stress and may alter behaviour

accordingly. In the future, studies are required to determine the extent to which individual differences in behavioural responses may affect oxidative status, and also to assess the biological relevance of different measures of oxidative stress.

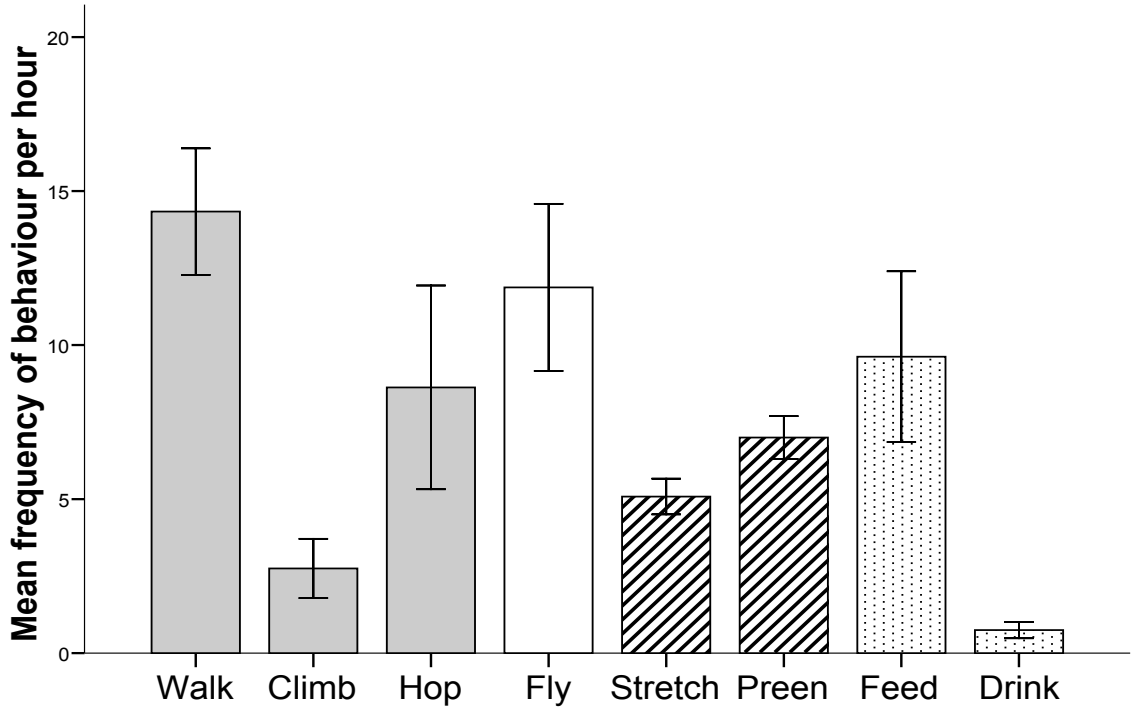

Figure 5.1 Mean (± S.E.) frequencies per hour of different behaviours for all birds. The four behavioural categories analysed were: Active behaviours (grey), Flying (white), Maintenance behaviours (cross hatching) and Feeding behaviours (dots).

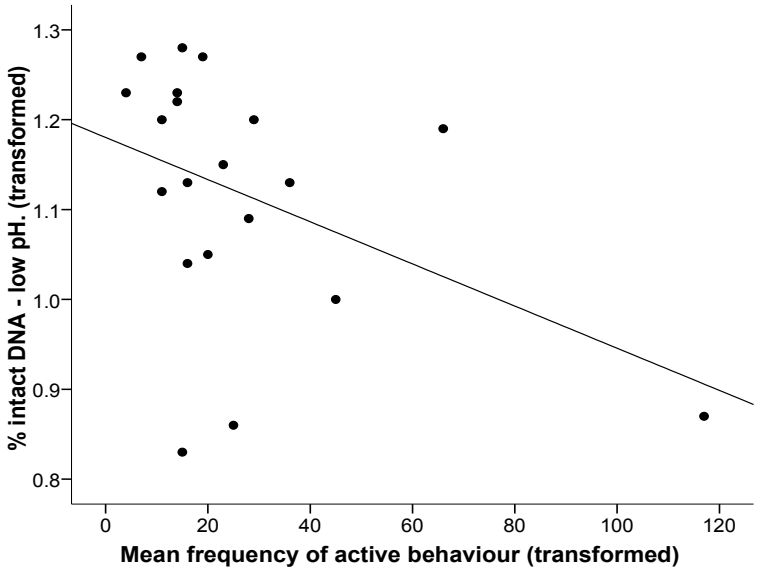

Figure 5.2 More active birds had significantly less intact DNA, measured by low pH Comet assay than more sedentary birds. When the very active outlier (see arrow) was excluded more active birds had marginally less intact DNA ($p = 0.069$) than idle birds. Proportions of DNA intactness were arcsine square root transformed and activity counts were square root transformed prior to analysis.

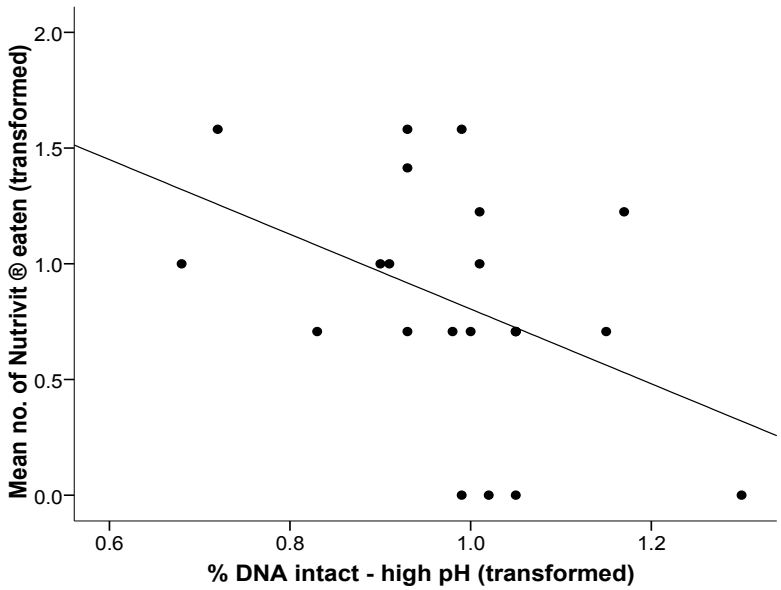

Figure 5.3 Birds with less intact DNA, measured by high pH Comet assay, consumed significantly more pieces of Nutrivit ® during food selection trials than those with more intact DNA. Proportions of DNA intactness were arcsine square root transformed and mean Nutrivit ® counts were arcsine transformed prior to analysis.

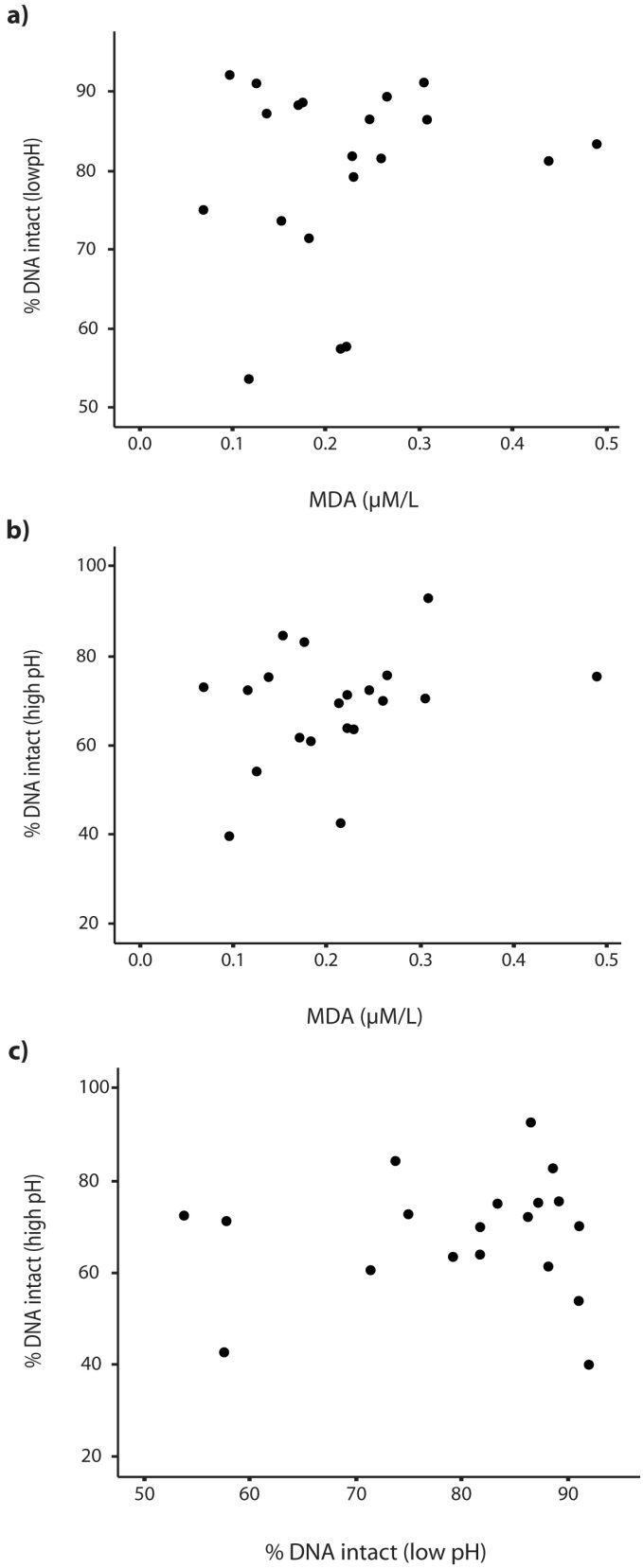

Figure 5.4 Lack of relationships between different measures of oxidative stress in each birds. **a)** MDA vs low pH comet **b)** MDA vs high pH comet, **c)** low pH comet vs high pH comet. Figure shows raw data rather than transformed data used in analyses.

Table 5.1 Correlations between different plasma antioxidants, and measures of oxidative stress (* = significant correlation after Bonferroni transformation).

		Retinol	Lutein	Zeaxanthin	MDA	Comet high pH	Comet low pH
Retinol	Spearman's rho	n/a	.347	.339	.226	-.114	-.018
	p =		.158	.169	.382	.663	.945
	n =		18	18	17	17	17
Lutein	Spearman's rho	.347	n/a	.959*	-.083	.244	.121
	p =	.158		<.0001	.745	.330	.633
	n =	18		19	18	18	18
Zeaxanthin	Spearman's rho	.339	.959(**)	n/a	-.171	.206	-.171
	p =	.169	<.0001		.498	.412	.498
	n =	18	19		18	18	18

Chapter 6. The effects of short term antioxidant supplementation on oxidative damage and flight performance in adult budgerigars *Melopsittacus undulatus*

6.1 Abstract

Antioxidants are known to play an important role in quenching reactive oxygen species (ROS), thus ameliorating oxidative stress. Since increased metabolism associated with exercise can increase oxidative stress, dietary antioxidants may be a limiting factor in determining aspects of physical performance. Here I tested whether oxidative stress levels differed after receiving a diet containing either enhanced (EQ) or reduced levels (RQ) of a nutritional supplement, rich in antioxidants (Nutrivit®) for four weeks. I also assessed differences in take-off escape time, a potentially fitness-determining physiological capability, of captive adult budgerigars, *Melopsittacus undulatus*. Oxidative stress was assessed using comet assay, to measure DNA damage, and analysis of malonidialdehyde (MDA), a by-product of lipid peroxidation. Flight exercise appeared to increase oxidative stress. Moreover, birds had a significantly higher percentage of intact DNA (fewer alkali labile sites) and lower levels of MDA after an EQ diet, than after an RQ diet. I found no difference in flight performance between the two diets. My results suggest that birds exerted maximum effort in escape flights, regardless of diet. However, they paid a cost of increased oxidative stress post-flight when on a reduced quality diet, but not an enhanced, antioxidant-rich diet. I suggest that dietary antioxidants may prove important in reducing exercise related costs through various physiological pathways. Further work is necessary to fully understand the effects of antioxidants and oxidative stress on exercise performance in the longer term.

6.2 Introduction

Within all animals an oxidative balance exists between production of reactive oxygen species (ROS) and the ability of endogenous and exogenous antioxidants to neutralise ROS. Oxidative stress is a term used to describe an imbalance whereby ROS production overpowers the detoxifying ability of the antioxidant systems. Oxidative stress can cause damage to proteins, lipids, and DNA and there is a growing understanding of the importance of oxidative stress in the development, health and ageing of animals (Knight, 1998). Levels of oxidative stress are therefore likely to be important fitness determinants, and identifying the factors influencing an individual's susceptibility to oxidative stress is of great interest. Physical activity is likely to be one factor influencing oxidative status since raising metabolism, as during exercise, will increase production of ROS (Urso and Clarkson, 2003). Indeed, increased ROS production during exercise has been demonstrated in both humans and other animals (Kanter et al., 1988; O'Neil et al., 1996). An endogenous antioxidant system, well conserved across all taxa, controls and neutralises the ROS produced during exercise. However, prolonged strenuous exercise may overwhelm this antioxidant system (Sjodin et al., 1990). In this case dietary antioxidants may assist in removing free radicals, and reducing oxidative stress. In birds, take-off is one of the most energetically demanding aspects of flight (Swaddle et al., 1999) and is likely to be associated with a burst of ROS production.

Since oxidative stress may cause damage to tissue (Bailey et al., 2007), there is growing interest in the possible role of dietary antioxidants in preventing exercise-induced oxidative stress (Powers et al., 2004). Accumulation of oxidative damage to tissues associated with exercise may eventually reduce locomotive function, antioxidant supplementation may decrease oxidative damage and improve exercise performance. Much of the interest in this area has focussed on the effects of antioxidant supplementation in human athletes, though results are far from clear (Urso and Clarkson, 2003). Data from other, particularly non-model, taxa have the potential to improve our understanding of these links between dietary antioxidants and exercise. In Chapter 5, I provided evidence of a link between unforced activity levels and oxidative stress in budgerigars *Melopsittacus undulatus,* and also for increased selection of an antioxidant rich food item in birds suffering greater oxidative stress. Using the same birds, in this chapter I will give each bird a diet containing high or low levels of the nutritional supplement consecutively. By measuring oxidative stress and escape flight time after each diet, I will further uncover the relationship between diet and

exercise related oxidative stress. In birds high velocity initial vertical take off, regardless of other escape strategies, is vital in avoiding predation (Kenward, 1978). Although morphological traits such as wingtip shape (Swaddle and Lockwood, 2003), and fat load (Kullberg et al., 1996) have previously been demonstrated as important in determining flight performance in birds, it is possible that antioxidant status may also mediate flight performance by limiting oxidative damage associated with exercise. The extent to which flight exercise affects oxidative stress in birds, and how dietary antioxidants may ameliorate this, is unclear. Recently, Costantini et al. (2008) demonstrated oxidative stress was positively correlated with flight duration in homing pigeons *Columba livia*, the first direct evidence of an oxidative cost to flight in birds. However, thus far no study has directly manipulated flight exercise and measured the effect of antioxidant supplementation on post-flight oxidative damage. In Costantini et al's study (2008), oxidative stress was measured after long endurance flights, whereas take off flight may have different oxidative costs. In birds, evidence suggests that short flights tend to use glycogen stores for energy metabolism, whereas during long flights free fatty acids, produced by hydrolysis of adipose tissue, are oxidised by flight muscles (Jenni-Eiermann and Jenni, 1991; Schwilch et al., 1996). When fed on an antioxidant-rich diet, an individual should have a greater supply of available antioxidants and will be better equipped to remove free radicals associated with flight activity, than when receiving a reduced antioxidant diet. Indeed, it has recently been demonstrated that male zebra finches *Taeniopygia guttata* fed supplementary carotenoids (nutrients displaying antioxidant properties) flew faster than controls, though oxidative stress was not measured (Blount and Matheson, 2006).

In this study, I fed individual birds both an enhanced and reduced quality diet. The main nutritional difference between these diets was in antioxidant concentrations. In all cases, each bird received each of the two diets consecutively, and acted as its own control. After receiving each diet, each bird's post-flight oxidative stress was measured. I examined two indices of oxidative damage; malonidialdehyde (MDA) and comet assay. MDA is one of the major products of lipid peroxidation, after transition metal decomposition of lipid peroxides. High performance liquid chromatography (HPLC) can be used to examine the prevalence of MDA in its free form. The comet assay was developed by Collins et al. (1997) for measuring breaks in the DNA of lymphocytes, and other single cells. I also calculated an average flight escape time across 4 days, in order to assess whether dietary antioxidants were capable of limiting flight performance. My specific aims were to

determine whether manipulating supplementary dietary antioxidants affected: 1) Post-flight oxidative stress levels; 2) Take-off flight escape time.

6.3 Methods

This experiment lasted 8 weeks (2 x 4 week blocks) and I employed a crossover design with each bird receiving either an enhanced quality (EQ) diet for four weeks diet followed by a reduced quality (RQ) diet for four weeks, or vice versa. In the fourth week of each dietary block, food intake and flight trials were performed, and all birds were blood sampled.

At the WALTHAM® Centre for Pet Nutrition, 12 male and 12 female green & yellow (wild type colour) budgerigars were selected from stock aviaries. The stock aviaries were large, allowing space for birds to fly, and interact with various behavioural enrichment toys. The domesticated budgerigars used have been selected for large body size, so skeletally are approximately 80% bigger than wild budgerigars. After removal from stock aviaries, each bird was weighed, health checked and randomly caged with a member of the opposite sex before being placed together in cages measuring 1002 x 545 x 410mm. These experimental cages were smaller than the stock aviaries, though had multiple perches allowing flight (see Chapter 5). Birds were not breeding, and temperature and daylength were held constant, during the experiment. Birds had *ad libitum* access to water and food throughout the experiment, except during intake trials. Grit was provided in a separate container. Birds were given ~10g of seed per bird each day, which was weighed in and out of the cage on a daily basis. Water and cage lining were also changed daily.

Diet manipulations

In this experiment, I manipulated the levels of an antioxidant-rich diet supplement within a standard seed mix for adult budgerigars. Nutrivit® is a nutritional supplement in the form of a small seed like grain that is mixed into the seed mix Trill® produced by Mars® (Mars, Csongrad, Hungary). The main nutritional effect of Nutrivit® is to provide a higher concentration of antioxidants than is present in seed alone. Thus, any differences between birds on the two diets are likely to be mediated by antioxidants. Every bird on the trial received a baseline diet consisting of seed mix (Trill®) with a standard inclusion of 3% Nutrivit® for 4 weeks prior to the start of the experiment. This was the same diet that the

birds usually received in their home aviaries. This ensured the birds were used to the Nutrivit® supplement before beginning the experiment. Then, two experimental diets were made up using identical proportions of seeds, but differing in the percentage inclusion of Nutrivit®. The enhanced quality diet (EQ), contained a 10% inclusion (by mass) of Nutrivit®. The reduced quality diet (RQ), contained a 1% inclusion (by mass) of Nutrivit®. Concentrations of antioxidants in the EQ diet were: α-tocopherol 0.1675 i.u. g^{-1}; retinol 22 i.u. g^{-1}; β-carotene 0114 μg g^{-1}; lutein 4.05μg g^{-1}; zeaxanthin 1.475 μg g^{-1}; and in the RQ diet were: α-tocopherol 174 i.u. g^{-1}; retinol 2.2 i.u. g^{-1}; β-carotene 0.0654 μg g^{-1}; lutein 4.17 μg g^{-1}; zeaxanthin 1.365 μg/g^{-1}. The birds were retained in their caged pairs and were randomly assigned to one of two groups. Half the pairs received EQ diet for the first experimental block, followed by the RQ diet for the second block. The other half of the birds received RQ diet for the first experimental block, followed by the EQ diet for the second block.

Flight escape time recording

Flight trials were conducted from days 23 to 27 of each experimental block. The flight apparatus and protocol were similar to those of a previous study (Veasey et al., 2001) with some modifications. The cage used was 2m high by 70cm wide by 70cm deep. The front wall of the cage was made of transparent Perspex. Coloured masking tape was taped horizontally across the cage at 10cm increments above the floor, on both the transparent front panel and the back wall. A hinged $20cm^3$ holding box was used to release the birds. The lid of the box was transparent, and was attached to a string. When pulled sharply this collapsed the side panels, and stimulated escape from ground level to a perch situated at the top of the cage. The loud sound of the sides hitting the ground elicited a standardised vertical escape flight from all birds.

On day 22 all birds were allowed to acclimate to the flight apparatus in groups of 6. On Day 23 of each experimental block, the flight cage was positioned in a fixed location in an enclosed procedures room, and video recording apparatus was fixed on a tripod 3m from the cage. Birds were then placed in the holding box and flown individually as in Veasey et al., (2001), but in this study I calculated escape time from 10cm to 50cm high. Following this, the birds were given two trial flights each day between day 23 and day 26, and 3 flights on day 27 (11 flights in total). Each bird had a 10 minute break between flights on the same day. By watching videos back frame-by-frame I could calculate escape time in

0.04 second intervals. Because of the holding cup design, I could not see the bird flying over the first few centimetres. I noted the flight as starting when the head passed a line 10cm from the ground, and finishing when the head passed the line at 50cm high. I used a mean escape time across all 11 flights in my analysis.

Blood and plasma analysis

Every bird was blood sampled prior to the experimental phase, after 28 days receiving their baseline diet, then on day 28 of each experimental block, around 24 hours after the final exercise trial. Individual birds were weighed and tarsus, wing and mass measurements were taken. A small blood sample (~250µl) was taken from the jugular vein via a syringe. 50µl of the whole blood was diluted in 1ml PBS immediately in a sodium citrate tube for comet assay. The rest of the blood was transferred to 75µl capillary tubes. The capillary tubes of blood for antioxidant and MDA analysis were centrifuged and haematocrit readings were taken from each. Plasma was stored at -70^{o}C, prior to antioxidant and MDA analysis.

Antioxidant extraction and analysis

At the University of Glasgow, I analysed levels of α-tocopherol, lutein, zeaxanthin, and retinol in order to uncover any effect of feeding treatment or exercise on plasma antioxidant profile. To extract the antioxidants, 40µl ethanol was added to 20µl of plasma and vortexed thoroughly. 50µl of hexane was then added and vortexed before the hexane layer, containing the antioxidants was drawn off. This was repeated with 40µl of hexane, before the hexane extract was placed in a SpeedVac for 20 minutes. The final antioxidant extract was then dissolved in 20 ml of methanol.

A Spectra Model 8800 HPLC pump system with a Phenomenex 250mm x 2mm id column was employed to determine antioxidant composition of each sample. I used HPLC at a flow rate of 0.2ml/min with a mobile phase of water/acetonitrile (2.5:97.5), and water/ethyl acetate (2.5:97.5) in a gradient elution. Using a Diode array absorbance detector type Thermo model UV6000, carotenoids were detected by absorbance at 445nm, α-tocopherol at 295nm, and retinol at 325nm. Peaks were identified by comparison with chromatography and retention times of several standards (Sigma, Poole, UK; Fluka, Gillingham, UK).

MDA analysis

The MDA method was based on that of Young and Trimble (1991). Thiobarbituric acid (0.044M,100µl) and phosphoric acid (1.22M, 100µl) were mixed together and added to 50µl of plasma (per bird) in a test tube. An inert atmosphere was created by applying a nitrogen blanket, and the test tubes were sealed and vortexed prior to heating (60 min, 70-75^{o}C). Samples were cooled in water, then 200µl was transferred to a centrifuge tube containing sodium hydroxide (1M, 100µl). Methanol (500µl) was added and mixed. Samples were centrifuged (10 min, 12000 g) and the supernatant analysed on a Summit HPLC system (Dionex, Idstein, Germany) using Chromeleon software (Dionex). An Acclaim 120 C18 5µ 4.6 x 250 mm column (Dionex) and guard were used with fluorescence detection (excitation 532nm and emission 553nm). The mobile phase was isocratic, 40:60 methanol:phosphate buffer (40mM, pH 6.5), with a flow rate of 1ml/min, and a run time of 7 min. Samples were assayed against a standard of malonidialdehyde bis (dimethyl acetal), (Sigma Aldrich, Poole, UK) that was simultaneously taken through the same procedure.

Comet assay

The alkaline comet assay procedure was performed according to Tice et al. (2000) at two different pHs for each bird. Electrophoresis at low pH (0.03M NaOH) to reveal single stranded breaks and electrophoresis at high pH (0.3M NaOH) which converts alkali labile sites into single strand breaks. Slides were made and analysed on the same day as blood sampling. 50µl SYBR Gold was used in each gel for visualisation of comets. Slides were viewed by epifluorescence microscopy using an Olympus BX-51(Olympus Optical Co., Tokyo, Japan) with a 460nm UV filter for SYBR Green. Komet software (v.6, Kinetics Imaging, Nottingham, UK) was used for image analysis on 100 randomly selected cells for each bird and treatment. Cells were scored according to % DNA in the comet head, as a measure of DNA intactness.

Intake trials

In order to ensure that the birds were eating the Nutrivit® supplement, and also to assess selection or otherwise of the high antioxidant food, I performed an intake trial during each experimental block. Each morning at 8.00am on days 24, 25 and 26 of each experimental block, feeding dishes were removed from each cage for a period of 2 hours to standardise hunger, and cages were cleaned. After 2 hours without food, pairs were separated with the female on the left of the cage in all cases. Individual budgerigars were presented with a food bowl containing a prepared 10g sample of their experimental diet containing very precisely weighed seed, and known numbers of Nutrivit®. The dish and tray were removed after 2 hours, along with any spilled seed. The contents of the dish and cage floor were then sorted and weighed, and the number of uneaten Nutrivit® particles was counted and subtracted from the original total.

Statistics

Oxidative damage, and flight escape time data from the EQ and RQ diet experiments were analysed individually using general linear mixed models (Proc Mixed in SAS version 9) with a normal error distribution. Individual identity was entered as a random factor into every model to control for the non-independence of repeated measures from the same individual. The order in which each bird received the diets, diet and sex were entered into each model as fixed factors, and morphometric, escape time and blood measures as covariates. All models used a Satterthwaite correction which can result in degrees of freedom that are not integers. Models were developed using backward elimination starting with the highest order interaction term. I tested for all two-way interactions between main effects and covariates, and removed non-significant factors stepwise from the full model beginning with the interaction terms. All significance tests were based on the F distribution. Comet data were proportions and so were arcsine square root transformed. Count data were square root transformed prior to analysis. Means with standard errors (S.E.) are reported throughout the text. In some cases problems during blood sampling or repeated failure to fly in some birds resulted in a sample size unequal to 24 birds. One bird was omitted from all analyses since it was observed to exhibit abnormal behaviour throughout the experiment and subsequently had atypical results.

6.4 Results

I found no effects of sex on any variable ($p > 0.1$) so data from males and females were pooled.

When individuals were maintained on the RQ diet they had significantly higher levels of MDA, than when on the EQ diet (GLMM $F_{1, 37} = 8.37$, $p = 0.0064$, see Figure 6.1a). Birds also had a higher proportion of intact DNA, measured by high pH comet assay, which assesses strand breaks and alkali labile sites, on the EQ diet than on the RQ diet (GLMM $F_{1, 35} = 5.3$, $p = 0.0273$, see Figure 6.1b). There was no effect of diet on the low pH comet ($p>0.5$, see Figure 6.1c). There was no significant effect of diet order on any measure of oxidative stress, showing there were no carry-over effects between experimental blocks. Plasma oxidative stress levels prior to the start of the experiment with a 3% Nutrivit® inclusion and no exercise, were: MDA, 0.237 μM/L ± 0.026; High pH comet, 67.83 % ± 2.64; Low pH comet 79.81 % ± 2.62 (see Chapter 5). All measures showed higher levels of oxidative damage during the experimental phases regardless of diet, than prior to the start of the experiment (Figure 6.1).

There was no effect of diet on flight escape time (means in seconds = EQ: 0.581±0.038; RQ: 0.568 ± 0.026, GLMM $F_{1, 33} = 0.02$, $p = 0.88$). There was a significant, positive relationship between mass and flight escape time (GLMM $F_{1, 32} = 7.39$, $p = 0.011$, see Figure 6.2), but no relationships between comet assay, plasma MDA or plasma antioxidant concentrations, and flight escape time. There was no effect of diet on blood plasma concentrations of plasma α-tocopherol, lutein, zeaxanthin or retinol ($p > 0.1$ in all cases), and no significant effect or general trend of diet order on plasma levels of the different antioxidants, demonstrating that there was no carry-over effects of antioxidant supplementation (see Figure 6.3). Levels of plasma antioxidants were unrelated to any measure of oxidative damage ($p > 0.08$ in all cases).

Birds consumed significantly more Nutrivit® per 2 hour feeding trial on the EQ (mean =1.07±0.23) diet than on the RQ (mean = 0.08±0.039) diet (GLMM $F_{1,44} = 19.32$, $p<0.0001$). However, there were no differences in the mean mass of seed eaten over a 2 hour trial (means: EQ 1.17g± 0.16, RQ 1.04g± 0.16 GLMM $F_{1,44} = 0.321$, $p=0.574$).

6.5 Discussion

Dietary antioxidant availability impacted individual levels of oxidative stress. I found a significant effect of diet on both comet assay and MDA analysis. First, using the high pH comet assay there was a higher proportion of intact DNA on the EQ diet, than on the RQ diet. In addition, birds had significantly higher levels of plasma MDA after receiving the RQ diet, than after the EQ diet. I compared levels of oxidative damage during both experimental diets, with mean values of oxidative damage prior to the start of the experiment when birds were not exercised (see also Chapter 5). All tests of oxidative damage showed damage was higher following the flight procedure on both diets than when receiving a standard diet and no exercise. Thus, flight exercise seemed to impose an oxidative burden on the birds which was eased by the consumption of a nutritional supplement rich in antioxidants. Oxidative stress levels appeared to be lower when birds were not exercising. It appears that the flight exercise imposed did lead to increased oxidative stress, which is in accord with other studies of DNA damage and exercise in humans and animals (Hartmann et al., 1995; Aniagu et al., 2006). Increases in MDA after exercise have also been shown in other taxa (for review see Vollaard et al., 2005). The high pH comet assay is thought to reveal alkali labile sites in DNA, representing several different forms of oxidative damage, whereas the low pH comet does not (Rojas et al., 1999). Although there was no difference between EQ and RQ diet in the low pH comet assay, the higher abundance of alkali labile sites on a reduced antioxidant diet suggests that dietary antioxidants prevented some exercise-induced oxidative damage of DNA.

Although in a short term study it is difficult to assess the biological importance of exercise induced damage, I suggest that increases in DNA damage may have long term consequences. Lymphocytes are the most prevalent cells used in comet assay of avian blood (C. Tregaskes, unpublished data). Avian lymphocytes are only produced early in development, so can be repaired but not replaced during adulthood, and circulate for a high proportion of an animals life (Glick, 1979). Increases in DNA damage, including alkali labile sites, can eventually lead to apoptosis (Monti et al., 1992). Long-term DNA damage may induce a reduction in lymphocyte number and therefore immunocompromise an individual. The preventative action of dietary antioxidants on exercise-induced oxidative stress may thus have important effects besides those pertaining to exercise performance. Hartmann et al. (1995) previously reported that dietary vitamin E prevented oxidative damage of DNA in human subjects after treadmill running, though to my knowledge, this

is the first study to report dietary antioxidants preventing exercise-induced DNA damage in birds.

Increased oxidative stress was predicted to be one of the factors involved in limiting strenuous exercise in animals, where there may be a trade-off between minimising damage to tissue, yet maximising the chances of escape from a predator. Over four days, I calculated average take-off escape time, an important aspect of fitness, of each bird when on EQ and RQ diets. I found that there was no effect of dietary antioxidant intake on escape time. In contrast, Blount and Matheson (2006) reported that male zebra finches *Taeniopygia guttata,* a small passerine bird, receiving a carotenoid enhanced diet flew faster than controls. In their study oxidative stress was not measured, and it is possible that the difference was due to an effect of carotenoids aside from antioxidative protection. Of course, the difference may also represent a species-specific difference in behaviour. In this experiment I expect that birds exerted maximal effort in flight and paid a cost through increased oxidative damage. The consequences of this increased oxidative damage are unclear from a short-term experiment; though there is some evidence that oxidative stress can cause muscle damage (Bailey et al., 2007), other studies have found no effect of oxidative stress on muscle performance. Indeed, ROS production may actually be necessary to optimise muscle performance (Reid, 2001). Measuring flight speed repeatedly over a longer course of time on each diet may have revealed differences in performance, and it is possible that oxidative damage accumulating in muscle tissue is more important in affecting muscle stamina, rather than burst exercise. For example, one recent study showed the length of flight in homing pigeons *Columba livia* was directly related to oxidative stress after flight (Costantini et al., 2008). In this study, though I expect the birds exerted maximal effort, the flight procedure was quick, and birds were rested between each flight. It has been shown that energy metabolism in short flights is quite different from long flights (Schwilch et al., 1996). Thus flying birds for long periods, perhaps using a wind tunnel, or flying birds more often without rest, would reveal further differences in oxidative stress and flight performance.

Another limitation of this study is in the resolution of different flight escape times. I filmed flights using a standard video camera. This meant flight duration could only be measured to the nearest 0.04 seconds. Given the short flight distance, any difference in within-individual flight performance may have required a more sensitive filming technique. In order to properly assess flight take-off, a high speed camera would attain a more effective

measure of performance (e.g. Williams and Swaddle, 2003). This is especially true over the first few centimetres, potentially those most important in predator evasion (Kenward, 1978). However, in this study, the flight protocol I employed was certainly suitable to encourage exercise and there were individual differences in oxidative damage between the bird's baseline levels of oxidative damage, and those during this experiment, apparently mediated by the flight procedure. In this context the design was successful, and similar apparatus has been used to assess differences in vertical flight escape times before (Veasey et al., 1998; Veasey et al., 2001; Blount and Matheson, 2006). One important consideration is that prior to the experiment, birds were housed in small cages. Though these were large enough to allow flight, they were not sufficiently large to allow continuous vertical flight as was encouraged by the flight procedure here. The effects of exercise fitness, and training on are likely to be important in mediating post-exercise oxidative stress. For example, in rats endurance treadmill running has been shown to increase by-products of lipid peroxidation post exercise (Senturk et al., 2001). Conversely, it has recently been demonstrated that endurance training, treadmill running 5 days a week for 8 weeks, attenuates this increased peroxidation (Oztasan et al., 2004). Also, activity of antioxidant enzymes was increased during exercise in trained rats. In wild birds, especially nomadic flocking birds such as budgerigars, constant exposure to exercise training and oxidative stress may increase antioxidant enzyme activity to maximal performance. How dietary antioxidants would affect oxidative stress in this case, after what is effectively exercise training remains untested.

In spite of the effect of dietary treatment on oxidative stress, I found that there was no significant difference in plasma levels of lutein, zeaxanthin, α-tocopherol or retinol after each 4 week block. This does not indicate a failure in the dietary treatment. I found that during the two hour intake trials, intake of Nutrivit®, but not seed, was significantly increased on the EQ diet, compared to the RQ diet. Of course, it is possible that the EQ birds had greater levels of antioxidants in other organs, though not in blood plasma. For example, it has been shown repeatedly (e.g. Surai et al., 2002, Karadas et al., 2005a) that birds are capable of storing antioxidants in their liver at levels far greater than those seen in plasma. Although birds may have been storing antioxidants, there was no evidence of a carry-over of stored antioxidants between experimental blocks. The experimental oxidative stress measures were lower after receiving the RQ diet, regardless of the diet order. If antioxidants from the EQ diet were stored, I would have expected a lesser effect of exercise on oxidative stress after the RQ diet on birds that received the RQ diet in the

second experimental block, which was not the case. Indeed, there was no effect of diet order on any plasma antioxidant measured. Other recent studies of antioxidants in birds have found no effect of increased dietary antioxidants on plasma antioxidants levels (Biard et al., 2006). They did, however, find effects of the supplementation on other parameters, supporting the idea that although the antioxidants are not evident in plasma, they are being used in other physiological systems. While retinol and α-tocopherol are increased in Nutrivit® in relatively high concentrations, other antioxidants, which were not sampled, are possibly more important in reducing exercise-induced oxidative stress, for example endogenous antioxidant enzymes. In future the roles of dietary antioxidants should be considered together with those of antioxidant to attain a more effective estimate of antioxidant status.

Given the apparent beneficial effects of antioxidants, it could be expected that animals may selectively choose antioxidant-rich food items. Evidence for this is scarce (but see Schaefer et al., 2008). In contrast to Chapter 5, here I found no evidence that birds were selectively eating the antioxidant supplement. On the RQ diet Nutrivit® was seldom eaten at all, whereas on the EQ diet the average number eaten was around one piece during the 2 hour palatability trial. This suggests that rather than selecting Nutrivit®, the birds ate it by chance when mixed into their seeds. However, after a two hour deprivation it is conceivable that the birds selected foods based on their calorific content, rather than antioxidant status. By assessing differences in diet choice over a longer period, a more reliable indicator of differences in selection of different food items would be attained.

In this study, I found evidence for exercise-induced lipid peroxidation, and DNA damage. I also found that dietary supplementation with an antioxidant supplement was capable of countering this increased oxidative stress. Although I found no evidence of dietary antioxidants modifying exercise performance, longer term studies will be crucial in elucidating the roles of both oxidative stress and dietary antioxidants in determining physical capabilities in animals. DNA damage may have implications for immune function, as well as exercise performance. Thus access to dietary antioxidants may be an important fitness determinant, through various physiological pathways.

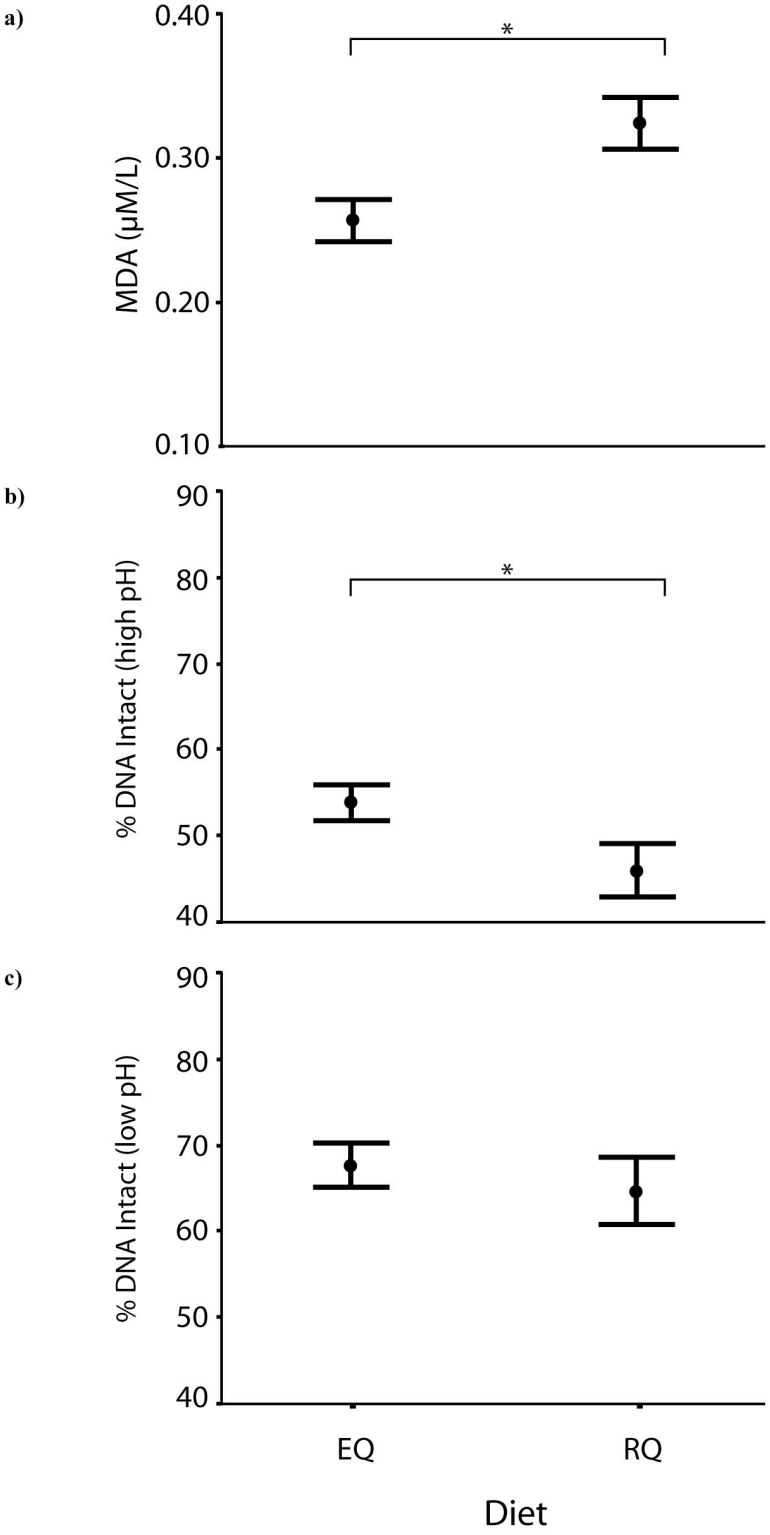

Figure 6.1 Measures of oxidative damage following each diet: enhanced quality (EQ, 10% Nutrivit® inclusion), and reduced quality (RQ, 1% Nutrivit® inclusion). **a)** Levels of MDA indicating degree of lipid peroxidation; **b)** Percentage of DNA intact after high pH comet showing alkali labile sites and single strand breaks; **c)** Percentage of DNA intact after low pH comet revealing single strand breaks. In all cases * indicates a significant difference (GLMM, $P<0.05$). Figure shows raw data rather than transformed data used in analyses.

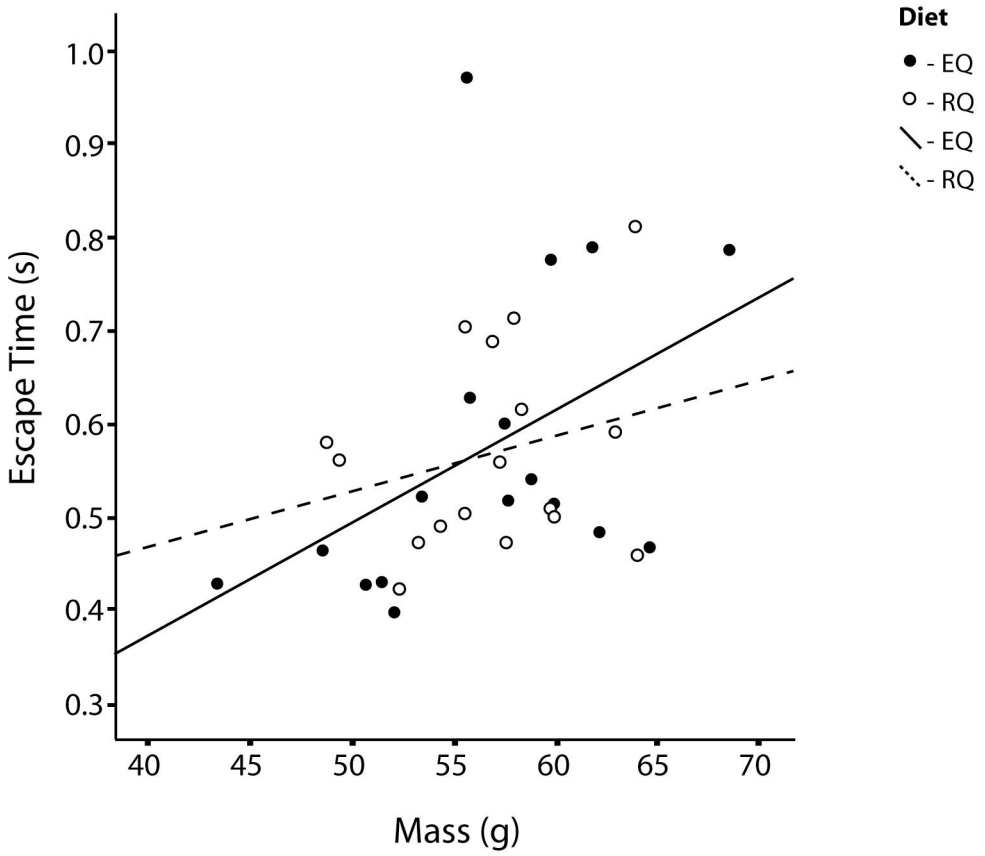

Figure 6.2 Relationship between body mass and escape time on each diet.

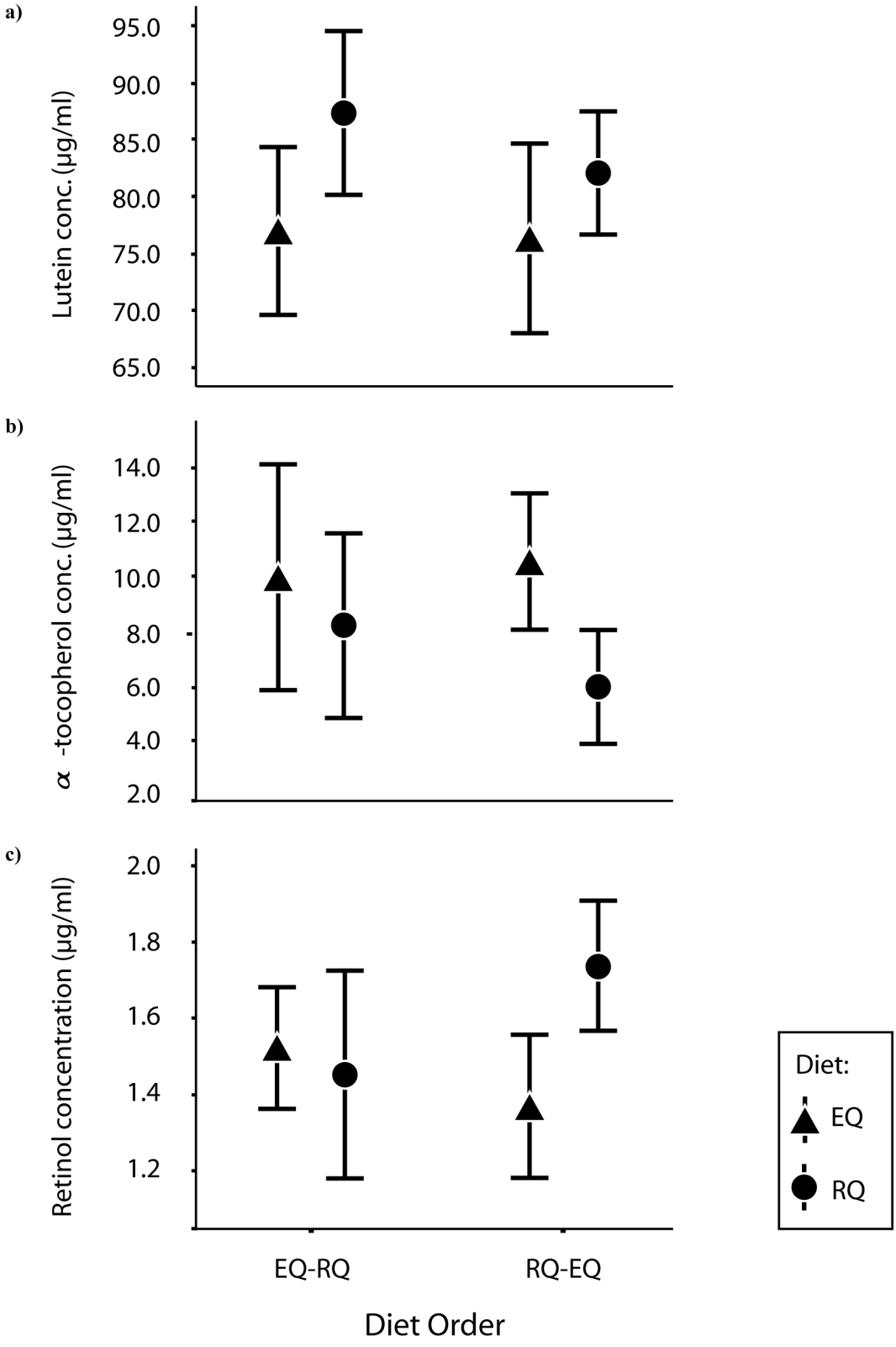

Figure 6.3 Plasma concentrations of a) Lutein, b) α-tocopherol and c) Retinol, following each diet: enhanced quality (EQ, 10% Nutrivit® inclusion), and reduced quality (RQ, 1% Nutrivit® inclusion). Data are presented by the order in which diets were received to demonstrate lack of carry-over effect.

Chapter 7. Flight training attenuates oxidative stress in captive adult budgerigars *Melopsittacus undulatus*

7.1 Abstract

The interactions between exercise, oxidative stress and antioxidant supplementation have attracted considerable interest. Antioxidants may protect muscle and cellular components against reactive oxygen species (ROS) produced during exercise. Thus they may also improve exercise performance. Previously, it was shown that short periods of intense exercise are capable of increasing oxidative stress in largely sedentary budgerigars *Melopsittacus undulatus*. In another study, captive male budgerigars on an enhanced quality diet, rich in antioxidants, improved in flight performance compared to those on a reduced quality diet This was attributed to dietary antioxidants protecting locomotive function, though oxidative stress was not measured. However, recent experiments have shown that exercise training, i.e. repeated performance of the same exercise task over time, is capable of improving activity of endogenous antioxidant enzymes. I retained budgerigars on enhanced quality (EQ) and reduced quality (RQ) diets for 12 months. After 9 months without strenuous flight activity, I flew captive adult budgerigars regularly for 9 weeks. I measured malonidialdehyde (MDA) levels, a by product of lipid peroxidation, after 1 week and 9 weeks of training. I found that levels of MDA were significantly lower after 9 week than after 1 week of flight training. This is the first indication of an exercise training mediated attenuation in oxidative stress in birds. Interestingly, there was no effect of diet quality on levels of MDA at any point. However, further experiments are required to elucidate the roles of dietary antioxidants in mediating exercise-induced oxidative stress. I suggest that these results may have implications for our understanding of the costs of flight in wild, as well as captive birds.

7.2 Introduction

The links between oxidative stress, antioxidant status and exercise are attracting considerable interest in a range of species (Powers et al., 2004). Strenuous exercise may increase metabolic rate by up to 100 times, and this is associated with an increase in many reactive oxygen species, including the highly reactive hydroxyl radical (Leeuwenburgh et al., 1999). If left unchecked these radical would damage all cellular components; lipids, DNA and proteins (Halliwell and Gutteridge, 1984). Instead, the body's endogenous antioxidant systems, together with exogenous dietary acquired antioxidants, may quench ROS and prevent damage to tissue. Flight activity in birds is an energetically expensive behaviour, and well known to result in an increase in metabolic rate (Bundle et al., 2007). In budgerigars, *Melopsittacus undulatus*, metabolic rate during flight is increased 6.7 times from resting levels, though the exact increase will depend on speed and direction of flight (Tucker, 1969; Schmidt-Nielson,1972). However, the oxidative costs of flight have only recently started to be explored (Costantini et al., 2008). In Chapters 5 and 6, I demonstrated that flight activity in captive budgerigars was associated with an increase in post-exercise oxidative stress; measured as DNA damage and lipid peroxidation. I also showed that supplementing dietary antioxidants was capable of ameliorating some of this oxidative stress.

Since antioxidants may protect muscle, and cellular components involved in muscle contractions and energy metabolism, against oxidative stress (Morris and Sulakhe, 1997; Lawler et al. 1998), they may also improve exercise performance (reviewed Powers et al., 2004). There is considerable interest in how antioxidant supplementation may affect exercise performance, though much of the focus is on human athletes (Vollaard et al., 2005). In wild animals exercise ability will have important roles, for example in determining food intake, escape from predators, attracting a mate, or in migration. Thus, antioxidant protection of locomotive function may represent a fitness determining characteristic. In Chapter 6, I did not find a difference in flight escape times in a study of domesticated budgerigars. However, in that study birds were flown irregularly, and the exercise was unlikely to have pushed birds to their physiological limits. At the University of Glasgow, an experiment similar to that described in Chapter 6 was conducted, measuring flight escape time over a longer course of time, with more regular and exhaustive exercise (Arnold, Larcombe et al., in review. See Appendix 1). Results revealed that males receiving an enhanced antioxidant diet did improve in the most strenuous test of

flight performance over time, while males receiving a reduced antioxidant diet did not. In addition, the faster flying, enhanced diet males were also more attractive to females in a mate choice test. These results seem to support the suggestion that an antioxidant rich diet ameliorated oxidative damage over time.

In mammals, there is increasing evidence to suggest an effect of exercise training in attenuating oxidative damage associated with exercise. Exercise training may improve antioxidant defences in cells associated with exercise. For example Sen et al. (1992) demonstrated that beagle dogs *Canis familiaris*, and male rats *Rattus norvegicus* trained on treadmills, had increased activity of the glutathione system in muscles associated with exercise, and in their livers. The glutathione system is an important endogenous antioxidant system involved in protection of cells (Surai, 2002). This upregulation of natural antioxidant defences with exercise training may also have occurred in the experiment by Arnold, Larcombe et al. (in review), in which birds were flown repeatedly over 9 weeks. However, oxidative stress was not measured in birds in that study. In treadmill trained rats, 8 weeks of exercise training, 5 days a week, resulted in increased activity of the antioxidant enzymes superoxide dismutase (SOD), and glutathione peroxidase after exercise, compared with untrained rats (Oztasan et al., 2004). This was associated with a decrease in levels of malonidialdehyde (MDA), a by-product of lipid peroxidation, in erythrocytes. In contrast, not all studies have found a protective effect of exercise training in resisting oxidative damage (Tiidus, 1998). So far, there are no studies investigating the effects of flight training on oxidative damage in birds.

In this study, I used a colony of captive wild-type budgerigars (as in appendix 1), to analyse the effects of long term exercise training on levels of oxidative damage in adult budgerigars. Birds were retained on reduced or enhanced quality diets for 12 months. Following a 9 month period without exercise, birds experienced one day of flight training, before plasma levels of MDA were measured to assess levels of lipid peroxidation. Following a further 8 weeks of flight training, plasma MDA was measured again. Overall, I tested whether flight training attenuates oxidative damage in budgerigars, though specifically I asked whether; 1) long term flight training in budgerigars decreases lipid peroxidation post-exercise, and 2) dietary antioxidant supplementation affects levels of lipid peroxidation after flight training.

7.3 Methods

This study was very similar in length and layout to a previous experiment, described in Appendix 1. I used the same colony of wild type budgerigars, composed of the offspring and grand-offspring bred from captured wild budgerigars, imported from Australia in 2002. Ages of birds ranged from 4-8 years. Birds were housed in cages measuring approximately 77 x 45 x 41cm. Thus the birds were relatively sedentary. The experiment ran from October 2006 till December 2006.

Diet manipulation

All birds in the study had previously been randomly assigned to one of two dietary treatments: Enhanced Quality (EQ) or Reduced Quality (RQ). The diets were made by mixing a standard seed mix, with identical proportions of each seed, with a different percentage (by mass) of Nutrivit® (Mars, Csongrad, Hungary). The main nutritional effect of Nutrivit is to provide more antioxidants than is found in seeds alone. The EQ diet contained a 10% inclusion of Nutrivit, while the RQ diet contained a 1% inclusion. The nutritional breakdown of these two diets can be found in Chapter 6. Each bird had received its diet since October 2005, and the previous flight experiment detailed in Appendix 1 finished in January 2006. This meant prior to commencing this experiment, birds had received EQ or RQ diets for a total of 12 months, and had not received flight training for the previous 9 months.

Flight procedure

I used the same flight apparatus detailed in Chapter 6. The flight trials were performed by flying each bird from a holding cup on the floor, to a perch 1m above. Escape time was calculated as the time taken for each bird to fly from a point 10cm above ground level to a point at 80cm above ground level. During the course of the experiment all birds were flown six times on one day per week for a total of nine consecutive weeks. In the morning, each bird was first weighed, then performed two flights with a 5-10 minute break in between, and was then returned to its home cage. Approximately, four hours later, the bird did four consecutive flights in quick succession without a break. Escape time was calculated as a mean of the 6 daily flights.

Blood sampling and morphometric measurements

One day, and 9 weeks after the commencement of flight training, all birds in the trial were blood sampled, measured and weighed. There was an interval of around 36 hours between a flight procedure and a blood sample being taken. A small volume of blood, approximately 300 µl, or less than 1% of the body mass, was taken from the brachial vein and collected in a heparinised capillary tube. The plasma was separated from the red blood cells by centrifuging for 5 minutes. Next the tarsus, winglength and mass were measured. The plasma and red cells were stored at -70ºC. Body condition was calculated using residuals from the regression of ln(mass) on 3*ln(tarsus).

MDA analysis

The MDA method was based on that of Young and Trimble (1991). Thiobarbituric acid (0.044M,100µl) and phosphoric acid (1.22M, 100µl) were mixed together and added to 50µl of plasma (per bird) in a test tube. An inert atmosphere was created by applying a nitrogen blanket, and the test tubes were sealed and vortexed prior to heating (60 min, 70-75^{o}C). Samples were cooled in water, then 200µl was transferred to a centrifuge tube containing sodium hydroxide (1M, 100µl). Methanol (500µl) was added and mixed. Samples were centrifuged (10 min, 12000 g) and the supernatant analysed on a Summit HPLC system (Dionex, Idstein, Germany) using Chromeleon software (Dionex). An Acclaim 120 C18 5µ 4.6 x 250 mm column (Dionex) and guard were used with fluorescence detection (excitation 532nm and emission 553nm). The mobile phase was isocratic, 40:60 methanol:phosphate buffer (40mM, pH 6.5), with a flow rate of 1ml/min, and a run time of 7 min. Samples were assayed against a standard of malonidialdehyde bis (dimethyl acetal), (Sigma Aldrich, Poole, UK) that was simultaneously taken through the same procedure.

Statistics

All statistical analysis used SPSS version 14. Training and diet induced changes in MDA levels and escape time were analysed using repeated measures general linear models. Sex and treatment were added to each model as fixed factors, to assess the effect of antioxidant availability and sex on change in oxidative stress after flight training. Body condition was added to each model as a covariate to control for an effect of relative body mass on the

difference in oxidative damage or flight performance. Models were simplified by dropping non-significant factors until only significant terms remained. Of 25 birds in the study, 7 birds failed to fly sufficiently high or straight to accurately calculate escape time, thus they were omitted from analyses of flight performance. The birds still exerted maximal effort in the flight procedure thus were included in analyses of oxidative damage.

7.4 Results

There was no effect of flight training on body condition (repeated measures GLM: $F_{1,24}$ = 0.003, p = 0.957), therefore all results below reflect changes specifically related to exercise training, not a by product of a training-induced change in morphology.

Effect of flight training on post-flight oxidative stress

Using a repeated measures GLM I found a significant effect of flight training on plasma levels of MDA (repeated measures: training $F_{1,24}$ = 9.133, p = 0.006). MDA levels were significantly lower after 9 weeks flight training, than before flight training started (Figure 7.1). However, there was no effect of sex (repeated measures GLM: $F_{1,24}$ = 0.44, p = 0.514) nor dietary treatment (repeated measures: $F_{1,24}$ = 0.338, p = 0.540) on the difference between untrained and trained levels of MDA (Figure 7.1). Body condition did not affect the change in levels of MDA before or after training.

Effect of training flight performance

Mean daily escape flight time decreased over the 9 weeks of flight training (repeated measures GLM: within subjects factor $F_{1,17}$ = 15.49, p = 0.002), with the greatest improvement tending to occur in EQ males (sex x diet $F_{1,17}$ = 4.38, p = 0.057).

7.5 Discussion

In this study, I found evidence for a significant effect of flight training on levels of oxidative damage in budgerigars. Levels of MDA were lower after 9 weeks flight training, than after a single bout of flight exercise. However, there was no effect of dietary treatment on the difference in levels of MDA between trained and untrained flight. This provides evidence that for captive birds, regular exercise training may attenuate oxidative stress, independently of diet.

In Chapter 5 and 6, I tested the idea that flight exercise, and activity, were likely to be associated with an increase in oxidative damage in budgerigars. I provided some evidence that this was the case, and that dietary antioxidants may help ameliorate the oxidative damage associated with exercise. I hypothesized that the long term exercise requirements of wild animals may necessitate sufficient dietary antioxidants to resist a decrease in exercise performance with increasing deleterious effects of oxidative damage. Here, I found that exercise training actually decreased levels of MDA, a result independent of diet. Interestingly, exercise training was also associated with a decrease in flight escape time, that is, an increase in flight performance in addition to a decrease in oxidative damage. This was particularly evident in EQ males. Exercise training can lead to an improvement of activity in endogenous antioxidant systems. Data on this phenomenon from trained and untrained human athletes often conflict in results (for review see Urso and Clarkson, 2003). In animals, model species have been used to assess the effects of training on antioxidant systems. In Wistar rats for example, treadmill training has been shown to increase activity of SOD and glutathione in muscle. However, results for these studies may be "strain" and tissue specific. Thus, whether exercise training always leads to an increase in antioxidant enzyme activity is unclear. In another study, Gul et al. (2006) found no increase in activity of SOD, and a decrease in the activities of glutathione reductase and peroxidise, in heart tissue of trained Sprague-Dawley rats. In this study I did not measure antioxidant status in each bird, before and after flight training.

It is possible the decrease in MDA after exercise training was due to an effect aside from an increase in antioxidant enzyme activity. The stress response in animals is known to produce an increase in ROS, which may increase oxidative damage (e.g. Şahın and Gümüşlü, 2007). In this study, birds may have been more stressed at the start of the experimental procedure than the end, when they had acclimated fully to the equipment,

procedure and handling. If physiological stress was responsible for an increase in ROS production at the beginning of flight training, though not at the end, then this might explain the reduction in MDA with training. I would argue that this was not the case because in Chapter 6, I showed that budgerigars had increased oxidative damage after flight exercise only when receiving a reduced quality diet, regardless of the order the diets were received. It is reasonable to assume that if a physiological stress response to handling was responsible for the increase in oxidative damage, then decreased oxidative damage after a bird's second exposure to the procedure would have been expected. This was not the case, and it should be noted that before this experiment, birds were regularly handled, and had become accustomed to the flight apparatus (see Appendix 1).

Interestingly, I found no effect of the two diets on attenuation of oxidative stress with exercise training. There are several possible reasons for this. Firstly, in this study I used plasma levels of MDA to quantify lipid peroxidation. This measure will have provided an assessment of the general status of lipid peroxidation throughout the body, though is not tissue-specific. Sampling tissue from flight muscles would achieve a more precise measure of lipid peroxidation specifically attributable to flight. Similarly, differences in oxidative stress may have become apparent by using a different measure of oxidative stress, for example comet assay of DNA damage (see Chapters 5 and 6). In addition to finding no effect of diet on the attenuation of oxidative stress with training, I found no effect of diet on levels of MDA when birds were untrained. This was in contrast to my findings in Chapter 6, where budgerigars suffered less oxidative damage after receiving an EQ diet than an RQ diet (including lower levels of MDA). In Chapter 6, birds were relatively untrained in exercise before every blood sampling, as flight training lasted only 4 days prior to each sample, with three week breaks in flight training between. As in this study, birds were not sampled immediately after flying, though were sampled within 12 hours of final flight, not 36 hours as here. However, I decided allowing birds a rest period following exercise before blood sampling was of benefit to the study since the implications of a transitory rise in oxidative stress after exercise would be unclear.

The results from this experiment may have exciting implications for our understanding of the oxidative costs of exercise in birds. I provided some evidence that captive budgerigars may be able to reduce levels of oxidative stress after exercise by regular training. Since this result was independent of antioxidant content of diet, I suggest it is potentially mediated by increased activity of endogenous antioxidant systems. In this experiment I aimed to

manipulate long term oxidative costs by repeatedly flying birds. In wild birds, in which exercise is an important part of daily life, rather than an enforced semi-regular activity, whether or not an upregulation of antioxidant activity is possible is unclear. It is likely that endogenous antioxidant systems will operate at maximal activity at all times. Thus in wild birds the requirement for exogenous dietary antioxidants to prevent a decline in motor function will probably be higher than in relatively sedentary laboratory animals. In future, capture of wild birds and assessment of oxidative stress and antioxidant status following a series of demanding exercise tasks, may reveal the extent to which antioxidant activity can be increased in nature. Then, the true benefits of dietary antioxidant availability on oxidative stress and exercise performance can be assessed.

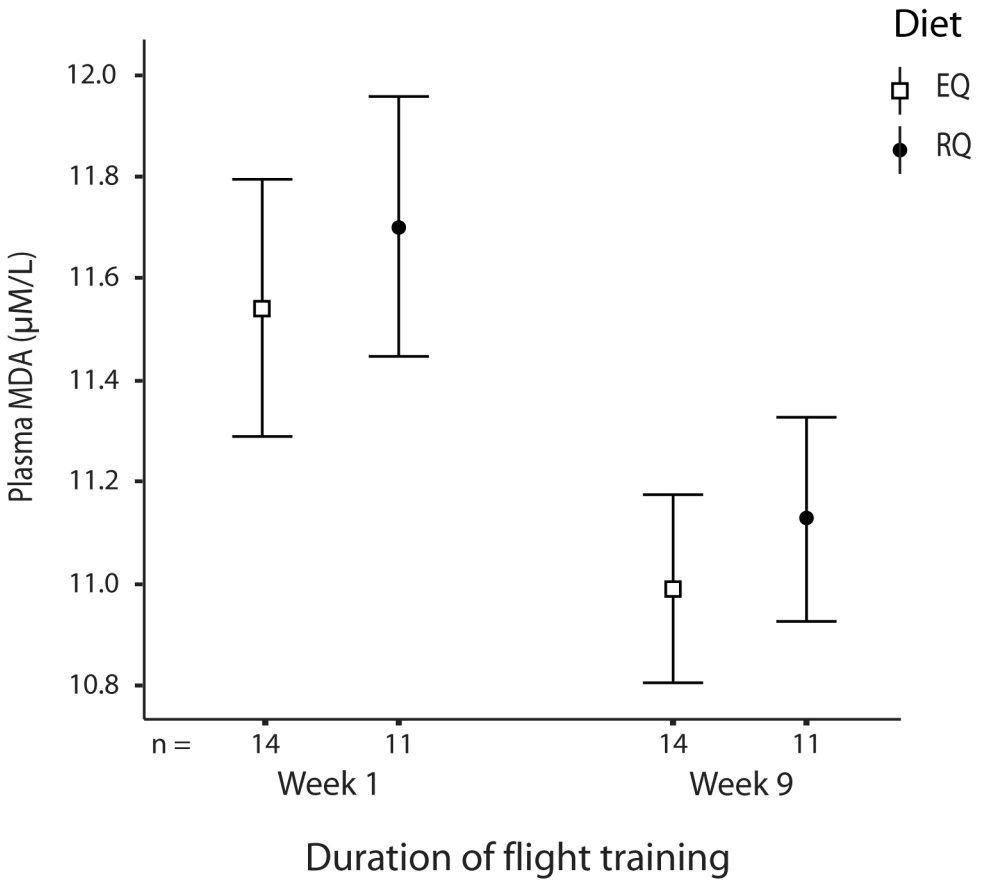

Figure 7.1 Mean (± SE) MDA levels after 1 week, and 9 weeks of flight training.

Chapter 8. General Discussion

Throughout this thesis, each discussion has described and interpreted the results for that chapter. In this general discussion, the key findings from each chapter will be linked in order to show how understanding of the subject has been increased by this PhD. In addition, I will discuss the limitations of each study and discuss how future research may draw upon the results. Although the overall aims of the PhD were linked by assessing the effects of dietary antioxidants on individual performance and fitness, in this discussion I will discuss the two central themes of the PhD separately: firstly dietary α-tocopherol and maternal effects in blue tits, and secondly, the effects of antioxidant availability on oxidative stress and exercise performance in budgerigars. Finally, I will bring these two strands together, and discuss their implications for our understanding of how dietary antioxidants can impact upon fitness related traits.

Maternal α-tocopherol availability and reproductive output, chick development and phenotype in blue tits.

In Chapters 2-4, I was interested in the effects of dietary α-tocopherol availability for blue tit reproduction, and chick development and phenotype. Carotenoids have long been used in behavioural ecology studies, owing to their ubiquity in plumage colour and sexual ornaments, and their myriad of functions, including as antioxidants, immunomodulators, precursors of vitamin A, and cell signalling agents (Blount, 2004). In particular, there has been some recent interest in the adaptive maternal effects of carotenoids in female diet, being incorporated in egg yolks (e.g. Blount et al., 2002; Saino et al., 2003; Biard et al, 2005). Often carotenoids are thought of as important antioxidants in eggs, however, α-tocopherol is a more potent antioxidant, present in egg yolks in even greater concentrations than carotenoids (Surai et al., 1998; Biard et al., 2005). Therefore manipulating α-tocopherol levels in the diet allowed an assessment of reproductive traits mediated specifically by antioxidant function. In **Chapter 2**, I found no evidence that females differed in their reproductive effort in terms of egg size, yolk volume, clutch size, or incubation time depending on whether or not they received an α-tocopherol supplement. Perhaps more surprisingly, I also found no difference in yolk concentrations of antioxidants (including α-tocopherol) in the fifth egg between control or α-tocopherol supplemented birds. At first glance this may suggest that the treatment was ineffective, but birds did appear to eat the α-tocopherol supplement. Since antioxidant levels in yolk

generally decline across the laying sequence (Hõrak et al., 2004; Biard et al., 2005), antioxidants may have differed in the yolks of eggs other than the fifth. I found that adult females in worse condition during chick rearing had invested in more yolk antioxidants, than females in good condition. As condition of each bird was measured just once, it is unclear whether this result means birds that invested in more yolk antioxidants had a subsequent decrease in condition, or that females already in poorer condition invested in more yolk antioxidants. Also, I demonstrated that mothers supplemented with α-tocopherol had significantly more female biased sex ratios than control supplemented birds. This was in direct contrast to my prediction that α-tocopherol supplemented birds may male bias their sex ratios, since males grow faster and are larger than females. Although several models for adaptive manipulation of sex ratios have been hypothesized in the literature, and several studies have shown results that fit with one or more of these hypotheses, demonstrating that sex ratio manipulation is adaptive is very difficult. In this study, with a small sample size, it is possible that the difference in sex ratio was overestimated, and represented a statistical artefact (Ewen et al., 2004). Secondly, the α-tocopherol may have interfered with adult females' reproductive system, producing a non- or maladaptive sex ratio bias. The exact method for sex allocation in birds is unclear (Pike and Petrie, 2003) but none of the existing hypothesis appear to be malleable to antioxidant status. If this sex ratio bias was in fact an adaptive response to α-tocopherol, one would expect that the daughters of α-tocopherol supplemented mothers may reap fitness benefits. Alternatively, the α-tocopherol supplemented mothers may have increased their own chances of future reproduction. Therefore, I suggested that the α-tocopherol supplement may have benefited the adult female (or male) in ways that were not manifested in the first stages of reproduction, though that might have influenced chick development or phenotype, or adult survival.

In **Chapter 3**, I tested these ideas by cross-fostering half broods between nests where parents had identical brood sizes, and hatching dates, though different dietary treatments during egg laying. I measured a suite of morphometric traits, and also plumage colouration for each bird. In particular, I measured MDA, a by-product of lipid peroxidation. Since the main role of α-tocopherol *in vivo* is in the prevention of lipid peroxidation (Burton and Ingold, 1984), I predicted that chicks with greater access to α-tocopherol should suffer less lipid peroxidation than chicks from control nests. Chicks from α-tocopherol nests were smaller on day three, the day at which they were fostered, than chicks from control nests. However, I found chicks whose parents received α-tocopherol, grew faster than those

whose egg parents had received control supplement, and were no lighter at fledging. There was no effect of rearing environment on growth rate. This indicates that some aspect of chick development was determined prior to the cross fostering. Potential reasons for this were discussed, although it is hard to imagine any immediate benefits to chicks of increased growth from a smaller size. Indeed, given that the chicks from α-tocopherol eggs were faster growing it may be expected they should have higher MDA levels than controls, contrary to my initial predictions. Instead, I found no effect of treatment on levels of MDA in the chicks. Thus the chicks from α-tocopherol eggs grew faster, but did not pay the expected oxidative cost. Despite their increased mass gain, at day 14 chicks from α-tocopherol eggs were skeletally smaller than those from control eggs, though were no lighter i.e. were in better condition.

So far, the results from both chapters 2 and 3 do not appear clear cut. In contrast to studies of carotenoids in the same species and closely related species (Biard et al., 2005; Berthouly et al., 2007), I detected no clear benefit to chicks of α-tocopherol supplementation. I did detect differences between control and α-tocopherol supplemented birds, implying the treatment worked insofar as birds reacted to it. Assuming the α-tocopherol treatment was metabolized, why did it have no obvious benefits for adults or their chicks? Since early environment has long-term consequences for fitness, it is assumed that assuring one's offspring of the best start in life will benefit fitness of offspring and parent alike (Lindström, 1999). However, lifetime fitness for each adult will depend on future, as well as current reproduction. The following year to the feeding trials, all ringed females breeding in our field site were caught. Unfortunately this data concerned only females, and thus excludes any male-specific results. The results indicated that there was no difference in chick survival and recruitment, but there was a trend for more α-tocopherol supplemented females to return than controls, with a higher reproductive success. This offered some indication that the α-tocopherol treatment may have improved adult condition, and survival possibilities, without a cost to chicks. In addition, mistnetting birds at feeding stations in the winter of 2007/2008 showed that more F1 progeny of α-tocopherol of both sexes were caught. This may suggest that in spite of no major differences in phenotype at fledging, chicks from α-tocopherol treated eggs may have had increased survival. It should be emphasized that a larger sample size is required to test this.

Even if adult survival and reproduction could partly demonstrate a benefit of α-tocopherol supplementation, it is still interesting that this study showed different results to similar

studies manipulating carotenoids. This may be due to a genuine difference in responses to α-tocopherol and carotenoids, related to the relative benefits of each to offspring or parent. This is interesting, as α-tocopherol is a potent antioxidant, and I predicted antioxidant function was unlikely to be the main role of carotenoids antioxidants in nature. It is possible that the benefit of antioxidant protection during development in this species may be less important than the benefits carotenoids infer through their additional roles in cell signalling, and immuno-enhancement. Alternatively, different antioxidants may be more or less important at different life history stages. Several studies have recently shown that carotenoids are relatively poor antioxidants, or certainly are not used as such, in some birds (Costantini et al., 2007b; Isaksson et al., 2007a; Isaksson and Andersson, 2008). However, carotenoids appear most effective as antioxidants in low oxygen concentrations (Burton and Ingold, 1984) such as those found in avian eggs. To my knowledge, there are no published papers revealing the effect of yolk carotenoids in specifically reducing oxidative damage in embryos (studies examining susceptibility to peroxidation, or antioxidant capacity are not considered, as they are not measuring *in vivo* oxidative stress *per se*). Evidence that carotenoids did reduce oxidative stress in egg bound embryos may increase our knowledge of their antioxidant roles during development. Where information is available, α-tocopherol appears to be a major constituent of egg yolk, moreso even than carotenoids (e.g. Surai et al., 1998). Of course the investment of each nutrient in egg yolks, will depend on the relative costs to females of obtaining and processing them. Assessing levels of both carotenoids and α-tocopherol in the food and tissues of adult birds, and relating these to levels found in egg yolks would allow a better understanding of the investment of each into eggs.

Red herring hypothesis, plumage colouration and oxidative stress in nestlings

In **Chapter 4**, the effects of α-tocopherol and carotenoids during chick development were assessed by feeding chicks within a brood one of three treatments: control, carotenoids or α-tocopherol. There has been much interest in the information revealed by carotenoid pigmented plumage, an apparently honest signal of individual quality (Olson and Owens, 1998). Hartley and Kennedy (2004) suggested that antioxidant protection of carotenoid pigments could result in an increase in plumage colour, a method by which carotenoid plumage may reveal antioxidant status. By using a within-brood design I was able assess the contributions of provisioning of different antioxidants to chick development and phenotype. Although this allowed an examination of differences in growth and oxidative

damage mediated by different antioxidants, the main aim of this experiment was to elucidate the roles of carotenoids or α-tocopherol in determining plumage colouration of nestlings. Carotenoid supplemented birds had a marginally higher chromatic signal of the carotenoid mediated portion of their plumage. However, there was no significant difference in carotenoid chroma, a measure of colour specifically relating to the carotenoid content of plumage, between treatment groups (e.g. Isaksson et al., 2005). I had predicted that protection of carotenoid pigments by α-tocopherol would allow α-tocopherol supplemented birds to increase in plumage colouration compared to a control, but this was not the case. Since α-tocopherol is a more effective biological antioxidant than carotenoids, I anticipated that MDA levels would be lower in α-tocopherol supplemented birds, than both other groups. Again, I found no differences in MDA levels between any treatment group. This is interesting, since in Chapter 3 I showed that rearing environment explained some of the variation between broods in MDA levels. I suggested that this may reflect differences in food, and hence antioxidant, provisioning by parents. That in this study I found no effect of either carotenoid or α-tocopherol supplementation on MDA suggests another difference between broods must be responsible. This demonstrates that levels of oxidative damage are likely to depend on factors aside from dietary antioxidant availability. Oxidative stress will also be affected by hormones (Alonso-Alvarez et al., 2007a), immune status and parasitism (de Lope et al., 1998), and importantly by endogenous antioxidant systems. In future, more integrative studies manipulating, or measuring more of these factors may help assess the role of dietary antioxidants in mediating oxidative stress and ultimately fitness.

One of the most obvious difficulties in comparing these results with those from any similar supplementary feeding studies is that results may be different both between populations, and between years. Even within this thesis, the experiment carried out in Chapter 4 was carried out a year before the experiments in Chapters 2 and 3. Supplementation with dietary antioxidants in wild birds is obviously susceptible to environmental differences in many factors including: the availability of prey, nutritional breakdown of different prey species, disease and parasite prevalence, and weather conditions amongst others. Indeed, differences between populations in plasma and feather antioxidants, even within small geographic ranges, have been reported (Isaksson et al., 2007b) All of these will probably affect the oxidative status, antioxidant demands and antioxidant availability of birds. Results from Chapters 2 and 3 are interesting in their own right, as they represent the first test of the effect of α-tocopherol availability during reproduction on different aspects of

chick growth and phenotype. The lack of a clear benefit in these studies, does not rule out a major role for α-tocopherol in mediating offspring fitness. In a year or area with low food abundance, in which natural α-tocopherol intake is deficient then experimentally evaluating the role of α-tocopherol in avian development would be easier. For example, despite the evidence that carotenoid supplementation of females improves offspring quality in tits, one recent study found no effect of increased carotenoid availability for great tits in a different population (Remeš et al, 2007)

The information gained in chapter 2-4 represents one of the first experimental tests of the effects of α-tocopherol supplementation on phenotype, development and fitness in a wild bird (but see de Ayala et al., 2006). As such it represents a valuable increase in our knowledge of the importance of dietary antioxidants in birds. Though none of the experiments showed demonstrable benefits of α-tocopherol supplementation, this does not diminish the viewpoint that α-tocopherol is an important nutrient. However, if α-tocopherol supplementation genuinely has no benefit for development or fitness of blue tits, this suggests the widespread benefits of carotenoids in this species may relate to one of their roles other than antioxidant protection. Until recently, within ecological literature carotenoids and antioxidants were synonymous with each other. Several papers have challenged the paradigm of carotenoids as antioxidants (e.g. Hartley and Kennedy, 2004, Costantini and Møller, 2008; Isaksson and Andersson, 2008), not only in birds (Olsson et al., 2008). If the biological actions of these molecules is related to cell signalling, or immunomodulation then this represents a fascinating topic for future research. However, this thesis is only a small start in assessing the different roles of different antioxidants within birds, and results are likely to be dependent on many different factors. More studies are required to elucidate the importance of dietary antioxidants in mediating fitness in birds.

Antioxidant manipulation in wild birds

In chapters 2-4 the reason for manipulating α-tocopherol was to specifically elucidate the role of *antioxidant* availability during development and reproduction. However, there are several reasons why manipulating one antioxidant or the other, may not yield the desired result. In Chapters 2-4, in addition to the supplementary α-tocopherol, birds were receiving other antioxidants through their natural diet. In this case, the diet of blue tits is already particularly rich in α-tocopherol and carotenoids (Arnold and Ramsay, unpublished data),

which may have several effects. Firstly, absorption of antioxidants is dependent on a number of factors (Surai, 2002). Both α-tocopherol and carotenoids are lipophillic antioxidants, and thus have similar modes of absorption. So far, there is some evidence that α-tocopherol absorption may be impaired by carotenoids, through competition (Woodall et al 1996; Parker 1996). This may have resulted in α-tocopherol not being absorbed in the quantities I had hoped. Calculating dosage for experimental supplementation in wild birds is particularly difficult. I calculated approximate intake of each substance based on known concentrations in prey species, but the bioavailability of these nutrients from invertebrates was unknown. During the breeding season the blue tit diet comprises a large amount of caterpillars, meaning this calculation will likely be more accurate than for generalist feeders. However, absorption efficiency for each substance will probably be diminished when all of a nutrient is supplied in one feed, as in Chapter 4. Although in Chapter 4 I found increased plasma concentrations of lutein in carotenoid supplemented birds, I did not find increased plasma α-tocopherol, in α-tocopherol supplemented birds. It is also unclear whether the difference in lutein represents a small increase in absorption of the substance, or the absorption of all of the supplementary nutrient. A small rise in availability of a specific nutrient may not be enough to trigger a difference in oxidative damage, and the phenotypic traits measured. One method to circumvent this problem in adult birds is to provide feeders, and allow birds to eat the supplement "at leisure". This probably allows a more natural increase in antioxidant levels. In wild populations assessing how much of the supplement a given bird has eaten is very difficult, since other species will use the feeders. In addition, some birds will have access to the feeder for longer owing to differences in duration of nest building, and egg laying. Though in Chapter 2, I aimed to control for this by including duration of treatment in models, the amount of supplement consumed will depend on a combination of duration of treatment, and propensity of feeder use. This may depend on individual differences in personality traits (Herborn and Arnold, unpublished), sympatric species, and local habitat quality. Perhaps by providing the supplement within the nestbox itself, a more accurate impression of feeder use would be attained (e.g. Biard et al., 2005). However, in the process of designing the experiment, I was concerned birds would not use a feeder within the nest regularly.

A further problem caused by blue tit's natural diet comes when attempting to disentangle the effects of one antioxidant, from those of another: viewing different antioxidants as discreet may be flawed. There is plenty of evidence that α-tocopherol and carotenoids may act synergistically in resisting oxidative stress (reviewed Surai, 2002). Also, carotenoids

may assist in vitamin E recycling (Bohm et al., 1997). If vitamin E can be recycled in adult birds, then α-tocopherol may be more expendable than other non-recyclable antioxidants. On the other hand additional dietary α-tocopherol, may diminish the requirement for recycling, freeing other molecules. The costs of vitamin E recycling, are unknown, and this remains a fascinating area of research. These non-exclusive sparing effects, and synergistic interactions render experimental tests of the roles of different antioxidants difficult, especially in studies of wild birds, with limited knowledge of the differences in oxidative balance, and internal antioxidant systems between individuals.

In chapter 2-4, although I had *a priori* reasons to suspect that growth rate, oxidative damage and plumage colouration would be affected by the dietary treatments, it is naïve to suspect that dietary antioxidant availability would be the only determining factor. Hormones are another important constituent of eggs, and are likely to have profound implications for growth, and also oxidative stress in chicks (Alonso-Alvarez et al., 2007b). The results from chapter 4 also hint at a role for hormones in plumage colouration, as previously suggested (Peters, 2007). Just as considering one antioxidant without another is misleading, so considering antioxidants without hormones in chick development does not reveal the whole picture. Indeed, patterns of hormone and antioxidant deposition in eggs have shown a positive (e.g. Navara et al., 2006b) or a negative (e.g. Royle et al., 2001) relationship between antioxidants and hormones in eggs. In Chapter 2 I did not find differences in yolk antioxidants in the 5th laid egg between the treatment groups. There are reasons to suspect antioxidant levels may have differed in other eggs, though I do not know whether other yolk constituents, including hormones, differed within the eggs. I predicted that increased α-tocopherol may *allow* faster growth, though whether or not it could cause faster growth is unclear. A more integrative approach, assessing the effects of dietary antioxidants, on both the hormonal and antioxidant systems in developing chicks may assist our understanding of the roles of both in determining growth rate and body size.

Blue tits appeared to be a good model to study the effects of different antioxidants. As wild birds, they have been constantly acted upon by natural selection, ensuring selection for efficient antioxidant absorption and mobilisation has been retained. In domesticated or laboratory species, such adaptations may be lost (Sol, 2008). Also, blue tits are easy to manipulate in a nestbox population, and are generally hardy towards disturbance caused by such experiments. Although I have discussed that the antioxidant rich diet of blue tits may have caused problems, the fact they encounter α-tocopherol and carotenoids in relatively

high concentrations naturally, ensures that both are biologically relevant. Although being small birds, unfortunately I was limited in the amount of measures I could use from one small blood sample. MDA which formed a large part of these experiments requires a relatively large volume of blood. This meant that the relationships between internal dietary antioxidant reserves, and oxidative stress could not be assessed. Indeed, I could not gather a suitable amount of blood for MDA analysis for every bird, which reduced the sample size. A further complication caused by this species is that in this population, fledging success was very high every year. It is possible that this study site represents prime territory for blue tits, and during the breeding season birds are unlikely to display symptoms of nutritional deficiencies, at least those relating to antioxidants. It is more difficult to catch and analyse birds when they are not in nestboxes, but post-fledging environment will possibly represent a more demanding and fitness determining time for tits (Monrós et al., 2002). Although I did re-trap adults and chicks the year after the adult dietary manipulation, only 10 adult females and 13 F1 female chicks were recaught in 2007, and 1 adult females and 8 F1 chicks in the winter of 2007/2008. This does not yield a suitable sample size to statistically analyse survival rates. In future, by placing more emphasis on re-capturing birds after experimental supplementation, the true fitness benefits of α-tocopherol supplementation may be revealed.

Manipulating oxidative stress

A major limitation of nutrient supplementation experiments, is that the effects of additional antioxidants will depend on oxidative costs specific to individuals. It is my belief that experimental manipulation of oxidative costs must be considered alongside supplementation of antioxidants. In future, studies investigating the role of antioxidants in limiting oxidative damage must consider this. For example, Isaksson and Andersson (2008) manipulated paraquat, a weed killer known to produce ROS *in vivo,* in great tits. In this way they could specifically elucidate the adaptive role of specific antioxidants in mediating oxidative stress. There are two parts to demonstrating an adaptive role of dietary antioxidant in reducing oxidative stress. First, is demonstrating the negative effects of ROS production and oxidative stress. It is possible to manipulate oxidative costs in several ways. Firstly, an egg may be manipulated by changing the atmosphere during incubation. By removing eggs from a nest and artificially incubating, oxidative stress may be altered. Increasing the oxygen partial pressure above atmospheric levels can increase ROS production (Lawrence et al., 1996). Interestingly, hypoxia may also cause oxidative stress

(Blokhina et al., 2001), thus decreasing partial oxygen pressure below atmospheric levels may also induce oxidative stress. Similarly, oxidative stress may be altered by addition of an oxidizing agent (Isaksson and Andersson, 2008). By manipulating the oxidative status of a developing egg, the protective effects of different antioxidants can be assessed. Firstly, this may be achieved by experimentally manipulating levels of specific antioxidants in yolk (e.g. Saino et al., 2003). By manipulating antioxidant levels in eggs, then manipulating oxidative stress, differences in growth rate, condition, and importantly, measures of oxidative stress post-hatching can be attributed to the protective effect of antioxidants. An interesting experiment might be to divide clutches of eggs of unmanipulated females between two treatments: control and α-tocopherol, and injecting either α-tocopherol or a passive control into the yolks of these eggs. During incubation, these eggs could be removed for a period and subject to two treatments, high oxidative stress, and low oxidative stress, for example by manipulating oxygen pressure. By rearing the hatched chicks in a common environment and assessing the effects of both yolk antioxidants and oxidative stress treatment, a better understanding of the roles of antioxidants in resisting damage caused by oxidative stress specifically, and affecting chick development would be gained. Although this experimental design largely ignores the question of how maternal dietary α-tocopherol levels influence deposition in yolk, this merits experimental investigation of its own. Once the protective effects of an antioxidant have been demonstrated the same experimental manipulation of oxidative stress could be used to assess differences in oxidative stress between eggs laid by mothers receiving different dietary supplements. A subset of eggs could be used to assess yolk antioxidants.

In summary, I found little hard evidence to suggest that maternal α-tocopherol supplementation affected her condition, or reproductive effort. α-tocopherol supplementation did lead to differences in the phenotype and development of chicks, but demonstrating this was of adaptive benefit was more difficult. Provisioning of dietary antioxidants to chicks did not reduce levels of lipid peroxidation, and only carotenoid supplementation had an effect on plumage colouration. I found no evidence that α-tocopherol could improve plumage colouration in nestling blue tits. Further experiments are needed to elucidate the roles of dietary antioxidants and oxidative in mediating phenotype and fitness in wild birds.

Antioxidants, oxidative stress and exercise performance in budgerigars

The effects of dietary antioxidants are not limited to reproduction. Several physiological processes are associated with increased oxidative stress, and dietary antioxidants may play a crucial role in eliminating ROS and ensuring normal somatic function. Next, I studied the impacts of dietary derived antioxidants on oxidative damage and physiological performance in adult birds. I particularly focussed on escape flight, a potentially fitness-related trait. Flight is a metabolically expensive behaviour (Bundle et al., 2007), and is likely to be associated with an increase in ROS (Leeuwenbergh et al., 1999). However, in birds the oxidative costs of flight have been little studied (but see Costantini et al., 2008). Behavioural ecologists are interested in trade-offs between physiological systems for the same resource. In wild birds, where flight is likely to be a part of daily existence, there may be a high demand for antioxidants to ameliorate exercise-induced oxidative stress. Failure to protect tissue from the damaging effects of ROS and other radicals produced during flight, may lead to a decrease in flight performance, and consequently fitness. Therefore acquisition of dietary antioxidants may be an important determinant of flight ability, as well as important in maintaining genomic and cellular integrity, immune function and plumage colour. Flight performance may have serious implications for fitness, for example in migratory species, and prey and predator species relying on flight. In Chapters 5-7, I attempted to uncover some of the oxidative costs of flight in budgerigars, and assess the extent to which dietary antioxidants may help ameliorate this.

In **Chapter 5,** I used correlational data from budgerigars to assess whether individual differences in oxidative damage were related to differences in active behaviours, or diet choice. I evaluated oxidative damage in three different ways: MDA analysis as a measure of lipid peroxidation, comet assay at low pH to assess single strand DNA breaks, and comet assay at high pH to assess both single strand breaks and alkali labile sites in DNA. Diet choice was assessed by quantifying the selection of Nutrivit®, an antioxidant rich pellet added to seed mix. I found that budgerigars that were more active, engaging in more walking, hopping, and climbing had more DNA damage, measured as strand breaks, than less active birds. In addition, I showed birds that selected more Nutrivit® from their diet had higher levels of DNA damage. This indicates that individual levels of activity may result in differences in oxidative stress, and that such differences in oxidative stress can potentially be detected and acted upon by birds. Importantly, different measures of oxidative stress were not correlated with each other. Thus, different measures of oxidative

stress reveal different aspects of oxidative damage. For example, I revealed no differences in MDA, a by-product of lipid peroxidation, attributable to active behaviour in this study. Indeed, the two results showing differences in comet assay, revealed differences in specific forms of DNA damage. Throughout this thesis I have suggested that manipulating antioxidant levels in birds and assessing their effects without considering a measure of oxidative stress/damage does indicate an effect of antioxidant activity *per se.* However, the results from this study reveal that using any one measure cannot reveal total oxidative status (Dotan et al., 2004). In behavioural ecology, choosing an appropriate test of oxidative stress is important in order to reveal consequences of a treatment related to the aims of a study.

Escape flight and oxidative stress

Despite showing a link between oxidative damage and activity in birds in chapter 5, the causative nature of this association was unclear. In addition I found that the frequency of flying around the, albeit small, home cage was unrelated to oxidative damage. As flying is the most metabolically expensive exercise in birds, I could not rule out the possibility that the relationship between activity and oxidative stress was caused by individual differences in stress responses. In **Chapter 6**, I manipulated the oxidative costs to the same adult budgerigars, by enforcing flight exercise on birds. I also gave every bird two diets; an enhanced quality (EQ) diet, rich in antioxidants, and a reduced quality (RQ) diet, with reduced antioxidants. The birds received both diets sequentially (though in a randomised order). For every bird I assessed difference in MDA and DNA damage, and flight performance following each diet. I found that irrespective of diet, all birds had higher levels of oxidative damage of all kinds, after flight trials than before, during the phase outlined in Chapter 5. However, oxidative damage measures were lower after flight on the EQ diet than the RQ diet, regardless of diet order. This represents the first experimental evidence of an oxidative cost to flight, and also the first demonstration that dietary antioxidants may reduce this cost. This has important implications for our knowledge of the oxidative costs faced by wild birds. Furthermore, if flight exercise is a significant contributor to differences in oxidative damage, then species with different modes and function of flight may have varying requirements for antioxidants. In the study, despite finding evidence of exercise induced oxidative stress in birds, I did not find any effect of the diet on flight performance, i.e vertical escape time. If exercise-induced oxidative damage has no effect on locomotive function, then some of the costs to exercise caused by

antioxidant deficiencies I predicted may be biologically irrelevant. Nevertheless, there is reason to suspect that the costs of flight may yet lead to differences in flight performance. Indeed, another study revealed differences in flight performance with carotenoid supplementation in zebra finches (Blount and Matheson, 2006). I suggested that increasing the level of flight activity to exhaustive exercise, or flying birds over a longer period of time, may increase the oxidative burden of flight, leading to differences in flight performance.

Flight training and oxidative stress

In another study carried out at the University of Glasgow (Arnold, Larcombe et al., in review. See Appendix 1), we performed such an experiment. Using wild type budgerigars, I demonstrated that male budgerigars receiving an EQ diet significantly improved in flight performance, compared to RQ males and all females. In this study birds were flown repeatedly over 9 weeks and the flight trial was more strenuous than in Chapter 5. This suggests my prediction that longer, exhaustive exercise will increase the oxidative stress over time, may be correct. However, studies in mammals have demonstrated that exercise training, i.e. repetition of the same exercise task, may increase activity of endogenous antioxidant enzymes and decrease oxidative stress (e.g. Sen et al., 1992). In **Chapter 7,** I examined the role of exercise training in reducing oxidative damage in the same captive population of wild budgerigars used in appendix 1. The birds were retained on their experimental diets for 12 months. After being sedentary for 9 months, I resumed a strenuous flight procedure once per week for 9 weeks. MDA levels were measured after 1 week, and a further 8 weeks of training. I found that all birds had reduced levels of MDA after 9 weeks training, than after 1. This is the first experimental evidence of a training effect in oxidative stress attenuation in birds. Interestingly, there were no differences between birds receiving EQ or RQ diet in the level of this attenuation, but it should be noted that the sample size was small, given the individual variation in flight performance.

The results from Chapter 7, combined with those from Chapter 6 and Appendix 1, have important implications for our knowledge of the costs of exercise in birds. The fact that my evidence suggests birds are capable of increasing antioxidant activity with exercise training may appear to contradict earlier findings that flight is associated with increased oxidative damage and dietary antioxidants may alleviate exercise induced oxidative stress and improve performance (Chapter 6, Appendix 1). In the experiment described in Appendix 1,

it was suggested that a retardation of exercise-induced oxidative damage by dietary antioxidants was likely responsible for the improvement in flight of EQ males in strenuous flight tasks. In Chapter 7, I did not re-examine escape time, but my results did not suggest an effect of diet or sex on MDA levels. RQ birds may have reduced flight effort, resulting in slower flights but keeping oxidative costs at the same level as faster flying EQ birds. The multifaceted health benefits of antioxidants mean the possibility that the effect was due to another role of antioxidants cannot be ruled out. For example, an antioxidant mediated improvement of immunity or cognition may have improved the ability to perform well in flight tests. However, evidence that training may increase activity of antioxidant enzymes, this does not rule out a role for dietary antioxidants in reducing exercise-related oxidative stress. In most studies of exercise training and antioxidant enzymes, dietary antioxidant status is not measured. In a recent study, Chang et al. (2007) demonstrated an interaction between dietary α-tocopherol and the activities of glutathione and SOD in rats, though the results for each enzyme were complex, and differed between muscle types. In general, rats fed a diet deficient in α-tocopherol had decreased α-tocopherol in muscle, though elevated activity of glutathione peroxidise, compared to non α-tocopherol deficient rats. However, activity of SOD was reduced, and overall α-tocopherol deficient rats suffered more oxidative stress (Chang et al., 2007). My results represent the first thorough experimental tests of oxidative stress and flight in birds. The potential ability of birds to up- or downregulate antioxidant activity has important implications for the study of dietary antioxidants in all birds. Future work is required to assess whether birds can regulate activity of antioxidant enzymes, in response to diet.

Though several mammalian studies have demonstrated an increase in antioxidant activity with exercise training, and my results suggest this is possible in birds, whether these results are ecologically relevant or not is still to be determined. No studies of wild birds have yet addressed the effects of exercise on oxidative stress. Indeed, whether or not wild animals are capable of upregulating antioxidant enzymes is unknown. It could be expected that since wild animals have a constant requirement for exercise, endogenous antioxidant systems will operate maximally at all times. In future it would be beneficial to assess the effect of dietary antioxidants on exercise induced oxidative stress in wild caught individuals. For example Kullberg et al. (2002) studied take off flight ability in wild caught blue tits. If birds can be caught and brought into aviaries (allowing flight), then levels of oxidative stress may be assessed, and related to antioxidant levels between individuals. By

investigating differences in oxidative stress following exercise in wild individuals, the preventive role of dietary antioxidants in nature can be assessed.

Measures of oxidative stress

As discussed above, different measures of oxidative stress may yield different results, and choosing an appropriate measure of oxidative stress is vital when considering the results of any experiment. Oxidative stress is defined as the imbalance where production of ROS, outweighs the potential of antioxidant systems to cope. As such, the concentration of reactive oxygen species, and also the antioxidant capacity of a given tissue can be measured, and the ratio of these can be used to assess oxidative stress (e.g. Costantini et al., 2006). This offers perhaps the most precise measure of "oxidative stress". However, an implicit assumption when discussing oxidative stress, is that the production of ROS beyond the organisms ability to remove them will lead to deleterious effects. In particular, the overproduction of ROS will lead to lipid peroxidation, causing damage to cells, DNA and other tissues. It is this oxidative damage that will ultimately impact on an organism's fitness, and I believe that measures of oxidative damage provide the most ecologically relevant measure for studying the effects of antioxidants and oxidative stress. Throughout this PhD I have used MDA analysis, an assessment of by-products of lipid peroxidation. Prevention of lipid peroxidation appears to be the main function of dietary acquired lipophillic antioxidants, especially vitamin E (Burton and Ingold, 1981), which were manipulated in this study. Thus, MDA analysis seemed a suitable method for assessing differences in oxidative stress specifically related to my different treatments. Nevertheless, using the same technique may still produce varying results, depending on the timing, and tissue of sampling. Results of analysis of oxidative stress will depend on the turnover and removal of biomolecules used as correlates of increased oxidative damage, and this will probably depend on tissue. Unfortunately the data from MDA analysis in this thesis do not allow a proper assessment of interspecific differences, or differences attributable to timing of sampling. In Chapter 7 wild type budgerigars were sampled 36 hours after flight, and in chapter 6 domesticated budgerigars were sampled 12 hours after flight. In addition all the budgerigars sampled were adults whereas the blue tits used in chapters 3 and 4 were pre-fledging chicks. In addition to choosing the correct measure of oxidative stress, future experiments will also have to choose an appropriate time to sample study animals. For example, in Chapter 7 I may have found a difference in MDA levels caused by diet, if birds had been blood sampled immediately after flight. However, transitory rises in post-

exercise correlates of oxidative stress, may be of little ecological relevance. The correct time to sample birds will depend on the aims of the experiment in question.

The broad aim of this PhD was to investigate the roles of dietary antioxidants and oxidative stress in mediating fitness related traits. There can be little doubt that dietary antioxidants are of vital importance in the health and fitness of animals. So far, within behavioural ecology, the study of antioxidant function in life history trade-offs is still in its infancy. As dietary antioxidants offer an identifiable limited resource, the functions of carotenoids in particular in trade-offs in birds have received most attention. However, at present almost nothing is known the role of endogenous antioxidants systems in nature (but see Isaksson et al., 2005). What little has been studied in this respect often relates to poultry science, or the effects of pollutants and contaminants (e.g. Hilscherova et al., 2003). The amount of oxidative stress suffered by an individual will depend not just on the ability to prevent the damaging chain reactions of ROS but also on halting production of radical formation, which is achieved by SOD, and glutathione and thioredoxin systems amongst others (Surai, 1999). Finding no effect of a given dietary antioxidant on oxidative stress does not mean that nutrient is not an antioxidant: there is some evidence that dietary antioxidants and antioxidant enzymes will interact to determine an individual's oxidative status (e.g. Sodhi et al., 2008). For example, supplementation of dietary antioxidants may allow a downregulation of endogenous antioxidant systems, resulting in the same level of oxidative stress. I previously suggested that antioxidant enzymes may operate at full capacity in wild animals, though the relative costs and benefits to using dietary and/or endogenous antioxidants is unknown. Nor are the relative contributions of genetics, age and diet to the activity of antioxidant enzymes clear for most animals. Interestingly, Isaksson et al. (2005) showed that there were some differences in activation of the glutathione system between urban and rural great tits *Parus major*, however the relationship between endogenous antioxidants and environmental and dietary factors is unclear. Future studies may wish to consider the impacts of dietary antioxidants alongside the action of endogenous antioxidant systems, in order to ascertain the relative contributions of both to minimising oxidative stress in wild animals.

Conclusions

Experimental supplementation of maternal α-tocopherol produced effects on the phenotype and development of their offspring in blue tits. Demonstrating a fitness benefit of these effects was difficult, and it is unclear whether α-tocopherol availability limits reproductive success in this species. There was no evidence to suggest that α-tocopherol was capable of increasing expression of a carotenoid mediated plumage trait in blue tit nestlings, nor of decreasing lipid peroxidation during growth. I did show some evidence that oxidative stress was correlated with growth rate in blue tit chicks, though the effect of differences in oxidative stress, and nestling phenotype for fitness remain to be fully explored. Future studies are required to assess the true fitness consequences of dietary antioxidants, and oxidative stress in wild birds. Using captive budgerigars, for the first time I demonstrated experimentally that flight in birds is associated with an increase in oxidative stress. It was also shown that dietary antioxidants are capable of ameliorating exercised-induced oxidative stress, and potentially improving flight performance. In addition, I showed that training captive birds may attenuate exercise-induced oxidative stress, perhaps mediated by endogenous antioxidant enzymes. In wild birds whether or not antioxidant enzymes can be upregulated in response to environmental conditions is still to be tested. Dietary antioxidants alone cannot fully explain differences in oxidative stress between individuals. Hormones, disease, age, behaviour, diet and endogenous antioxidants will all contribute to an individual's oxidative status. In future more integrative studies are required to assess the contribution of different antioxidants to oxidative stress, and ultimately fitness in birds.

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Appendix 1: Antioxidant supplementation of wildtype budgerigars impacts male flight performance and attractiveness

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Abstract

Antioxidants can ameliorate the effects of oxidative stress. As physical activity can increase oxidative stress in the body, an antioxidant rich diet is suggested to improve exercise capabilities. Such a diet is also predicted to enhance sexual attractiveness, as some antioxidants have multiple roles including the mediation of colourful ornaments. In this study, we assessed the effects of antioxidant supplementation on interrelated traits in adult budgerigars *Melopsittacus undulatus:* antioxidant concentrations and total antioxidant capacity of blood plasma, escape flight time, plumage colouration and sexual attractiveness. A colony of wildtype male and female budgerigars were provided with either an enhanced (EQ) or reduced quality (RQ) diet varying in antioxidant concentrations for several months. HPLC analysis showed that birds on the EQ diet showed significantly higher blood plasma concentrations of retinol and α -tocopherol and a tendency for higher levels of the carotenoid zeaxanthin, than those on the RQ diet. The total antioxidant capacity of the blood, which is an index of both endogenous and dietary-derived antioxidant activity, was greater in males than females and marginally higher in EQ males than other birds. Males, but not females, on the EQ diet showed a significant improvement in stamina escape time, the most strenuous exercise test, but not rested escape time compared with the RQ group. In mate choice trials, females showed a preference for EQ males over RQ males. Dietary-mediated divergence in sexual attractiveness was not due to differences in the chromatic signals of plumage ornaments. However, EQ males that were relatively fast in the escape flight experiment were more strongly preferred in the mate choice arena than their RQ competitors. Our results show that dietary-derived antioxidants can influence sexual attractiveness and other fitness-related traits through multiple pathways, for example by enhancing the capacity for physical activity.

Introduction

Antioxidants can fulfil several roles in the body: as vitamins, as pigments, and as protectors against oxidative stress (Hartley and Kennedy, 2004; McGraw, 2005; Olson and Owens, 1999). Thus, multiple physiological systems may compete for the same pool of antioxidants. As their name suggests, one of the main roles of antioxidants is in protecting molecules in the body against oxidation. Organisms are under constant assault from reactive oxygen species (ROS) such as free radicals, which are produced as a byproduct of metabolism. ROS can cause oxidative damage or stress to DNA, protein or the polyunsaturated fatty acids (PUFAs) that form cell membranes. The body has both endogenous and exogenous, i.e. dietary-derived, antioxidant defences against ROS. Particularly efficacious of the dietary-derived antioxidants responsible for antioxidant defence within the body is α-tocopherol (vitamin E), although other important antioxidants include retinol (vitamin A), vitamin C, and carotenoids (Surai, 2002). Physical activity increases the generation of ROS via a number of routes including the increased mitochondrial consumption of oxygen, the release of ROS- generating metabolites and processes involved in damaged tissue repair (Urso and Clarkson, 2003). Antioxidant deficiencies lead to a curtailment of exercise performance in mammals, including humans (Lukaski, 2004; Powers et al., 2004), thought to be indicative of the detrimental effects of oxidative stress (Powers et al., 2004). However, despite commonly held beliefs among the sporting community, the evidence for a positive benefit of antioxidant supplementation on exercise performance in individuals without deficiencies is at best ambiguous (Urso and Clarkson, 2003; Vollaard et al., 2005).

In birds, the most strenuous exercise performed is take-off flight, which is why it is potentially a good standardised test of physiological state. Given the imperative of a fast take-off for predator avoidance and thus survival, it is proposed that ‘startled’ birds should always invest maximal effort in ‘escape flight’ (Kullberg et al., 1998). Thus, the time of such flights should be an honest signal of condition relatively independent of individual differences in motivation. If antioxidants do have positive effects on exercise performance then, birds on enhanced antioxidant diets are predicted to have faster escape times than those on reduced antioxidant diets. The mechanisms are likely to be multifaceted, because dietary-derived antioxidants can protect against ROS damage, enhance immune function and are involved in cell signalling thus enhancing muscular response time (Surai, 2002).

As in mammals, the impacts of antioxidants in birds may only be seen when exercise is strenuous.

As well as protecting against ROS, in many avian species, some antioxidants, are also used for the pigmentation of plumage and integument (McGraw, 2005). Since antioxidants such as carotenoids have multiple functions, may be a limiting resource (Olson and Owens, 1999) and/or are energetically costly to use (McGraw, 2005), carotenoid-mediated colouration is though to provide an honest display of an individual's quality (but see Hartley and Kennedy, 2004). In mate choice tests, carotenoid supplemented birds have been shown to be more attractive to the opposite sex than controls (e.g. Blount et al., 2003b). The strength of this preference has generally been attributed to the change in the colour of a carotenoid-mediated ornament. However, if antioxidants have multiple roles in the body then antioxidant supplementation might change other traits and behaviours, which potential mates pay attention to. Parrots provide a model for testing this hypothesis because this family do not directly use carotenoids to pigment feathers red, yellow or orange, but instead utilise psittacofulvins (McGraw and Nogare, 2004; Stradi et al., 2001). These pigments are synthesised endogenously, probably in the feather follicle (McGraw and Nogare, 2004; Stradi et al., 2001). More data are needed to determine whether short term antioxidant supplementation can improve sexual attractiveness, even when it has no direct effect on colouration.

The budgerigar *Melopsittacus undulatus* belongs to the parrot family Psittaciformes. The wildtype budgerigar is a nomadic, colonial, cavity breeding species native to the dry grasslands of interior Australia. They are socially monogamous and exhibit obligate biparental care (Brockway, 1964). Budgerigars feed on a variety of different seeds in the wild, so maintaining a steady food intake despite irregular rainfall (Wyndham, 1980). The budgerigar's (human visible) sexual dichromatism is limited to the cere. In this study, we investigated whether a manipulation of dietary antioxidants over several months influenced interrelated phenotypic traits that can potentially signal individual quality. Specifically we measured: 1) concentrations of dietary-derived antioxidants and total antioxidant capacity of the blood plasma; 2) escape flight performance; 3) plumage colouration; 4) male sexual attractiveness.

Methods

To outline the study: In the first month, all birds were kept on the same baseline diet during which birds were trained to use the flight recording apparatus. In week 4 of the baseline diet, take-off escape time was recorded for each bird. Two days later, baseline blood samples and morphometric measurements were taken. The experimental diets then commenced with half the birds receiving an enhanced (EQ) and the rest a reduced quality (RQ) diet, varying significantly in antioxidant levels. Weekly flight recordings were made until week 8. Next, blood samples and morphometric measurements were taken once again. In the following month, mate choice trials were carried out. The study presented here consists of the first four months of a longer term project which commenced in October 2005 and finished in January 2007.

The study population of adults aged 3-7 years consisted of the offspring and grand-offspring of wild budgerigars bred in captivity and imported from Australia in 2002. The birds are behaviourally quite different, skeletally much smaller and less prone to obesity than the familiar 'British budgerigar'. Birds were maintained at a temperature of 20°C and provided with 14 hours of light per day with a mixture of UV-emitting and visible-light emitting bulbs that were turned off / on gradually every day to simulate dawn and dusk. Same sex pairs of birds were housed in cages sized 77 cm x 45cm x 41cm, so they were relatively sedentary. This work was carried out under licence from the UK Home Office and subjected to ethical reviews by both the University of Glasgow's and WALTHAM® 's ethical committees. No birds became ill or injured as a result of this experiment.

Diet Manipulation

All birds were provided with approximately 10g daily of mixed seed containing specific proportions of red, yellow and white millet, oats and canary seeds (Larcombe et al In Review). At the start of the experiment (Oct 2005), birds were put on a baseline-feeding regime consisting of *ad libitum* commercial bird food Trill (seed mix with a 3% inclusion of Nutrivit ®), plus water and cuttlefish. Nutrivit ® is a small seed-shaped nutritional supplement rich in antioxidants, which can be mixed with the seed mixture. After four weeks on the baseline diet, birds were randomly assigned, in same sex pairs, to either the

enhanced (EQ) or reduced quality (RQ) diets. The enhanced diet quality treatment group received the seed mix with a Nutrivit ® inclusion of 10% and the reduced quality group was given an identical seed mix with a Nutrivit ® inclusion of 1%. Nutrivit ® contains retinol (unlike seed) and around one hundred times the concentration of α-tocopherol and 2-10 times the levels of carotenoids compared to the same mass of a standard seed mixture (see Larcombe et al. (In Review) for a nutritional breakdown of the diets).

Blood sampling and morphometric measurements

Blood samples and morphometric measurements were taken after four weeks on the baseline diet and again after seven weeks on the experimental diets. Both samples were taken two days after a flight trial. A small volume of blood, approximately 300 µl, or less than 1% of the body mass, was taken from the brachial vein and collected in a heparinised capillary tube. The plasma was separated from the red blood cells by centrifuging for 5 minutes and stored at -70 ºC. Next the tarsus, winglength and mass were measured. Wingloading was calculated as mass (g) / winglength (mm).

Analyses of blood plasma antioxidants

In order to determine whether the diet manipulation changed the levels of antioxidants in the blood plasma, samples were analysed using a HPLC technique. Antioxidants were extracted from the plasma using the methods outlined in Larcombe et al. (In Review). A HPLC pump Spectra Model 8800 was employed for HPLC analyses. Sample analysis was carried out using Phenomenex PHLC column type 250mm x 2mm id. Data were acquired between 220-600nm with a Diode array absorbance detector type Thermo model UV6000. Xcalibur computer software was used to control the Autosampler and Diode array detector and to record the level of individual carotenoids. Concentrations were calculated with reference to α-tocopherol, retinol, lutein and zeaxanthin standards (Sigma-Aldrich). Concentrations are given in µg per ml.

Total antioxidant capacity of blood plasma (TAC)

The total antioxidant capacity of the blood provides an index of the ability of the plasma sample to quench free radicals. In addition to dietary-derived antioxidants, non-enzymatic endogenous antioxidants such as uric acid contribute to the total antioxidant capacity

(TAC). A spectrophotometric method was used to analyse the TAC of the blood plasma after 7 weeks on the experimental diets. The methods used as similar to those in (Re et al., 1999), so are only described here briefly. The pre formed 2.2'azinobis-(3-ethylbenzothiazolina-6-sulfonic acid) ($ABTS^{++}$) is generated by oxidation of ABTS with potassium persulfate and is reduced in the presence of hydrogen donation antioxidants. Trolox, an α-tocopherol analogue, was used as a standard. After dilution and preparation, blood plasma was added to a clean glass cuvette, containing ABTS and placed in the spectrophotometer and a reading was taken after 60 seconds. The proportional reduction in absorbance at 734 nm was first calculated by comparing the reading from the ABTS dilution before and after the plasma sample was added. The Trolox equivalent of this reduction was then taken by comparing the value to a concentration response curve, this provides a index of the TAC of the blood plasma.

Escape flight performance

Vertical take-off is the most strenuous part of flight, so escape time is a good measure of physiological condition (Veasey et al., 2001). The methods used are fully described elsewhere (Veasey et al., 2001); Larcombe et al. In prep), so are only described here briefly: For each flight, one bird was placed in a wooden holding box at the base of the flight chamber. When a string was pulled the sides of the wooden box collapsed outwards startling the bird and stimulating it to fly straight up to a perch 2m high. The time taken to fly between markers at 20cm and 80 cm above the base of the flight chamber was recorded using a video camera. By watching videos back frame-by-frame, we could calculate escape time over this 60 cm distance.

Two weeks before the first recorded flight, birds were acclimated to and trained to use the equipment. During training, birds were flown from the holding box individually at least six times over the course of a day, or until no further improvement in escape time could be detected.

During the experimental phase, all birds were flown six times (using procedures outlined above) on one day per week for a total of eight consecutive weeks. One flight trial was during the baseline diet phase and the other seven during the experimental diet phase. In the morning, each bird was first weighed, then performed two flights with a 5-10 minute break in between, and was then returned to its home cage. The mean of these two flights was termed the ‘rested escape time’. Approximately, four hours later, the bird did four

consecutive flights in quick succession without a break. The mean of these four afternoon flights was designated the 'stamina escape time' as they were done without a break so were more tiring for the birds than the rested escape flights. Only flights in which the bird flew straight up to the perch unimpeded were included in the analyses.

Plumage colouration

Next, we tested whether dietary-derived antioxidants had indirect effects on plumage colouration, for example by protecting plumage pigments from oxidation. However, it should be noted that this experiment was probably not long enough for a full moult (Wyndham, 1981). Adult budgerigars moult over the course of the year, so feather growth is not focussed into a short period as in many temperate zone species. After the birds had been on the diets for 3 months, we measured the reflectance of the crown and cheek patches between 300nm to 700nm using a spectrophotometer (Oceanoptics S2000). Reflectance data from plumage patches with pin feathers were excluded. Using the SPEC package (http://www.bio.ic.ac.uk/research/iowens/spec), and available data on the spectral sensitivities of the four single cones in a budgerigar's retina (Bowmaker et al., 1997), we analysed the spectral data. The SPEC program multiplies cone sensitivities by the reflectance spectrum from the plumage patch (Hadfield and Owens, 2006). This is done for every wavelength to which the cones are sensitive, and these values are then summed for each cone type, to give four quantal cone catches, denoted UVS (Ultraviolet sensitive), SWS (Short wavelength sensitive), MWS (Medium wavelength sensitive) and LWS (Long wavelength sensitive) (Vorobyev, 2003). A mean quantal cone catch was calculated from the two readings taken for each plumage area.

As this project was investigating the component of plumage colouration specifically involved in mate choice, we focussed on the fluorescent component of the crown and the UV and blue components of the cheek patches (Arnold et al., 2002; Pearn et al., 2001). The fluorescent yellow of the budgerigar's crown differs from 'normal' yellow plumage in several areas of the spectrum: First, in the region of the spectrum that coincides with the MWS cones compared with the SWS and the LWS compared to the MWS cones (Arnold et al., 2002). Second, fluorescent yellow lacks a UV component compared to, for example, a carotenoid-mediated yellow (Bleiweiss, 2005). This is because fluorescence occurs when UV wavelengths are absorbed and re-emitted at higher wavelengths, e.g. yellow. For the blue cheek patches, we analysed the relative UVS chromatic signal and the SWS (blue)

chromatic signal. Analyses of chromatic cues permit the distinction of stimuli of different spectral composition regardless of intensity, typically achieved by chromatic opponency of signals from photoreceptors (Osorio et al., 1999). We calculated chromatic signals with the following formulae (Osorio et al., 1999): 1) Crown chromatic signals: a) MWS chromatic signal = (MWS - SWS) / (MWS + SWS); b) LWS chromatic signal = (LWS - MWS) / (LWS + MWS); c) UVS chromatic signal = (UVS – SWS) / (UVS + SWS); 2) Cheek patch chromatic signals a) UVS chromatic signal = (UVS - SWS/(UVS + SWS); b) SWS chromatic signal = (SWS – MWS) / (SWS + MWS).

Mate choice trials

Female preferences for males kept on RQ and EQ diets were tested using standard mate choice experiments (Arnold et al., 2002). In month 4 of the project, a two-way choice apparatus was used (see Jones et al., (2002) for the choice chamber layout), which consisted of a central cage into which the test female was placed, and two stimulus cages located behind the central cage into which we placed the stimulus males. Lighting of the stimulus and central cages was provided by a mixture of 60cm 18W ‘black’ lights (providing mainly UV wavelengths) and 120cm 62W ‘daylight’ lamps (mainly human visible wavelengths). The stimulus cages were separated from the central cage by wire mesh, and the two stimulus cages were separated by wooden panels, sothe males were prevented from seeing each other. A few days prior to the experiment, small groups of birds were allowed to explore the choice cage for two hours.

Each choice trial consisted of two 90 minute observation periods between which the males swapped sides to control for side-bias. The experimental female was placed in the choice chamber the afternoon before the choice trial in order to acclimate fully to the set-up. One male bird from each of the treatment groups, matched for mass, was added to each stimulus chamber the following morning within two hours of dawn. A recording of the sound in the home cage room was played back during the trials to settle the birds. A successful trial was when a female moved into both sides of the choice cage and had observed both males. In total seventeen trials were conducted during January and early February 2006, with seven trials being discarded because the female stayed in one side of cage throughout the trial. Of the ten successful females, half were from the RQ group and the rest from the EQ group. From videos of the trials, preference was determined as the mean time spent with the each of the males across the two phases of the trial.

Statistics

Data were analysed using General Linear Models (GLMs) in SPSS. The change in escape time over the 8 week flight testing phase was analysed using a repeated measures GLM. The values for retinol and α-tocopherol could not be transformed to meet the assumptions of the repeated measures GLM, so Mann-Whitney U-tests were applied to the change in the concentrations of all antioxidants (concentration after 7 weeks on experimental diets minus concentration during the baseline phase). Paired-T-tests were used to analyse the mate choice data. Proportional data, for example the TAC of plasma, were transformed to fit assumptions of models. The mass of individuals did not change significantly over the course of the experiment (repeated measures GLMs within subjects effects $p > 0.2$). Body mass was also unaffected by diet treatment and sex (repeated measures GLMs between subjects factors $p > 0.3$ in all cases), so could be excluded from subsequent statistical models. Means ± standard errors (S.E.) are presented. Sample sizes vary between analyses due to missing data or failed flights.

Results

Blood plasma antioxidant levels

Blood plasma concentrations of lutein increased significantly from the baseline measurement to seven weeks after the start of the diet manipulation (repeated measures GLM; within subjects factor $F = 12.970$, $df = 7, 30$, $p = 0.01$), but this increase did not differ between the treatments (between subjects effects $F = 0.665$, $df = 1, 37$, $p > 0.4$). Zeaxanthin levels tended to increase only in the EQ group (repeated measures GLM; between subjects effects $F = 4.012$, $df = 1, 27$, $p = 0.055$; Figure 1a). The increases in the plasma concentrations of retinol (Figure 1b) and α-tocopherol (Figure 1c) were significantly higher in birds on the EQ than the RQ diet. Sex and body mass were non-significant in all cases. After 7 weeks on the experimental diets, the plasma concentrations of all antioxidants were correlated with each other (Spearman's correlation $p < 0.001$ in all cases). So, an individual with high plasma retinol concentrations also had high levels of lutein, zeaxanthin and α-tocopherol

Total antioxidant activity of blood plasma

After two months on the experimental diets, there was a tendency for males on the RQ diet to have lower TAC than all other birds (GLM $F = 3.41$, $df = 3, 31$, $p = 0.074$). Overall, males (mean = 19.36 % ± 2.28) had significantly lower plasma TAC than females (mean = 24.89 % ± 1.39; $F = 4.54$, $df = 1, 34$, $p = 0.041$). The total antioxidant capacity of the blood plasma was significantly negatively correlated with plasma levels of lutein (Spearman's rho = -0.360, $N = 35$, $p = 0.021$) and zeaxanthin (Spearman's rho = -0.512, $N = 35$, $p = 0.001$). TAC was uncorrelated with α-tocopherol or retinol concentrations ($p > 0.1$ in both cases).

Escape flight performance

Prior to the commencement of the experimental diets in November 2005, neither stamina escape time nor rested escape time with diet treatment, sex or wing-loading (GLM $p > 0.2$).

First, we investigated whether antioxidant supplementation affected the rested escape time (mean of two flights with a 10 minute break between). Training improved escape time over

8 weeks (repeated measures GLM within subjects effects $F = 2.64$, $df = 7$, $p = 0.014$) but this did not vary with sex ($p > 0.8$) or diet ($p > 0.4$), just wing-loading ($F = 7.62$, $df = 1,23$, $p = 0.011$). Birds with a lower wingloading were faster and showed a greater improvement in rested escape time than those with a higher wingloading.

Next, we determined whether our diet treatment altered stamina escape time (mean of four consecutive take-off flights without a rest period). Stamina escape time declined over 8 weeks (repeated measures GLM within subjects effects $F = 2.58$, $df = 7$, $p = 0.017$). Birds with a lower wingloading showed a greater improvement in escape time than those that were heavier for their wing size in week 8 ($F = 4.68$, $df = 4,15$, $p = 0.046$). In addition, the interaction between sex and diet significantly affected the change in stamina escape time over the 8 week flight trial (repeated measures GLM sex x diet $F = 4.72$, $df = 1, 19$, $p = 0.045$; sex NS; diet NS; Figure 2). Males on the EQ diet (Figure 2a) showed a significantly greater improvement in stamina escape time than all other birds (Figure 2b).

No aspects of flight performance were correlated with blood plasma concentrations of any antioxidants ($p > 0.2$ in all cases). The TAC of blood plasma in the final flight trial was not correlated with rested escape time (Spearman's rho = - 0.275, $N = 35$, $p > 0.1$) but was marginally negatively correlated with stamina escape time (Spearman's rho = - 0.323, $N = 35$, $p = 0.059$).

Plumage colouration

From the fluorescent crown, the LWS chromatic signal (GLM $F = 0.993$, $df = 3, 19$, $p > 0.3$), MWS chromatic signal (GLM $F = 1.591$, $df = 3, 19$, $p > 0.2$) and the UVS chromatic signal (GLM $F = 2.288$, $df = 3, 19$, $p > 0.1$) did not differ with sex, diet or the interaction between them. Moreover, the UV chromatic signal (GLM $F = 0.438$, $df = 3, 19$, $p > 0.5$) from the cheek patches did not vary with the interaction between diet and sex. Males did have a greater UV chromatic signal (mean = 0.57, ± 0.018) than females (mean = 0.41, ± 0.040; GLM $F = 15.97$, $df = 3, 19$, $p = 0.003$). Finally, the SWS chromatic signal from the cheek patches did not vary with diet, sex or the interaction between them (GLM $F = 0.384$, $df = 3, 13$, $p > 0.5$).

Mate choice

During the mate choice trials, females showed a strong preference for males on the enhanced antioxidant diet, spending significantly longer with EQ than RQ males (Paired T-test, $t = 2.80$, $N = 10$, $p = 0.021$; Figure 3a). Within these pairs of males matched for size, the EQ males had significantly faster stamina escape time in week 8 than RQ birds (Paired T-test $t = 2.86$, $N = 10$, $p = 0.019$). The difference between the time spent with the EQ male and the RQ male was negatively correlated with the difference between the stamina escape time of the EQ and RQ males (Spearman's rho = -0.706, $N = 10$, $p = 0.022$; Figure 3b). Females showed a stronger preference for EQ males that had a faster mean stamina escape time in week 8, than EQ males that were relatively slow compared to their RQ mate choice rival. There were no relationships between mate choice preference and mean rested escape time at the end of the flight period ($p > 0.1$) or the differences in plasma concentrations of lutein, zeaxanthin, retinol or α tocopherol ($p > 0.1$ in all cases) between EQ and RQ birds.

Discussion

Manipulating the supply of antioxidants to wildtype adult budgerigars altered blood plasma levels of antioxidants in both sexes and in males influenced TAC, escape time and sexual attractiveness. Our results demonstrate that dietary antioxidants have the potential to mediate a number of avian life history traits. Although there were no sex differences in plasma antioxidant levels, as seen in other species (McGraw and Nogare, 2004), there were differential consequences of supplementation for males and females. As predicted, the raised antioxidant intake of EQ birds (10% inclusion of Nutrivit ®) did result in a significant improvement in the escape time but only in the most strenuous test, i.e. the mean stamina escape time, and only in males. No such improvement was seen in EQ females or birds receiving the RQ diet (1% inclusion of Nutrivit ®). Rapid acceleration is an important component of prey escape in many bird species and the most energetically expensive part of flight (Veasey et al., 1998). Assessment of escape time is therefore an evolutionarily relevant measure of a bird's condition. Our results are in line with the one other study investigating the effect of antioxidants on avian flight performance, in this case in male zebra finches *Taeniopygia guttata* (Blount and Matheson, 2006). However, the evidence from other taxa that antioxidants improve exercise performance or recovery, is so far inconclusive. It has been suggested the disparity in the literature is due to differences in exercise level (e.g. mild versus strenuous or long distance running versus sprinting), the dosage level and duration of supplementation and type of oxidative stress index used (reviewed in Urso and Clarkson, 2003). Studies that report positive effects of supplementation tend to examine more strenuous exercise, and supplement antioxidants for larger periods compared to those studies which do not (Urso and Clarkson, 2003; Vollaard et al., 2005). Indeed, here we discovered effects of diet on the most strenuous flight test, but not on rested escape time. Also, in a similar experiment with just four weeks of supplementation and a less demanding exercise challenge, we found no effect of antioxidants on flight performance, but higher levels of oxidative stress in the RQ versus EQ birds (Larcombe et al., 2007). Our results suggest that escape flight is strenuous, potentially resulting in muscle damage and increased ROS production. This damage, and resultant increase in escape time, might be tempered by the actions of dietary antioxidants, but only if they are provided over several months.

In our mate choice experiment, female budgerigars significantly preferred to associate with EQ than RQ males. So, which phenotypic traits might have been affected by nutritional

status of the males? First, we investigated the chromatic signals from plumage regions known to be associated with mate choice in the budgerigar (Arnold et al., 2002; Pearn et al., 2001). No evidence was found that diet or the interaction between diet and sex affected chromatic signals from the crown or cheek patches. It should be noted that budgerigars undergo moult throughout the year (Wyndham, 1981). So, over the course of the trial the birds were unlikely to have re-grown enough of their feathers to significantly influence the colouration of plumage ornaments. Most studies investigating dietary influences on avian ornaments have focussed on carotenoids (Blount et al., 2003b; Hadfield and Owens, 2006; McGraw, 2005; Olson and Owens, 1999). As the red and yellow colour of parrot feathers are due to psittacofulvins rather than carotenoids (McGraw et al., 2005; Stradi et al., 2001), any effects of dietary-derived antioxidants on feather colouration would have had to have been indirect, for example via the protection of pigments in the feather follicle from oxidation (Arnold and Larcombe Unpublished data). In the one other study looking at the honesty of plumage signals in parrots, the size of the red abdominal plumage patch of male burrowing parrots *Cyanoliseus patagonus* was correlated with their paternal care abilities (Masello et al., 2004). It still remains to be tested experimentally whether Psittaciform plumage colouration is an honest signal of antioxidant status and individual quality.

Next, we investigated whether an individual's capacity for physical exercise, which in males was modulated by diet, could be judged by potential mates. We discovered that the faster an EQ male flew in the final flight take-off performance trial compared with the RQ male, the more the female preferred him in the mate choice arena. Thus, exercise capacity seems to be an honest and assessable signal of quality in male budgerigars. In a monogamous species with biparental care, such as budgerigars, the physical condition of males will be of paramount importance to females looking for mates. Courtship displays and other physically tiring behaviours are often the basis of mate choice. However, for a long lived species with opportunistic breeding, continuous assessments of flight performance and other active behaviours are likely to provide females with more information about male quality than simply ornament size and/or courtship display rate.

Providing the nutritional supplement at the 10% level resulted in significant rises in retinol and tocopherol concentrations and a marginal increase in circulating concentrations of zeaxanthin. There were no such effects in birds receiving Nutrivit ® at the 1% level. In contrast to sexually dichromatic passerines (Badyaev et al., 2002; Saks et al., 2003) and other parrot species (McGraw and Nogare, 2004), male budgerigars did not have higher

circulating levels of carotenoids or indeed retinol or α-tocopherol, compared to females, irrespective of diet. However, we suggest that the positive effects of our EQ diet on male flight performance and attractiveness did originate from the actions of dietary-derived antioxidants. It is highly likely that males and females differ in the way that they physiologically utilize dietary antioxidants (Blount et al., 2003a, 2006; McGraw et al., 2002), even in a sexually monomorphic species. This is supported by the sex differences in TAC we observed.

Lutein plasma concentrations were not changed in the EQ group compared with the RQ group, probably because birds in both treatment groups acquired abundant lutein from seed. Interestingly, despite discovering that our EQ diet manipulation increased circulating levels of retinol and α-tocopherol and resulted in a tendency for higher plasma TAC in EQ males, there was a negative relationship between TAC and carotenoid levels. This unexpected result has been found previously in a lutein supplementation experiment in laying chickens (*Gallus domesticus*), but remains unexplained (Cohen et al., 2007). Most of the sexual selection literature asserts that in birds, carotenoid-mediated ornament colouration is an honest signal of individual quality, reflecting antioxidant status (Blount et al., 2003b; McGraw, 2005; Schantz et al., 1999). If the positive effects of carotenoids are due to their antioxidant properties as proposed, a positive correlation between carotenoid concentrations and TAC should have been found here. Lutein, the dietary supplement most often used in sexual selection studies, is one of the more abundant carotenoids available to birds but is now considered to be a weak antioxidant (Hartley and Kennedy, 2004). Also, carotenoids, when oxidised repeatedly, form toxic end products, which attack cells (Hartley and Kennedy, 2004). Moreover, oxidised carotenoids cannot be recycled, unlike α-tocopherol and retinol (Surai, 2002). Thus, carotenoids may not be important biological antioxidants for birds and their levels in the blood not indicative of TAC or individual quality. Alternatively, but not exclusively so, under certain conditions carotenoids have been shown to have pro-oxidant properties, which could diminish the TAC of blood plasma. Parrots might be particularly vulnerable to the pro-oxidant effects of circulating carotenoids because they do not use carotenoids in plumage pigmentation (Stradi et al., 2001), so are unable to 'dump' excess carotenoids in a biologically inert form. Further studies are needed to determine whether carotenoids can have pro-oxidant actions *in vivo* at the levels commonly experienced in ecological studies.

Experimentally manipulating the levels of antioxidants in the diets of adult budgerigars over the several months significantly altered circulating levels of physiologically important nutrients. These in turn impacted upon the total antioxidant capacity of blood plasma, flight performance and sexual attractiveness in males. The mechanism by which male attractiveness was influenced by diet, was not a result of plumage colouration, but seemed to involve an individual's capacity for physical activity. Thus, escape time and other indices of exercise capabilities are potentially honest signals of individual quality. Dietary-derived antioxidants mediate a range of life history traits and their multifaceted roles in underpinning physiological trade-offs deserve further exploration beyond the current focus on ornament colouration.

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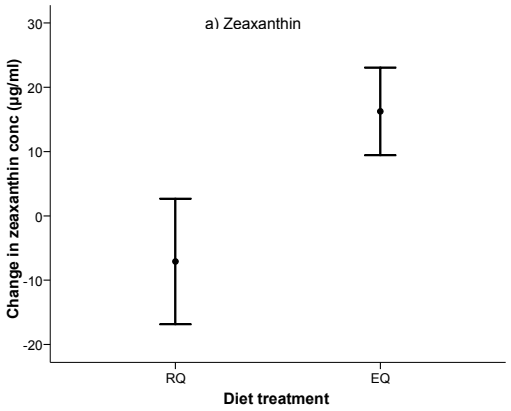
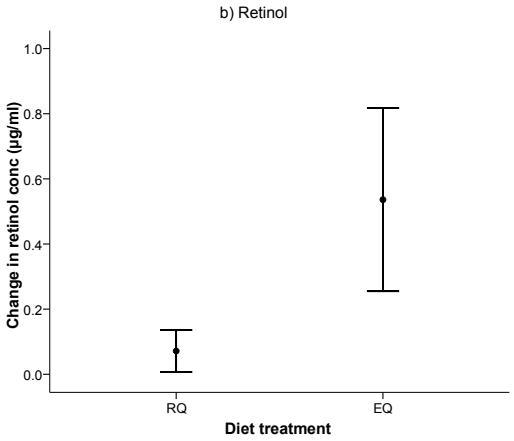
Figure 1 Changes in blood plasma concentrations of antioxidant concentrations after 7 weeks of either an enhanced (EQ) or reduced quality (RQ) diet. Levels of the following antioxidants significantly increased from baseline in the EQ (N = 14) group and either decreased or did not change in the RQ group (N = 15): a) Zeaxanthin ($z = -2.575$, $p = 0.010$); b) Retinol ($z = -2.859$, $p = 0.004$); and c) α-tocopherol ($z = -2.227$, $p = 0.026$). Statistics given are for the Mann-Whitney U-test. Note the different scales on each of the figures. Means ± S.E. shown.

Figure 2 Mean stamina escape time (mean of four take-off flights in quick succession) by adult budgerigars on an enhanced (EQ) or reduced (RQ) quality diet. a) Males on an EQ diet showed a significant improvement in stamina escape time over the 8 weeks. b) Females' change in stamina escape time did not differ between treatments. Means ± S.E. shown.

Figure 3 Female mate choice for males: a) Mean time spent by females with males on enhanced quality (EQ) diet was significantly longer than that with males on the reduced quality (RQ) diet during a three hour mate choice trial. b) The difference in the time spent by the female with the EQ and RQ males during the mate choice trials (Time spent with EQ male minus time spent with RQ male) was negatively correlated with the relative difference in stamina escape time of the males in the final flight trial (stamina escape time of EQ male minus stamina escape time of RQ male).

Figure 1


a) Zeaxanthin
Change in zeaxanthin conc (µg/ml)
30
20
10
0
-10
-20
RQ
EQ
Diet treatment


b) Retinol
Change in retinol conc (µg/ml)
1.0
0.8
0.6
0.4
0.2
0.0
RQ
EQ
Diet treatment

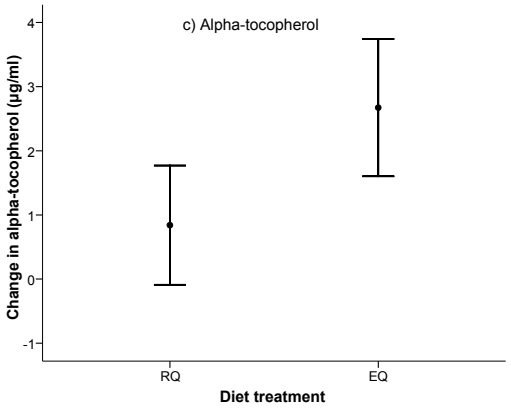

c) Alpha-tocopherol
Change in alpha-tocopherol (µg/ml)
4
3
2
1
0
-1
RQ
EQ
Diet treatment

Figure 2

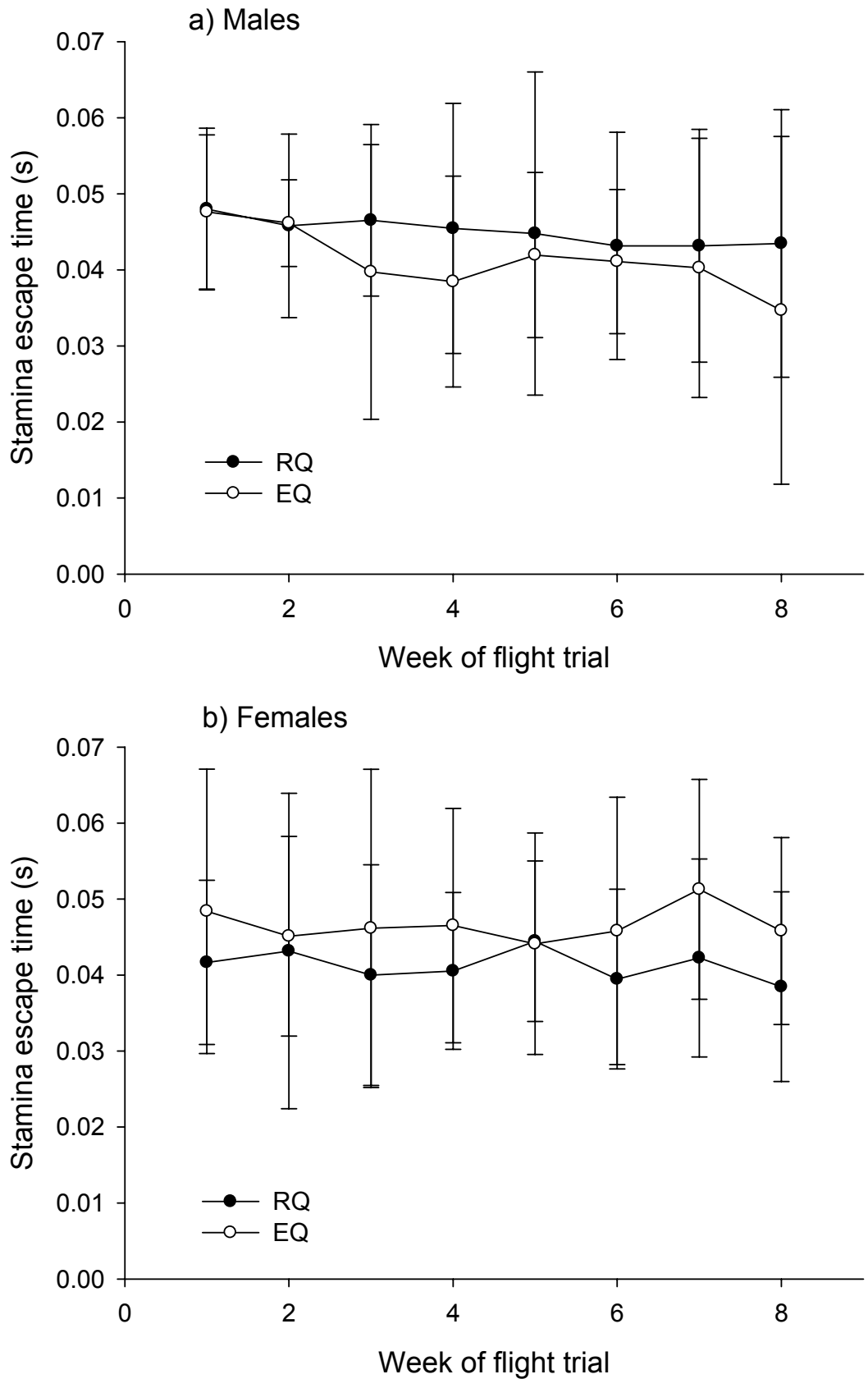
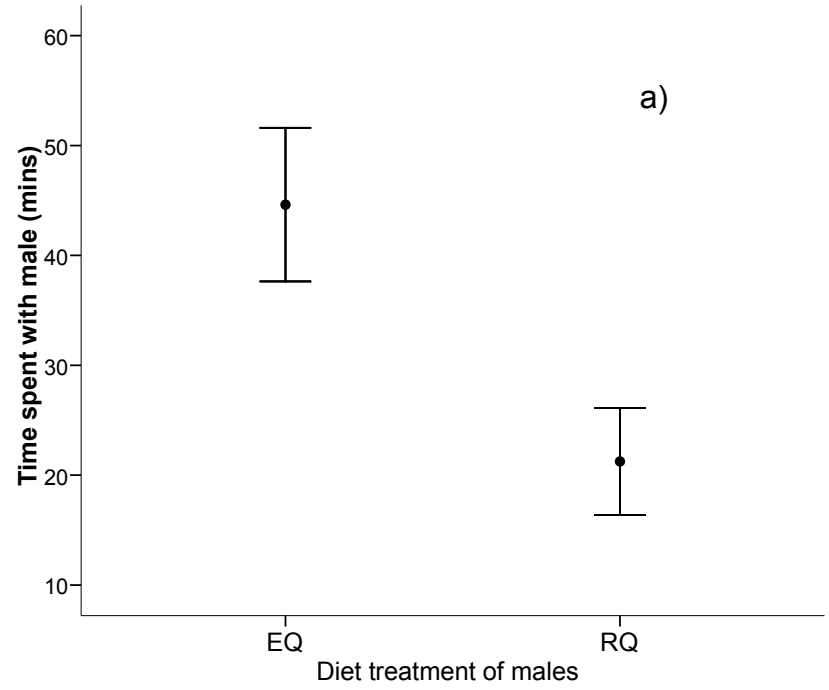
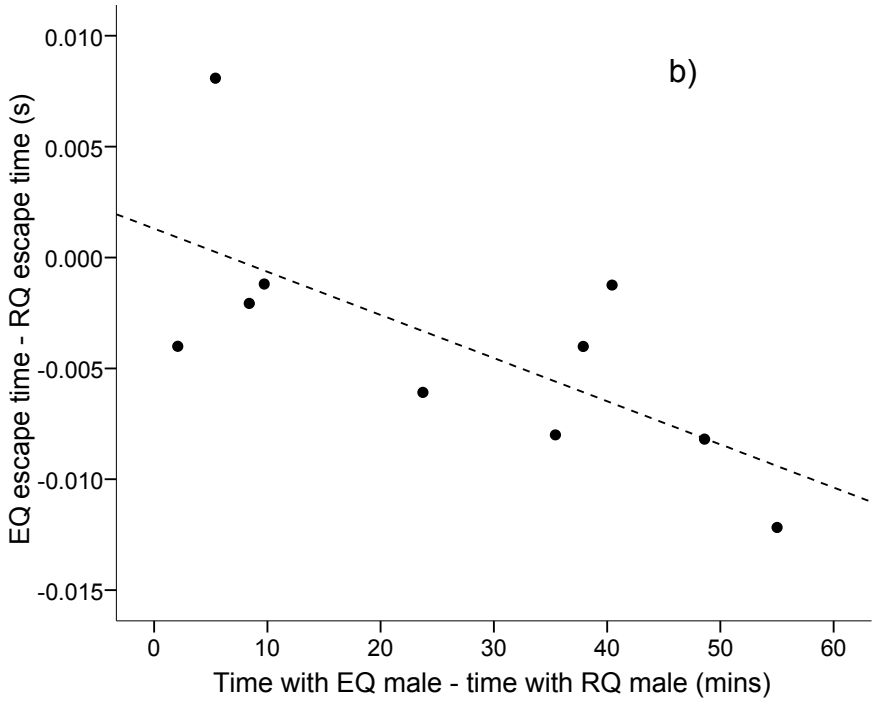

a) Males
Stamina escape time (s)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
RQ
EQ
0
2
4
6
8
Week of flight trial
b) Females
Stamina escape time (s)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
RQ
EQ
0
2
4
6
8
Week of flight trial

Figure 3


a)
Time spent with male (mins)
60
50
40
30
20
10
EQ
RQ
Diet treatment of males


b)
EQ escape time - RQ escape time (s)
0.010
0.005
0.000
-0.005
-0.010
-0.015
0
10
20
30
40
50
60
Time with EQ male - time with RQ male (mins)