The effects of over-winter dietary provisioning on health and productivity of garden birds

Submitted by

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

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ABSTRACT

Food supply plays a crucial role in regulating bird populations. For many small passerines, both in the UK and globally, winter food availability is substantially increased through the provision of supplementary food. Garden bird feeding is a popular and growing phenomenon. Yet there remains a distinct lack of understanding of the ecological impacts this may be having on wild bird populations. Using a three year landscape-scale study I have investigated the carry-over effects of winter supplementary feeding on the health and productivity of resident blue tit populations (*Cyanistes caeruleus*) during the breeding season. Replicating the diffuse nature by which food is provisioned in gardens, I have examined the importance of energy (fat) and antioxidants (vitamin E) as carry-over effect mediators. Females showed greater resource allocation through a proportional increase in yolk mass, whilst males exhibited an improved oxidative status during the brood-rearing period in response to vitamin E provisioning. But in addition, significantly lower feather carotenoid concentrations were seen in individuals from vitamin E fed woodlands, suggesting that birds of poorer condition prior to feeding were able to survive winter and recruit into breeding populations as a result of antioxidant provisioning. This indicates that winter supplementary feeding has the capacity to perturb natural selection and alter the phenotypic quality of breeding populations. Furthermore, over-winter provisioning led to a reduction in fledging success across both treatments, which suggests that it may give birds false cues as to natural food availability and encourage them to make an unsustainable investment in nestling numbers, thereby acting as an ecological trap. With garden bird feeding promoted as a method for conserving declining wild bird populations, these new insights suggest much more needs to be done to fully understand its impacts.

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AUTHOR'S DECLARATION

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Appendix 1: Habitat surveying conducted by S. Vandome.

CHAPTER 1

General introduction

1.1 FOOD AS A LIMITED RESOURCE: AN OVERVIEW

Food supply plays a crucial role in avian ecology; directing community structures, regulating population sizes and influencing individual behaviour and life-history traits (Newton 1998). Justifiably, therefore, much time has been invested into understanding the survival and fitness consequences of limited food availability (Lack 1954; Newton 1980; Martin 1987; Robb et al. 2008a). For birds in temperate climates, access to sufficient dietary resources is likely to vary seasonally, leading Lack (1954) to hypothesise that populations would be regulated by food availability during the period of least abundance; the 'winter food limitation hypothesis'. Indeed, subsequent studies testing Lack's theory found food supply and resource competition to be key determinants of over-winter survival (Jansson et al. 1981; Brittingham & Temple 1988); and further correlations between winter food availability and subsequent breeding densities have also been made (Krebs 1971; Kallander 1981).

To optimise fitness, animals cannot invest solely in survival. In the face of limited resources, life-history theory predicts that trade-offs should exist in their allocation to competing biological functions (Stearns 1989). A classic example of this is the investment conflict between self-maintenance and reproduction. A wealth of literature has addressed the consequences of food resource variability on avian breeding performance (Martin 1987; Jonsson 1997; Christians 2002; Nager 2006; Robb et al. 2008a). However, previous studies detailing the effects of food supply and nutritional value have predominantly focused on limitations during the breeding season. In a seasonal environment, decisions made during one season may impact upon events in a later time or place (Fretwell 1972). Therefore, an individual's previous ability to resolve trade-offs can be 'carried-over' to affect future fitness and reproductive investment (Harrison et al. 2011). Whilst this has been recognised in a number of studies of migratory species, few have tested the effects of winter food availability on

reproduction in resident populations. Furthermore, the potential mechanisms by which carry-over effects might be derived have not been tested.

This thesis examines the carry-over effects of over-winter supplementary feeding, using populations of a resident UK bird species, the blue tit (*Cyanistes caeruleus*). In the following pages I expand upon the relevance of this investigation within the context of garden bird feeding; provide background into the candidate mechanisms which may be operating carry-over effects, namely energy and/or antioxidant supply; and explore the impacts that these might have during the subsequent breeding season. I conclude with an outline of the thesis aims and structure.

1.2 SUPPLEMENTARY FEEDING IN GARDENS

Provisioning of garden bird food is a popular, and growing phenomenon across the Western World (Jones & Reynolds 2008). Together, the UK and US purchase in excess of 500,000 tonnes of commercial bird food each year (O'Leary & Jones 2006), and the global market for bird seed is currently growing at an estimated 4% per annum (Lin 2005). In the UK alone, it is estimated that almost half of all householders provision bird food (Davies et al. 2009), spending *ca.* £200 million annually on peanuts, seeds and fat products (British Trust for Ornithology [BTO] 2006; Glue 2006). For species which utilise supplementary food, this equates to approximately one feeder for every nine individual birds, and enough to support 30 million great tits (*Parus major*) without the need to feed on natural resources (Robb et al. 2008b; Fuller et al. in press). When accounting for the provision of home-made foods and household scraps, the scale at which garden bird feeding is occurring will undoubtedly become much greater.

Gardens are seen to support a considerable number of wild bird species within the UK (Gregory & Baillie 1998; Mason 2000; Bland et al. 2004). As urban land cover expands to accommodate a growing human population (United Nations 2010), gardens are expected to play an increasingly important role in conserving biodiversity (Chamberlain et al. 2004). With populations of many familiar garden bird species declining (Baillie et al. 2010), the provision of supplementary food has been actively encouraged, and the current recommendation is to provision food throughout the year (e.g. British Trust for

Ornithology [BTO] 2009; The Royal Society for the Protection of Birds [RSPB] 2009). However, this is based on very little empirical evidence of the potential ecological impacts (both positive and negative) this could have on wild bird populations (Jones & Reynolds 2008; Robb et al. 2008a; Jones 2011).

1.2.1 Supplementary feeding in gardens: impacts on avian ecology

It is apparent that supplementary food provides an enormous resource with potentially far-reaching consequences for avian ecology (reviewed in Robb et al. 2008a). For example, analyses of large, long-term datasets infer connections between food provisioning in British gardens and changes to avian species abundance and breeding population sizes (Chamberlain et al. 2005; Fuller et al. 2008). However, although promoted as a method of conservation, concern has also been raised about the possible implications of garden bird feeding. For instance, potential opposing effects of supplementation might include increased disease transmission, encouragement of feeder dependency, creation of 'ecological traps' (see below), provision of foods of poor nutritional value, the competitive exclusion of valued species and a change to population structure, thereby influencing evolutionary trajectories and quenching natural selection (Fuller et al. 2008; Jones & Reynolds 2008; Jones 2011).

1.2.2 Supplementary feeding in gardens: provisioning during winter

Supplementary feeding occurs in greatest abundance during the winter months (Chamberlain et al. 2005; Jones & Reynolds 2008). Furthermore, many garden bird species utilise gardens to a much greater extent at this time, gravitating towards feeders when temperatures are low (Cannon et al. 2005; Chamberlain et al. 2005). In winter small passerine birds are faced with increased energetic stress, as natural food availability is low relative to demand (Gosler 1996). Falling temperatures and reduced days lengths are likely to make it increasingly difficult for individuals to find food, and consequently many will perish before the start of spring. It is expected that the provisioning of supplementary food will alleviate foraging costs and compensate for a lack of natural foods. Indeed, supplementary feeding has been shown to improve survival during adverse winter months (Jansson et al. 1981; Kallander 1981; Brittingham & Temple 1988); and facilitate feather growth, an indication of improved

nutritional condition (Grubb & Cimprich 1990). Moreover, over-winter provisioning has the potential to influence future breeding success, as is explained in further detail below (Robb et al. 2008b). Nonetheless, it is worth noting that population responses to supplementary feeding during winter may vary.

1.2.3 Supplementary feeding in gardens: is natural food always limiting?

When studying the effects of supplementary feeding on great tit populations during two consecutive winters, Kallander (1981) observed that when weather conditions were mild and beech mast was abundant, the addition of supplementary food had no effect. However during the preceding severe winter, supplementary feeding had increased adult and juvenile survival 2-fold. Annual variation in response to supplementary feeding is often reported during the breeding season also (Arnold et al. 1991; Norris 1993; Nager et al. 1997). It is therefore acknowledged that natural food supply might not always be limiting. However, even if provisioning supplementary food only has an effect every few years, this may still have a substantial impact on avian communities (Robb et al. 2008a). This highlights a need to consider the effects of garden bird feeding over multiple years, and under varying conditions of natural food abundance, in order to have a clearer understanding of its potential impacts.

1.3 CARRY-OVER EFFECTS OF WINTER FOOD AVAILABILITY

It has been recognised that at any point in time, variation in resource availability, and investment decisions concerning their allocation, can have downstream fitness implications (Stearns 1989; Metcalfe & Monaghan 2001). Many of these consequences may unfold across an individual's lifetime, for example early nutrition and growth conditions have been shown to influence future adult fitness traits (Blount et al. 2003; Biard et al. 2009). However, downstream effects may equally be revealed across a much shorter timeframe, for example amongst seasons. Fretwell (1972) first theorised that the consequences of decisions made in one season may impact upon events occurring at a subsequent time or place. Inter-seasonal links such as these are generally referred to as carry-over effects; and have been explicitly defined as: *'events and processes occurring in one season that result in individuals making the transition*

between seasons in different states (levels of condition) consequently affecting individual performance in a subsequent period' (Harrison et al. 2011).

Carry-over effects are expected to be an abundant and varied phenomenon. Although empirical evidence is still relatively lacking across all taxa, they have commonly been described in migratory bird species (reviewed in Harrison et al. 2011). Notably, several studies have reported seasonal interactions between ecological conditions experienced in winter, and subsequent body condition or productivity on breeding grounds. For example, Marra et al. (1998) showed that American redstarts (*Setophaga ruticilla*) wintering in high quality mangrove habitats benefited from improved body condition and earlier arrival at breeding grounds, compared to individuals using poor quality scrub habitats in winter. Whilst Bearhop et al. (2005) found that European blackcaps (*Sylvia atricapilla*) wintering in the north arrived earlier, produced larger clutches and fledged more young than birds from southerly wintering grounds; stipulating that benefits may have arisen from improved feeding opportunities.

Given the building evidence of seasonal interactions across migratory species, and that resident birds similarly experience seasonal variation within temperate regions; it seems intuitive that over-winter supplementary feeding might also produce carry-over effects. Therefore, in addition to influencing survival and body condition (Jansson et al. 1981; Grubb & Cimprich 1990), it is anticipated that the effects of winter feeding might carry-over to affect health and productivity during the breeding season. Indeed, in a recent experimental study, carry-over effects of over-winter food supplementation have been demonstrated for the first time. Despite feeding ceasing six weeks prior to breeding, Robb et al. (2008b) found that within woodlands provisioned with peanuts over-winter, blue tits had an advanced laying date and increased fledging success compared with birds within un-fed sites.

1.3.1 Carry-over effects: the candidate mechanisms

It has been argued that carry-over effects might explain a significant amount of intrapopulation variance in fitness. However, few studies have explored such links (e.g. Marra et al. 1998; Bearhop et al. 2005; Robb et al. 2008b). Moreover the mechanisms by which carry-over effect operate remain unclear. Carry-over effects are expected to

occur if individuals make the transition between seasons in different states, either in terms of stored reserves or in terms of body condition (Harrison et al. 2011). Therefore, one might hypothesise that food sources provide either energy and/or nutritional benefits.

1.3.1.1 Carry-over effect mechanisms: energy benefits

It is widely recognised that macronutrients, and particularly energy supply, play a key role in determining over-winter survival (Koivula et al. 1995; Forsman & Monkkonen 2003). Furthermore, birds employing a capital breeding strategy ('capital breeders') utilise stored reserves built up in the months prior to the breeding season to fuel reproduction (Drent & Daan 1980). For example, Prop et al. (2003) have shown that in barnacle geese (*Branta leucopsis*), the probability of successful breeding is correlated with the amount of fat accumulated at migratory stop-over sites. Whilst Inger et al. (2008) have since shown that these fat reserves are a function of the quality of food availability at stop-over (staging) sites. As such, carry-over effects are often attributed to previous fat and energetic gains. Many small passerines, however, are considered to be 'income breeders', which cannot lay down sufficient endogenous reserves to meet daily requirements during breeding. Instead, such species are thought to adjust food intake concurrently to meet the energetic demands of reproduction (Drent & Daan 1980; Jonsson 1997). Therefore, condition-based benefits rather than capital gains are likely to be of primary importance. In this instance, the extra energy provided by winter dietary provisioning could enable birds to maintain a higher body mass, fuelling metabolism and reducing the need to catabolise stored nutrient reserves, and may also enhance their ability to forage for natural foods and to maintain health, thus enabling them to enter the breeding season in a superior body condition. In either case, the possible carry-over effects of winter energy supply have not previously been studied.

1.3.1.2 Carry-over effect mechanisms: antioxidant benefits

Certainly in income breeders, but equally in capital breeders, it seems unlikely that energy is the only currency driving carry-over effects (Harrison et al. 2011). Vitamin E and carotenoids, for example, can only be obtained through the diet and may be scarce within the natural environment (Goodwin 1984). As such, they are considered to be a limiting resource in avian ecology, and important mediators of life-history trade-offs

(Olson & Owens 1998; Catoni et al. 2008). It is hypothesised that antioxidants might also be a potentially important resource influencing the carry-over effects of winter food availability.

Vitamin E and carotenoids play a fundamental role in the antioxidant defence system, by limiting the propagation of harmful reactive oxygen species (ROS) and therefore reducing oxidative stress (Catoni et al. 2008; Monaghan et al. 2009). ROS, comprising a collection of pro-oxidant molecules, are the highly reactive by-products of normal metabolic activity (Finkel & Holbrook 2000). If ROS production exceeds an individual's capacity to neutralise them via the antioxidant system, oxidative stress will be incurred. As such, when accumulated, ROS can cause serious damage to protein, lipid and DNA macromolecules, eventually leading to physiological dysfunction, disease progression and aging processes (Finkel & Holbrook 2000; Halliwell & Gutteridge 2007). To provide protection, vitamin E functions as a chain-breaking antioxidant, neutralising ROS and reducing lipid peroxidation (Burton 1994). The term vitamin E describes a group of eight lipid-soluble tocopherols and tocotrienols; of which α-tocopherol is the most bioactive and ubiquitous in nature (Burton 1994; Brigelius-Flohe & Traber 1999), and is thought to play a valuable role in avian ecology (Costantini 2008). Carotenoids, on the other hand, are biologically active pigments primarily responsible for sexually-selected colouration, but which additionally function as antioxidants through the quenching of ROS (Sies & Stahl 1995). The importance of carotenoids as effective antioxidants is disputed in birds (Hartley & Kennedy 2004; Costantini & Møller 2008), but their capacity to recycle vitamin E still affords them a valuable position in avian antioxidant defence systems (Monaghan et al. 2009).

Antioxidants may induce carry-over effect via two pathways. Firstly, metabolic activity is heightened during harsh winter conditions, therefore increasing susceptibility to oxidative stress. Antioxidants acquired through the diet are expected to assist in combating a ROS build up (Alonso-Alvarez et al. 2004a; Larcombe et al. 2008), and therefore might enable birds to enter the breeding season in a superior condition and having suffered less debilitation due to parasites, diseases and oxidative damage during winter. Secondly, and alternatively, as lipophilic micronutrients of low weight and a high capacity for biological effects, there is potential for antioxidants to be stored at

important levels in lipid-rich tissues and called upon during periods of increased demand (Negro et al. 2001; Surai 2007; Metzger & Bairlein 2011). This is true of all species and therefore, in the case of micronutrients, the capital versus income species dichotomy may be irrelevant. Antioxidants acquired during winter could therefore be directly invested into reproductive activities during the breeding season; an intriguing possibility for species classically defined as income breeders such as tits.

1.3.2 Carry-over effects: individual variation and understanding negative effects

Variation in natural food availability, access to and competition at feeders and differences in individual foraging or assimilation efficiency may all lead to intra-population variation in the uptake of provisioned food. Therefore, in order to gain a better understanding of the effects of supplementary feeding across populations it is important to also consider inter-individual differences in supplement uptake. This could prove particularly beneficial when considering the potential negative impacts of winter feeding on breeding populations. For instance concern has been raised that winter feeders might act as an 'ecological trap', by encouraging birds to settle in areas which have insufficient natural food resources once supplementary food is removed (Jones & Reynolds 2008; Robb et al. 2008a). But equally, depressed breeding success might be observed at a population level if uptake of supplementary food enables relatively low quality birds to survive, recruit and breed but with lower success than other members of the population. These two effects could be teased apart by considering the variation in supplementary food use between different individuals within the population.

Stable isotope analysis has proven to be valuable tool for interpreting carry-over effects in birds at an individual level (e.g. Marra et al. 1998; Bearhop et al. 2004b; Norris et al. 2004; Bearhop et al. 2005). Stable isotope ratios expressed in body tissues reflect the diet of that individual at the time of tissue synthesis (Inger & Bearhop 2008). Therefore, by either using isotope ratios as a proxy for consumption of a particular food item, or assessing the relative proportions of different food sources, inferences can be made about the an individual's diet during tissue assimilation (e.g. Bearhop et al. 2004a; Davis et al. 2005; Robb et al. in press). Claws, for example, are metabolically inert and have a slow growth rate, and therefore may be used to infer diet up to 5 months prior to sampling (Bearhop et al. 2003; Inger & Bearhop 2008). This can provide a direct link

between the reliance of individual birds on supplemented food in the winter and their reproductive performance during the following spring.

1.4 THE EFFECTS OF FOOD AVAILABILITY ON AVIAN REPRODUCTION

Much of our understanding of the effects of variable food availability, and quality, on avian reproduction has been attributed to studies of supplementary feeding. For example, advancements in the timing of breeding; increases in clutch size and offspring numbers; and improvements in parental care and future survival have all been described in response to food provisioning (Martin 1987; Boutin 1990; Robb et al. 2008a). Furthermore, the importance of macronutrients and antioxidants as potentially limited resources has also been well documented. Although all of these studies have focused upon resource abundance at specific stages of the breeding cycle, they offer valuable insights into the potential carry-over effects winter feeding might also have on events during the breeding season. These are summarised below, with emphasis on breeding parameters investigated within this thesis.

1.4.1 The effects of food availability: the egg laying period

Decisions about when to breed can have profound fitness consequences in birds (Perrins 1965; Nilsson 2000; Naef-Daenzer et al. 2001). Early breeders are expected to benefit from greater prey abundance with which to raise young, improved fledging numbers and an increased probability of offspring recruitment (Verhulst & Tinbergen 1991; Noordwijk et al. 1995). An advancement in the start of laying is often one of the most commonly reported responses to supplementary feeding during the breeding season (e.g. Arcese & Smith 1985; Kallander & Karlsson 1993; Harrison et al. 2010). Furthermore, carry-over effects of winter habitat quality and dietary provisioning on the start of laying have also been reported (Marra et al. 1998; Norris et al. 2004; Robb et al. 2008b). It is hypothesised that egg formation may be constrained by resource availability (Perrins 1965). As such, supplementary food may enable females to reach thresholds necessary for breeding early.

Egg formation carries high costs, both in terms of energetic demands and nutrient requirements needed to produce a whole clutch (Perrins 1996; Nager 2006). As such, supplementary feeding during or immediately prior to laying has also been seen to influence egg number, size and composition to varying degrees (Christians 2002; Robb et al. 2008a). The egg's lipid-rich yolk provides the main source of energy for the developing embryo, and is provisioned with a cocktail of lipophilic antioxidants, and maternally-derived hormones and immunoglobulins used to optimise offspring phenotype (Blount et al. 2000; Gasparini et al. 2001; Groothuis et al. 2005). Supplementary provisioning of antioxidants at the time of egg formation has demonstrated that a female's ability to resolve physiological trade-offs in antioxidant allocation are improved through increased carotenoid and vitamin E availability (Blount et al. 2002; Royle et al. 2003; Biard et al. 2005; Scheideler et al. 2010). Increased antioxidant deposition in the yolk may consequently have a profound effect on offspring fitness; for example, increasing hatching success, improving nestling immunity, and influencing survival and sexual-signalling in adulthood (Royle et al. 2001; Saino et al. 2003; Biard et al. 2005; McGraw et al. 2005). The effects of antioxidant availability outside of the breeding season on egg yolk maternal effects have not been tested, but it is hypothesised that antioxidants acquired from winter provisioning may be used by females to maintain a superior body condition, thereby enabling them to resolve trade-offs more effectively during the breeding season. Or alternately, females might store these additional resources in subcutaneous fat for direct capital investment into eggs several weeks or months later (McGraw & Toomey 2010; Metzger & Bairlein 2011).

1.4.2 The effects of food availability: the brood-rearing period

For many bird species the brood-rearing phase represents a period of intense energetic demand (Bryant & Tatner 1991). Consequently, high costs to current condition and subsequent survival may be incurred as parental effort increases to match the needs of a growing brood (Nur 1984a; Hõrak 1995; Wiersma et al. 2004). However, the energetic, nutritional and temporal costs of brood-rearing may be reduced through food supplementation. Indeed, it has been found that increasing food availability during brood rearing can relieve the pressures of prey location and provide an energetic boost to improve the foraging efficiency of natural food items (Simons & Martin 1990; Grieco 2002; Markman et al. 2002). Parent birds provisioned with antioxidants specifically in

the early stages of the breeding season have also been found to benefit from improved antioxidant defences in addition to improved provisioning performance (Biard et al. 2005; Remeš et al. 2007). It might also be possible, therefore, that benefits to antioxidant capacity and a subsequent reduction in oxidative stress at this time may have a sustained influence on health and performance during the breeding season. Furthermore, the benefits to both egg composition and parental behaviour and condition arising through supplementary feeding may have a combined influence on nestling quality. For instance, nestlings of provisioned birds have been reported to have faster growth rates, demonstrate improved immunity and brighter feather colouration (Arcese & Smith 1988; Biard et al. 2005; McGraw et al. 2005; Berthouly et al. 2008).

1.4.3 The effects of food availability: fledging success

Nestling survival until fledging provides a definitive measure of reproductive output, bearing indication of the sustained investment made by two parents into the very last stages of the breeding season. Variation in natural food availability, within brood sibling competition, and parental quality and effort may all affect fledging success (Nur 1984b; Senar et al. 2002; Lambrechts et al. 2004), with supplementary feeding expected to enhance fledging success. For example, Robb et al. (2008a) found that 63.3% of 28 supplementary feeding studies reviewed reported benefits to fledging success. In addition, influences of winter habitat and dietary provisioning have been carried-over to impact upon fledging success (Norris et al. 2004; Robb et al. 2008b). However, negative implications of feeding on reproductive output have also been highlighted. Kallander and Karlsson,(1993) for example showed that European starlings (*Sturnus vulgaris*) encouraged to lay too early suffered reduced fledging success. Whilst Nilsson (1994) found that removal of supplemented food at the start of laying resulted in a greater number of broods being abandoned in blue tits, even though supplemented females had produced eggs of the same size and number as un-fed birds. Furthermore, Jansson et al. (1981), found that removal of additional food sources at the end of winter led to population densities greater than natural food availability could support. Findings such as this have raised concern that over-winter provisioning could generate an ecological trap, ultimately resulting in depressed breeding success (Jones & Reynolds 2008; Robb et al. 2008a).

1.5 SUMMARY OF THESIS AIMS AND METHODOLOGY

The primary aim of this thesis was to investigate the effects of over-winter supplementary feeding on health and productivity of garden birds during the breeding season. I have used the blue tit (*Cyanistes caeruleus*) as the focal species throughout (**Figure 1.1**).

Figure 1.1. A ringed blue tit visiting a custom-made fat ball feeder; K. Plummer, Feb 2010.

To date, much work has been conducted to assess the impacts of food availability, or supplementation of specific nutrients, on avian reproduction. However, whilst these studies are of considerable importance in broadening our understanding of resource-based limitations, the wider ecological impacts are often ignored. First and foremost, presentation of food items to specific individuals at the nest, or over restricted periods of the breeding cycle may result in misrepresentation of their true effects within a

natural context. Secondly, natural food availability might not always be limiting, and therefore birds are often seen to respond differently to supplementary food across spatial and temporal scales (Kallander 1981; Lambrechts et al. 2004). Failure to take these into consideration may therefore lead to inaccurate conclusions. Thirdly, it is apparent that reproduction may be limited by food supply not only at the time of breeding but also during the preceding winter. However, until now, the potential carry-over effects of winter food availability, and the mechanism by which these might influence breeding performance have rarely been considered in a resident bird species.

To address the main aim of this thesis and account for the issues raised above, I implemented a three year landscape-scale experimental study, investigating the carry-over effects of winter food availability on wild blue tit populations. With supplementary feeding of garden birds a popular and rapidly expanding phenomenon, an important focus of this work was to improve current understanding of its ecological impacts on wild bird populations. For this reason, I expressly aimed to manipulate food availability so as to replicate the widespread nature by which supplementary food is provisioned within gardens.

1.5.1 Detail of study system and study species

The study was conducted within nine woodland sites in Cornwall, UK from 2007 – 2010 (**Figure 1.2**). Within triplet groups, each woodland was randomly allocated to an over-winter feeding treatment to test the effects of energy (fat) versus antioxidants (vitamin E) as drivers of carry-over effects, compared to an unfed control. Treatment groups were rotated within triplets across years, so that every site received all three treatments over the course of the study. By replicating treatments across both sites and years, potentially confounding effects of habitat quality and between year differences have been prevented. In order to ensure that carry-over effects of winter food availability could be investigated with confidence, winter provisioning was stopped at least one month before laying commenced. An average of 38 (± 4.2 SD) nest boxes was provided at each site in order to monitor health and performance during the breeding season.

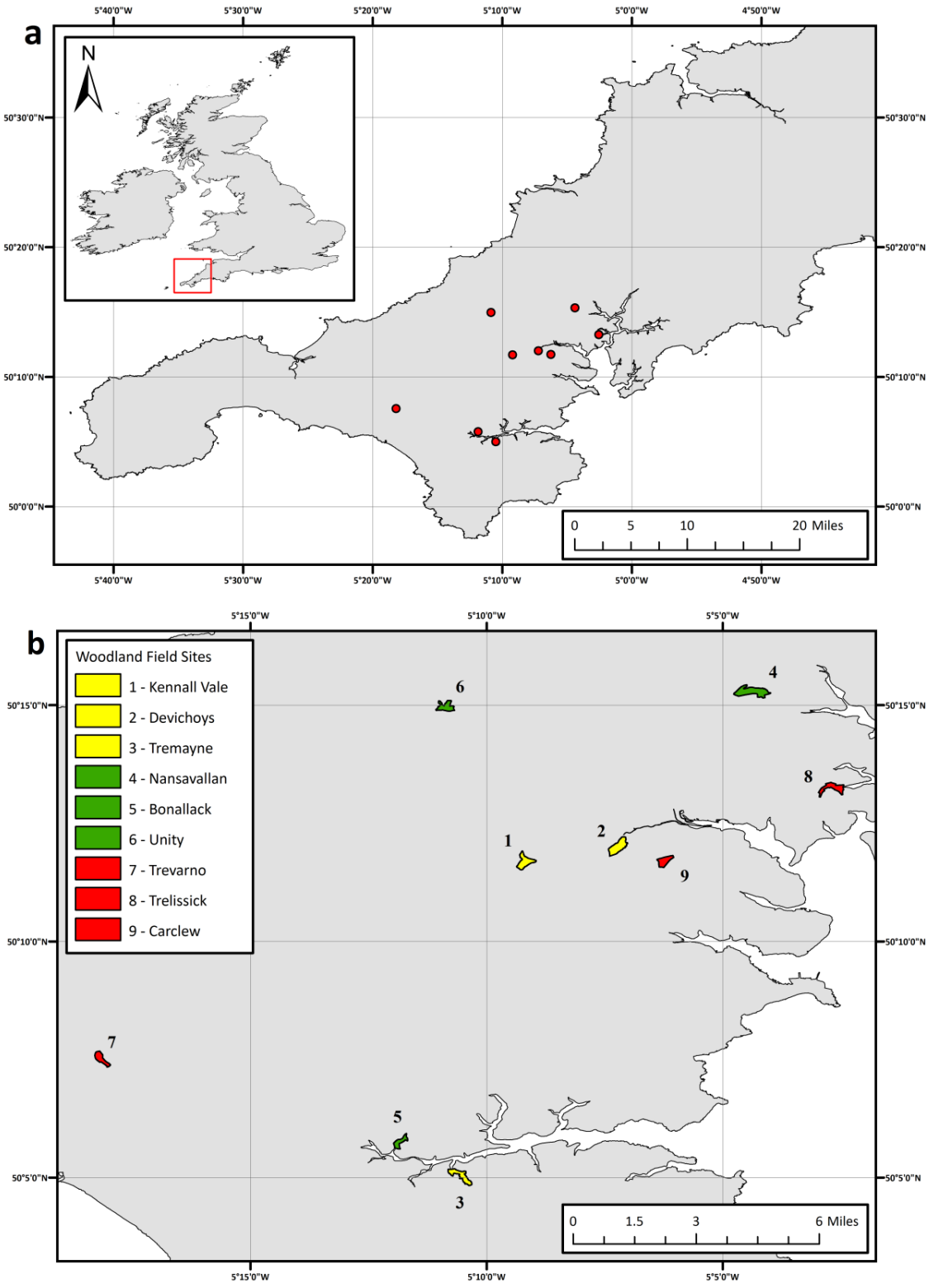

Figure 1.2. Maps detailing (a) Locations of the nine study sites in Cornwall, UK; and (b) The distribution of woodland field sites in west Cornwall coloured by triplet groups.

In all sites the majority of boxes were occupied by blue tits (mean ±SE per site: 89.7% ±2.9, 84.5% ±3.1 and 77.9% ±2.7 in successive seasons), and so further investigation was based on this species only (**Table 1.1**). The blue tit is a woodland resident in the UK, and commonly seen at garden bird feeders during the winter (Perrins 1979; Chamberlain et al. 2005) (**Figure 1.1**). As a cavity breeder, this species also takes readily to using nest boxes and much is known about their general biology (e.g. Betts 1955; Perrins 1979). Furthermore, the blue tit has been used as a focal species in a number of previous studies concerning resource availability and life-history trade-offs, making them an ideal candidate species for researching the impacts of supplementary feeding in this study.

Table 1.1. The number of nest boxes occupied by blue tits (BT), great tits (GT) and other species (coal tit or nuthatch) at woodland sites in 2008 – 2010, listed by triplet. See Appendix 1 for details of treatment allocations by year.

		2008				**2009**				**2010**			
	no. boxes	BT	GT	other	TOTAL	BT	GT	other	TOTAL	BT	GT	other	TOTAL
Kennall Vale	40	16	5	0	21	20	1	0	21	21	6	0	27
Devichoys	44	25	4	0	29	25	2	0	27	25	6	0	31
Tremayne	39	10	0	1	11	8	0	1	9	14	3	2	19
Nansavallan	43	20	0	0	20	21	3	1	25	24	4	0	28
Bonallack	32	13	0	0	13	17	4	0	21	18	6	0	24
Unity	41	30	3	0	33	25	6	0	31	27	6	0	33
Trevarno	34	9	2	0	11	13	2	1	16	18	2	0	20
Trelissick	39	13	0	0	13	9	5	0	14	14	9	0	23
Carclew	34	11	3	0	14	12	1	0	13	15	6	1	22
TOTAL	**346**	**147**	**17**	**1**	**165**	**150**	**24**	**3**	**177**	**176**	**48**	**3**	**227**

1.6 THESIS STRUCTURE

It was anticipated that carry-over effects of winter feeding may be mediated through benefits to egg production, or through benefits to parental health status. Therefore in **Chapter 2**, I begin by testing whether maternal investment in egg production is influenced by over-winter feeding; examining measures of clutch size, egg mass and proportional yolk investment, and antioxidant concentrations in the yolk. The possibility that carry-over effects of winter provisioning might arise via (1) benefits to maternal condition, or (2) direct deposition of vitamin E stored during winter is also explored, and within treatment group variance was evaluated. In **Chapter 3**, I go on to examine the effects of over-winter feeding on parental phenotypes during brood-rearing, to determine whether benefits to body condition potentially gained at winter feeders are still apparent in the later stages of the breeding cycle. The effects of feeding on population phenotypic structure are also inferred.

Chapter 4 brings together the findings from **Chapters 2 and 3** in order to determine the consequences of over-winter feeding for reproductive performance. This includes analyses of laying dates, hatching and fledging successes and nestling phenotypes. In **Chapter 5**, using stable isotope analysis, the potential mechanisms driving carry-over effects are examined in closer detail by accounting for individual variation in the uptake of supplementary food. Finally, in **Chapter 6** I summarise my findings and discuss them within the context of garden bird feeding, and provide further insights as to the broader, ecological implications of my work.

CHAPTER 2

Carry-over effects of winter food availability: the influence of vitamin E supplementation on egg quality in blue tits (*Cyanistes caeruleus*)

2.1 ABSTRACT

Maternal allocation of resources to eggs can have a fundamental effect on offspring fitness and survival. However it is hypothesised that egg quality is constrained by trade-offs in the allocation of potentially limiting resources, such as dietary antioxidants. In wild bird populations these constraints may be alleviated through supplementary feeding. The provisioning of garden bird foods is most prolific in the winter months, and as well as improving survival prospects, it seems intuitive that additional benefits may be carried-over to influence egg production. Here, I examine the importance of vitamin E (α-tocopherol) supplementation as a mechanism driving the carry-over effects of winter feeding on egg quality in wild populations of blue tits (*Cyanistes caeruleus*). Over three consecutive years, fat and fat-plus-vitamin E were provisioned and compared to an unfed control at a landscape-scale. In woodlands provisioned with vitamin E, females showed greater resource allocation through a proportional increase in yolk mass; whilst females from fat-supplemented woodlands invested greater than average levels of carotenoids in the yolk as the laying season progressed. There was little evidence to suggest provisioning influenced α-tocopherol deposition in the yolk, clutch size and mass, or egg and yolk mass independently. These results indicate that over-winter feeding does produce carry-over effects on egg quality in wild birds, and this is particularly notable given the diffuse nature at which food was provided. Three candidate mechanisms with the potential to influence egg production via carry-over effects are explored; and it is hypothesised that the benefits seen in egg quality are a consequence of an indirect effect on maternal condition. As wild bird feeding continues to grow in popularity, this study highlights the influential downstream effects provisioning can have on females during the early stages of the breeding season.

2.2 INTRODUCTION

Maternal effects arise when a mother's phenotype directly affects the phenotype of her offspring (Bernardo 1996), in such a way that the environmental conditions a mother experiences can have a definable and lasting effect on offspring fitness (Mousseau & Fox 1998). Maternal effects are particularly well exemplified in oviparous eggs. The formation of avian eggs, for example, carries high costs, both in terms of the energetic demands and the nutrient requirements which must be met in order to produce a whole clutch (Perrins 1996; Nager 2006). For a developing embryo, the egg's lipid- and protein-rich yolk provides the main source of energy, and variation in its mass has been identified as a determinant of offspring fitness (Williams 1994). But more specifically, females provision the yolk with an essential mix of lipophilic antioxidants and maternally-derived hormones and immunoglobulins, to optimise the phenotypic quality of their offspring (Gasparini et al. 2001; Groothuis et al. 2005; Surai 2007). As such, a female's condition during the onset of laying and her ability to acquire necessary resources will underpin the quality of her eggs, and ultimately influence her reproductive success.

Antioxidants, such as vitamin E and carotenoids, play a fundamental role in reducing oxidative stress (Catoni et al. 2008), by defending against an accumulation of reactive oxygen species (ROS) which result as a by-product of metabolism (Monaghan et al. 2009). In doing so, antioxidants limit the oxidative damage of macromolecules which might otherwise lead to physiological dysfunction, disease progression and aging processes (Halliwell & Gutteridge 2007). Vitamin E, for instance, functions as a potent chain-breaking antioxidant through the donation of ROS-neutralising hydrogen ions. The term vitamin E describes a group of eight lipid-soluble tocopherols and tocotrienols; of which α-tocopherol is the most bioactive and ubiquitous in nature (Burton 1994; Brigelius-Flohe & Traber 1999), and is thought to play a valuable role in avian ecology (Costantini 2008). Carotenoids, on the other hand, are biologically active pigments responsible for sexually-selected colouration, which additionally function as antioxidants through the quenching of ROS (Sies & Stahl 1995). The importance of carotenoids as effective antioxidants is disputed in birds (Hartley & Kennedy 2004; Costantini & Møller 2008), but they remain a valued part of the avian antioxidant defence system through their capacity to recycle vitamin E (Surai 2007).

Developing embryos are highly susceptible to oxidative stress, due to the elevated rates of metabolism experienced during rapid growth (Surai 2007). Therefore, increased deposition of antioxidants in the yolk can have a profound effect on offspring phenotype; from hatchability and survival (Royle et al. 2001; McGraw et al. 2005; Møller et al. 2008) to improved immunity (Saino et al. 2003; Biard et al. 2005), nestling colouration (Biard et al. 2005; Isaksson et al. 2006) and future secondary sexual signalling (McGraw et al. 2005; Biard et al. 2009). However, since vitamin E and carotenoids are only obtained through the diet (Goodwin 1984), their environmental availability is potentially limiting (Olson & Owens 1998). As a consequence physiological trade-offs in antioxidant allocation exist (Catoni et al. 2008), as individuals face fundamental decisions concerning self maintenance and reproductive investment.

For wild bird populations trade-offs presented by limited resource availability may be alleviated through supplementary feeding. Indeed, numerous studies have reported advantages of increasing food availability through artificial supplements (reviewed in Robb et al. 2008a). But more specifically, antioxidant provisioning studies have indicated the benefits of carotenoid and (to a lesser extent) vitamin E supplementation for maternal health and egg investment (Surai 2000; Blount et al. 2002; Royle et al. 2003; Blount et al. 2004; Biard et al. 2005; Scheideler et al. 2010). Supplementary food provisioned within gardens provides an enormous additional feeding resource to wild birds, particularly small passerines, not least during winter (Chamberlain et al. 2005; O'Leary & Jones 2006). As temperatures fall and day lengths are reduced, birds are faced with increased energetic demands during the winter months, at a time when natural food availability is at its lowest. Over-winter feeding can increase survival and improve body condition during these adverse winter conditions (Jansson et al. 1981; Kallander 1981; Grubb & Cimprich 1990), and there is also evidence to suggest that the benefits of supplemented winter foods are ‘carried-over’ to improve reproductive performance, even once feeding has ceased (Robb et al. 2008b).

Carry-over effects arise when events in one season influence an individual’s performance in a later time or place (Harrison et al. 2011). For example, in migratory birds, ecological conditions experienced during winter are found to influence

reproductive success in the subsequent breeding season (Norris et al. 2004; Bearhop et al. 2005). However, the potential mechanisms driving carry-over effects have not been tested (Harrison et al. 2011). Whilst increased survival has been attributed to the energy boost winter food provides (Jansson et al. 1981; Brittingham & Temple 1988), it seems intuitive that additional benefits to health and reproductive performance may result from greater antioxidant uptake, and that egg quality maternal effects might equivocally be influenced by resource availability before the breeding season has even begun. However, whilst many supplementary feeding studies have investigated the impacts of antioxidant provisioning during or immediately prior to egg laying, the potential for over-winter feeding to influence egg investment is yet to be evaluated.

It is anticipated that there could be three alternative explanations for the way in which over-winter provisioning of a valuable antioxidant, such as vitamin E, might influence maternal investment in egg production. Firstly, small passerine birds such as tits are predominantly income breeders (Drent & Daan 1980). Therefore they are not expected to rely on endogenous reserves during breeding, but instead adjust their current food intake to meet the demands of reproduction. This suggests that potential carry-over effects of winter feeding are likely to be condition-dependent. Since the provisioning of antioxidant-rich foods can help reduce an individual's susceptibility to oxidative stress, antioxidant-fed birds might enter the breeding season in a superior condition with less need to allocate newly-acquired resources to self-maintenance. As such it is predicted that birds provisioned with vitamin E through the winter should be able to afford a greater investment in egg quality during the breeding season. Secondly, and alternatively, as lipophilic molecules, antioxidants can be stored for long periods in subcutaneous fat (Negro et al. 2001). It has recently been hypothesised that this might enable carotenoids to be stored and called upon during periods of increased demand (McGraw & Toomey 2010; Metzger & Bairlein 2011). Antioxidant provisioning might therefore allow females to build up antioxidant reserves over-winter, for later capital investment into eggs. Thirdly at a population level, there is the possibility that vitamin E provisioning might not induce positive carry-over effects if it is in fact used as an indicator of natural food availability, or if it alters the phenotypic structure of the population by allowing poor quality individuals to survive and enter the breeding population. In these instances provisioning might lead to increased variation in egg

investment amongst individuals within a population. Furthermore, since feather carotenoid-based colouration is a good indicator of condition during moult (and therefore prior to winter feeding) (Møller et al. 2000), and also anticipated to be indicative of female reproductive capacity (Hidalgo-Garcia 2006; Doutrelant et al. 2008), over-winter provisioning might also produce evidence of females with lower carotenoid-based feather coloration entering the breeding population (see **Chapter 3**), resulting in a reduction in egg investment at a population level.

It seems apparent that despite the magnitude of over-winter garden bird feeding both in the UK and globally, there is a relative lack of knowledge of its implications and the mechanisms by which they are derived. Here, I examine the potential carry-over effects of over-winter dietary provisioning on maternal investment in egg production in wild populations of blue tits (*Cyanistes caeruleus*). The aims were (1) to determine whether over-winter feeding enabled individuals to increase the number, size and proportionality of their eggs, or increase deposition of antioxidants in the yolk (namely α-tocopherol and total carotenoids); (2) to investigate whether feeding lead to greater variation in antioxidant deposition within populations or evidence of a difference in female condition, indicative of a change in population phenotypic structure; and (3) to establish the importance of vitamin E supplementation as a mechanism in driving carry-over effects.

2.3 METHODS

2.3.1 Study site and experimental design

The over-winter supplementary feeding experiment was conducted over three years from 2007 to 2009, and the carry-over effects were measured during the subsequent breeding seasons, 2008 – 2010 respectively. The study took place in Cornwall, UK, across nine deciduous woodland sites (**Figure 1.2**), where oak (*Quercus* spp.), beech (*Fagus sylvatica*), sweet chestnut (*Castanea sativa*) and sycamore (*Acer pseudoplatanus*) were the predominant tree species. Sites studied averaged 10.7 hectares in size, and were situated at least 2 miles apart to restrict between-site movements (Greenwood et al. 1979). Sites were grouped into three triplets, according

to similarities in their woodland composition and other common features, such as proximity to settlements and amount of periphery woodland (**Appendix 1**).

In the first year of the study, each site within a triplet was randomly allocated to one of three feeding treatments. These treatments were: *(1) no supplement* (hereafter 'control'), *(2) fat only* (to test for energy effects; hereafter 'fat'), and *(3) fat-plus-vitamin E* (to test for effects of energy plus antioxidants, hereafter 'fat+VE') (see **2.3.2** for further explanation). Treatments groups were rotated within triplets across years, so that every site received all three treatments over the course of the study. Since feeding treatments were replicated three times using different triplet groups in a given year, any potential confounding effects of year have been prevented.

Within the six fed sites each year, squirrel-proof feeders (custom-designed to prevent access by grey squirrels (*Sciurus carolinensis*) and other non-target species) were hung *ca*. 4m from the ground at 100m intervals along parallel transects (100m apart), and at an average of 9 per site (**Figure 2.1**). A total of 346 nest boxes, with a 32mm entrance hole, were positioned across all sites. Boxes were erected at 25m intervals along transects and parallel to feeders, such that every box was no more than 50m from a feeder (**Appendix 1**). This design produced an equal density and distribution of feeders and boxes across individual woodlands, *ca*. 1 feeder and 4 boxes per hectare.

Figure 2.1. Provision of supplementary food; (a) Example of a squirrel-proof feeder; and (b) Example fat balls at the start and end of a 10 day provisioning period. K. Plummer; Feb 2010.

2.3.2 Supplementary feeding experiment

Food was provisioned through the winter only (14 December – 4 March 2007/08; 18 November – 11 March 2008/09 and 2009/10), leaving a gap of at least one month before laying commenced (8 April, 11 April, 15 April respectively), and thus allowing carry-over effects to be investigated with confidence. All feeding stations were provisioned with a fresh 150g fat ball every 10 days. Fat balls for the fat+VE treatment group were supplemented with α-tocopherol (≥96% DL-all-*rac*-α-tocopherol (HPLC), Sigma-Aldrich Ltd., Dorset) at a concentration of 10mg/ 100g fat, a level equivalent to that found to occur naturally in peanuts (Chun et al. 2005), a popular garden bird food (Glue 2006). α-Tocopherol cannot be provisioned to wild birds without the use of a 'carrier' and, as a lipophilic molecule, fat is required for vitamin E absorption (Blount et al. 2002; Jeanes et al. 2004). Therefore, the fat-plus-vitamin E treatment group provides an ecologically meaningful method of testing antioxidant effects.

All fat balls were produced from solid vegetable fat (Crisp 'n Dry, Princes Ltd., Liverpool, UK) 1 – 2 days in advance of provisioning, using standardised methods adapted from Blount *et al.* (2002). Fat was heated to 60^{o}C for *ca*. 1 hour until liquefied, then cooled on ice until viscous. When the fat reached 18 – 20^{o}C, yellow food colouring (0.125ml/ 100g fat; ASDA Natural Food Colouring, Asda Stores Ltd., Leeds) was added, to increase fat ball attractiveness to target species since carotenoid-pigmented species show preference for colourful foods (Rodd et al. 2002; McGraw et al. 2006), verified by a pilot study (KEP pers. obs.). α-Tocopherol was added to fat for sites in the fat+VE treatment group only, at the concentration specified above. 150g fat was weighed into a container and hardened at -20^{o}C overnight inside a sealed bag prior to provisioning. Fat balls were rarely fully depleted, and upon collection were weighed (±0.01g) to determine levels of consumption (**Figure 2.1**). Observations at feeders, beak markings on fat balls, and winter mist netting confirmed food use was dominated by Parid sp., including the target species, throughout the study (KEP pers. obs.). The uptake of provisioned food varied between sites and years, and was correlated to changing temperature and environmental conditions (KEP pers. obs.). Total consumption across all fed sites was: 7.68kg, 13.56kg and 38.60kg respectively during the three winter feeding seasons (**Appendix 1** provides a further detailed breakdown by site, treatment and year).

2.3.3 Breeding parameters

Nest boxes were inspected every 1 – 3 days from April to June to investigate effects of feeding on breeding performance. Lay date of the first egg was back-calculated by assuming one egg was laid per day, if more than one egg was present (Perrins 1979). After the first egg was laid, nests were visited every 1 – 2 days until clutch completion. To establish laying order new eggs were marked, using a fine tipped black permanent marker, with an increasing number of dots at each visit. Total clutch size was recorded upon clutch completion, as defined by a two day laying break and the onset of incubation, and total clutch mass weighed (±0.1g) using an electronic balance. One egg was then removed for measurement of mass and biochemical analysis. By way of standardising egg removal, an egg marked during the final visit, typically the last or second last egg laid, was always taken. Collected eggs were returned to the laboratory, where they were weighed (±0.001g) using an electronic balance, and then dissected, on the day of collection. The yolk was rolled over damp filter paper to remove traces of albumen, weighed (±0.001g) and stored at -80^{o}C until analysis. Given time constraints and lower levels of measurement accuracy in the field, it was not possible to draw averages for egg mass across the whole clutch. However, egg mass has previously been shown not to vary significantly within clutches (Ramsay & Houston 1998) nor to display general trends across the laying sequence (Nilsson & Svensson 1993a), and as such it is justifiable to use measurements taken from only the collected egg as a proxy for the whole clutch.

2.3.4 Female feather sampling

Yellow breast feathers were sampled from females in a subset of nest boxes each year under Home Office license (PIL 30/8161). Individuals were captured on the nest using spring traps (Amber Electronics Ltd, Daventry, UK) between day 5 – 18 of the nestling phase, and a feather sample plucked from a standardized position on the breast. This was stored in a sealed bag in the dark until later biochemical analysis (see **Chapter 3** §**3.3** for further details of parental sampling methodology).

2.3.5 Biochemical assays

2.3.5.1 Determination of egg yolk antioxidant levels

For extraction of antioxidants, egg yolk (0.040-0.050g) was vortexed in 0.7mL 5% NaCl for 5 seconds and then homogenised with 1mL EtOH for 20 seconds. 1.5mL hexane was added and further homogenised for 10 seconds. The sample was centrifuged for 4 minutes at 8000 × g, and the hexane phase containing the antioxidants drawn off. Extraction was repeated and both hexane extracts combined.

Total carotenoid concentrations in egg yolk were determined using spectrophotometry. Total carotenoid absorbance was measured at 450nm using a spectrophotometer (Nicolet Evolution 500), with total carotenoid concentration calculated using the extinction coefficient of lutein in hexane (2589, Craft & Soares 1992). 500µl hexane extract was dried and the residue re-dissolved in 150µl dichloromethane and 150µl methanol. For determination of α-tocopherol concentrations, samples (20µl) were injected into a high-performance liquid chromatography system (HPLC; Dionex Corporation, California, USA). Separation utilised a 3µ C_{18} reverse-phase column (15 cm x 4.6 mm) stationary phase (Spherisorb S30DS2; Phase separations, Clwyd, UK), with a mobile phase of methanol:water (97:3 v/v) at a flow rate of 1.1ml min^{-1}. Fluorescence detection (Dionex RF2000) was performed at 295nm (excitation) and 330nm (emission). The α-tocopherol peak was identified and quantified by comparison with a standard solution of α-tocopherol (Sigma-Altrich Ltd., Dorset) in methanol. Total carotenoid and α-tocopherol concentrations are reported as µg/ g yolk.

2.3.5.2 Feather total carotenoid level determination

Mechanical extraction was used to isolate carotenoid pigments from feathers (Stradi et al. 1995). Whole feathers were washed separately in ethanol (30 s) and hexane (30 s), then blotted dry to remove surface lipids. 3 – 5 mg of yellow barbules were trimmed, and then ground for 15 minutes at 30 Hz in the presence of 2ml methanol, using a Retsch MM200 micronizer equipped with zirconium oxide grinding jar and balls (Retsch UK Ltd., Castleford, UK). Samples were filtered by injection through a Sep-Pak Light C_{18} Cartridge (Waters Ltd., Dublin), and their absorbance measured at 450nm using a spectrophotometer (Nicolet Evolution 500). Total carotenoid concentration was

calculated using the extinction coefficient of lutein in methanol (2629, Craft & Soares 1992).

2.3.6 Statistical analysis

All statistical analyses were conducted using R version 2.12.0 (R Development Core Team 2010). To test the influence of over-winter feeding on maternal investment in egg production general linear mixed models (GLMM) were applied to a number of independent response variables; namely clutch size; clutch, egg and yolk mass; and α-tocopherol and total carotenoid concentration and total content of the yolk. A $\log_{10}$: $\log_{10}$ GLMM of yolk mass versus egg mass was used to examine proportional yolk investment. Nest box identity (252 levels: 129 used once, 85 used twice, 38 used three times over three years) nested within woodland site (9 levels) was specified as the random term, to control for temporal and spatial pseudoreplication. Clutches with laying breaks greater than two days were excluded from the analysis, as were clutches abandoned before the onset of incubation. To avoid bias resulting from embryo development, eggs showing evidence of incubation upon dissection were excluded from egg component analyses (16.0% of eggs collected).

2.3.6.1 Fixed effects

GLMMs were conducted using using 'lme' from the *nlme* package (Pinheiro et al. 2010). Sequential (type I) sums of squares (SS) were applied, which are more robust to unbalanced designs, with treatment fitted last as a three level factor (Hector et al. 2010). For all dependent variables tested, year was included as a fixed factor (2008, 2009 or 2010) to account for annual variation in breeding conditions (Svensson & Nilsson 1995), and lay date was included as a covariate accounting for seasonal variation (Norris 1993). Clutch size was additionally included as a covariate in analyses of egg components, since it may have trade-offs with individual egg investment (Perrins 1996) or be indicative of female quality (Slagsvold & Lifjeld 1990; Pettifor et al. 2001). Clutch size was also controlled for when analysing clutch mass. Although variation in egg components may be influenced by laying order (Hõrak et al. 2002), there was a strong correlation between egg number sampled and clutch size (Pearson's: $r= 0.82$, $n= 413$, $p< 0.001$), and therefore laying order was not included in egg component analysis to avoid collinearity. There was no evidence of a correlation between concentrations of

α-tocopherol and total carotenoids in the yolk (Spearman's rank: rs= 0.03, n=307, *p*= 0.557), and so total carotenoid concentration was not included as a predictor in analyses of α-tocopherol, and vice versa. All two-way interactions involving treatment were fitted to test for differences in main effects between treatment groups, and a clutch size × lay date interaction term was included in some analyses to improve model fit (see **Tables 1** and **3**). The $\log_{10}$: $\log_{10}$ GLMM model for proportion yolk content was fitted with main effects and a $\log_{10}$ (egg mass) × treatment two-way interaction only (see **Table 2**). Normality and heteroscedasticity of residuals were checked prior to model selection to test model fit. Concentrations of α-tocopherol and total carotenoids were subsequently log-transformed to correct normality.

2.3.6.2 Model section

An information-theoretic approach was used for model selection (Burnham & Anderson 2002; Johnson & Omland 2004; Symonds & Moussalli 2011), appropriate for complex large-scale field investigations as reported here (Whittingham et al. 2006). This approach uses Akaike's information criterion (AIC), a measure of model fit and complexity, to directly compare all models within a candidate set (Akaike 1973; Burnham & Anderson 2002). The model with the lowest AIC value represents the best fit of the data, and remaining models are ranked according to their relative support, using AIC difference (ΔAIC_i) between model *i* and the model of best fit. For each separate analysis, all possible models, given the predictor variables, were fitted as part of the candidate set alongside a null model fitted with only the intercept, using the 'dredge' function implemented in the package *MuMIn* (Bartoń 2011). In all cases the ratio of the number of observations to model parameters (*k*) was <40, so AICc, which applies a bias correction for small sample size, was used to rank models (Burnham & Anderson 2002). Akaike weights (w_i) were calculated for each model (following Burnham & Anderson 2002), where the weights of the candidate set sum to 1.00. A model's weight provides a measure of selection probability, interpreted as the likelihood that model *i* would be selected as the best fitted model were the data to be collected again.

2.3.6.3 Model averaging for multimodal inference

Model averaging was applied in the absence of a singularly well supported model (i.e. $w_{best} < 0.90$), across a reduced set of the most strongly supported models (the confidence set) using the 'model.avg' function in *MuMIn* (Bartoń 2011). Only models with an evidence ratio of <10 (*ER*; calculated w_{best} / w_i for model *i*) were included within the confidence set (Burnham & Anderson 2002; Lukacs et al. 2007). The evidence ratio of the null model was additionally used to evaluate the level of support for the confidence set (Dochtermann & Jenkins 2011; Mundry 2011). Coefficient of determination (R^2) values cannot be calculated for mixed models. However a pseudo-R^2 value was generated for the top ranking model to estimate variance explained by the model and to evaluate model fit, following Nagelkerke (1991). This formula extends the calculation of R^2 to models fit using maximum likelihood and can be interpreted in the same way as traditionally calculated coefficients of determination (Nagelkerke 1991; Dochtermann & Jenkins 2011).

Adjusted Akaike weights were calculated for the reduced confidence set, and parameter estimates (β) and their associated standard errors (SE) averaged, such that the contribution of each model in the set is proportional to its Akaike weight. Model-averaged estimates benefit from better precision and reduced bias compared to the estimates of a single model (Burnham & Anderson 2002). The selection probability of explanatory variables (*w*) was estimated by summing Akaike weights across models in which the variable appears, and used to estimate the probability that variable *k* featured in the best model. Where treatment (or a specific treatment interaction) was well supported for inclusion in the best model, *post hoc* testing was used to assess between treatment group differences. Such that, for each model in the confidence set featuring treatment (or the specific treatment interaction) *post hoc* pairwise comparisons were carried out, using comparisons of the original ranked GLMM model with models in which treatment groups were paired. Averaged parameter weights ± unconditional SE are reported throughout.

2.4 RESULTS

A total of 476 nest boxes were occupied by blue tits from 2008 to 2010, of which 63 were excluded from the investigation due to pre-incubation abandonment (n= 32), laying breaks greater than 2 days (n= 22) or late nesting attempts (n=9; first egg laid >7 days later than others breeders within same woodland). Blue tit nest box occupancy rates increased annually (147, 150, 176 respectively of 346 total boxes), but this was not found to be significantly different between years or between treatment groups (binomial GLMM with site/ nest box random factor; year: χ^2_2= 5.54, p= 0.063, treatment χ^2_2= 3.34, p= 0.19, interaction NS). For all measures of maternal egg investment there were a number of competing models with similar weights, indicating a level of uncertainty in distinguishing a 'best model'. Model averaging was employed across each confidence set (i.e. models with ER_i <10, see **Tables 2.1, 2.2** and **2.4**), to evaluate the relative support of predictor terms and produce unbiased parameter estimates and errors.

2.4.1 Maternal investment in egg production

The probability of treatment explaining clutch size, excluding possible interactions, was poorly estimated as indicated by relatively high standard errors (fat: β= -0.07 ±0.13, fat+VE: β= -0.14 ±0.19), and in fact treatment explained less than half the variation of year (2009: β= -0.33 ±0.18, 2010: β= 0.30 ±0.18) or lay date (β= -0.09 ±0.01) (w= 0.435, **Table 2.1 (a)**, models 1 and 2 only). An interaction between treatment and lay date also received a low level of support (w= 0.232) (**Table 2.1 (a)**), whereby fat supplementation helped to negate the seasonal effect of lay date on clutch size (fat × lay date: β= 0.012 ±0.018), whereas there was little difference between the control and fat+VE treatment groups (fat+VE × lay date: β= -0.003 ±0.008). But again, imprecision in the parameter estimates indicates this interaction should be judged with caution.

Total resource deposition across feeding treatments was investigated in an analysis of clutch mass, controlling for clutch size (w= 1.00; β= 1.04 ±0.043 (**Table 2.1 (b)**). The probability of treatment being able to predict variation in clutch mass was small in comparison to lay date (w= 1.00; β= -0.02 ±0.001) and year (w= 1.00; 2009: β= -0.29 ±0.09, 2010: β= -0.07 ±0.09), primarily involving an interaction with lay date (treatment × lay date w= 0.081, treatment main effect w= 0.076) (**Table 2.1 (b)**). This followed

Table 2.1. Confidence sets of ranked models (in descending order) for analyses of (a) clutch size, (b) clutch mass and (c) egg mass, and selection probability of fixed effects (*w*). + denotes fixed effects included in model. See §2.3.6 for description of terms and text for details of parameter estimates (β).

	Fixed effects											
Rank	Tr	Yr	LD	CS	Tr :† LD	Tr :† Yr	LD:† CS	*k*	Log-likelihood	AICc	ΔAICc	w_i
(a) Clutch size												
1		+	+	n/a			n/a	7	-756.8	1527.9	0.000	0.372
2	+	+	+	n/a			n/a	9	-755.0	1528.4	0.530	0.286
3	+	+	+	n/a	+		n/a	11	-753.3	1529.3	1.443	0.181
4	+	+	+	n/a		+	n/a	13	-751.7	1530.3	2.439	0.110
5	+	+	+	n/a	+	+	n/a	15	-750.3	1531.9	3.975	0.051
***9*				*n/a*			*n/a*	*4*	*-787.6*	*1583.3*	*55.377*	*0.000*
w	0.628	1.000	1.000		0.232	0.161						
(b) Clutch mass												
1		+	+	+				8	-439.14	894.7	0.000	0.605
2		+	+	+			+	9	-439.02	896.5	1.857	0.239
3	+	+	+	+	+			12	-436.93	898.7	4.025	0.081
4	+	+	+	+				10	-439.12	898.8	4.159	0.076
***46*								*4*	*-798.54*	*1605.7*	*710.502*	*0.000*
w	0.156	1.000	1.000	1.000	0.081		0.239					
(c) Egg mass												
1		+		+				7	294.3	-574.2	0.000	0.496
2		+	+	+				8	294.5	-572.5	1.746	0.207
3	+	+		+				9	295.0	-571.3	2.915	0.116
4		+						6	291.6	-571.0	3.206	0.100
5		+	+	+			+	9	290.4	-570.6	3.621	0.081
6				+				5	294.8	-571.0	3.621	0.068
***10*								*4*	*288.1*	*-568.1*	*6.085*	*0.018*
w	0.105	0.926	0.279	0.909			0.090					

Tr, treatment (control, fat, fat+VE); Yr, year (2008, 2009, 2010); LD, lay date; CS, clutch size; †, interaction
**Intercept-only models (reported in *italics* with the unadjusted Akaike weight)

Each global model included all listed terms. Treatment × clutch size did not feature in any confidence sets.
(a) Candidate set= 13 models, n= 413, ER_{null} >1000, pseudo-R^2 = 0.142.
(b) Candidate set= 49 models, n= 388, ER_{null} >1000, pseudo-R^2 = 0.870.
(c) Candidate set= 49 models, n= 312, ER_{null} = 20.96, pseudo-R^2 = 0.046.

the same pattern described for clutch size, although small and imprecise estimates reveal that this is not in fact an accurate predictor of clutch mass (fat × lay date: β= 0.002 ±0.004, fat+VE × lay date: β= -0.001 ±0.002).

Feeding treatment was also a poor predictor of egg mass variation (w= 0.156; fat: β= -0.0014 ±0.0020, fat+VE: β= -0.0011 ±0.0026) (Table 2.1 (c)). By comparison, differences in egg mass were considerably better explained by year, showing females laid comparatively smaller eggs in 2009 (w= 0.926; 2009: β= -0.0302 ±0.0142, 2010: β= -0.0251 ±0.0137) and clutch size, which had a small negative effect (w= 0.909; β= -0.0080 ±0.0049) (**Table 2.1 (c)**). However model 1 explained only a small proportion of the variation (pseudo-R^2= 0.046, **Table 2.1 (c)**). Furthermore, a variance components analysis of model 1 (**Table 2.1 (c)**), using restricted maximum likelihood (REML), revealed that 71.7% of variation in egg mass could be attributed to inter-clutch variation, with woodland site accounting for 3.5% and nest box for 24.9%, indicating that inherent female difference may be the greatest predictor of egg mass variation. No models were well supported in an analysis of yolk mass, where the intercept-only model ranked within the confidence set (null model, ER= 6.12, ΔAIC= 3.62, w_i= 0.036), and again the top ranking model represented a poor fit of the data (pseudo-R^2= 0.019). As such, further inference could not be drawn, and the model set is not reported.

Model averaging of proportional yolk investment revealed strong support for an effect of treatment on the relationship between egg mass and yolk mass (treatment × $\log_{10}$ (egg mass) interaction w= 0.972, **Table 2.2**, **Figure 2.2**). The model selection probabilities for year, clutch size and lay date were relatively smaller (w= 0.475, 0.354 and 0.337 respectively, **Table 2.2**). Whilst control and fat-fed females similarly had proportionally small yolks (control slope= 0.8701 ±0.0992; fat: β= -0.1834 ±0.1369), females from vitamin E-fed woodlands allocated an increasing proportion of total egg mass to yolk as egg mass increased (fat+VE: β= 0.2194 ±0.1413) (**Figure 2.2**). *Post-hoc* comparisons between treatment pairs reveal that this difference was consistently significant, and always in the same direction, between fat and fat+VE treatment groups across all models in the confidence set. Control and fat+VE groups were not significantly different (**Table 2.3**). These results suggest that whilst over-winter provisioning has not had an overwhelming influence on the number or size of eggs

produced, the provisioning of vitamin E enabled females to allocate more resources into individual eggs, through a proportional increase in yolk mass.

Table 2.2. Confidence set of ranked models for analyses of proportional yolk investment. Includes fixed effects selection probabilities (*w*). + denotes fixed effects included in model. See §2.3.6 for description of terms and text for details of parameter estimates (β).

	Fixed effects										
Rank	Tr	Yr	LD	CS	Log_{10} (EM)	Tr: † log_{10} (EM)	*k*	Log-likelihood	AICc	ΔAICc	w_i
1	+				+	+	9	581.7	-1144.8	0.000	0.197
2	+	+			+	+	11	583.8	-1144.7	0.027	0.194
3	+		+		+	+	10	582.4	-1144.1	0.636	0.143
4	+			+	+	+	10	584.3	-1143.8	0.993	0.120
5	+	+		+	+	+	12	584.3	-1143.6	1.215	0.107
6	+	+	+		+	+	12	584.3	-1143.4	1.344	0.101
7	+		+	+	+	+	11	582.7	-1142.5	2.219	0.065
8	+	+	+	+	+	+	13	584.5	-1141.8	2.964	0.045
9		+			+		7	577.6	-1140.9	3.913	0.028
***27*							*4*	*487.4*	*-966.7*	*178.067*	*0.000*
w	0.972	0.475	0.354	0.337	1.000	0.972					

Tr, treatment (control, fat, fat+VE); Yr, year (2008, 2009, 2010); LD, lay date; CS, clutch size; log_{10}(EM), log_{10}(egg mass (g)); †, interaction. **Null model (*italics*, with the unadjusted Akaike weight).

Candidate set = 40 models, n= 299, ER_{null} >1000, n= 299, pseudo-R^2 = 0.486.

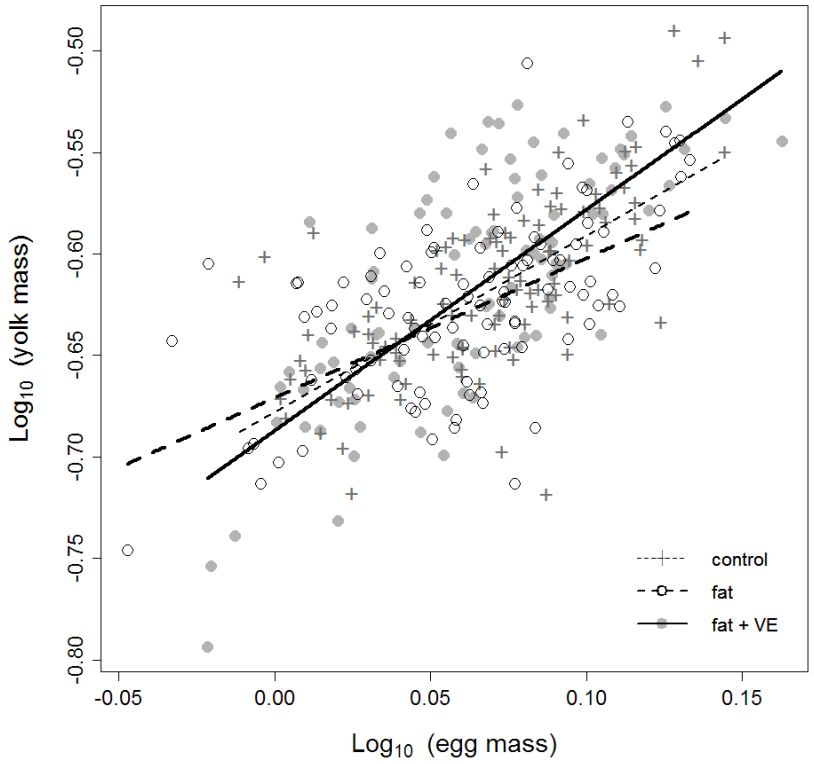

Figure 2.2. The linear relationship between yolk mass and egg mass differed between feeding treatment groups. Lines fitted using model averaged parameter estimates. See Tables 2.2 and 2.3 for statistical findings and text for details.

Table 2.3. Pairwise comparisons of the effect of feeding treatment on the linear relationship between yolk mass and egg mass. Comparisons are made for all models in the confidence set featuring the treatment × $\log_{10}$(egg mass) interaction (see Table 2.2).

	control v fat		control v fat+VE		fat v fat+VE	
Rank	χ^2_2	*p*	χ^2_2	*p*	χ^2_2	*p*
1	2.57	0.277	4.65	0.098	13.07	0.001**
2	3.22	0.200	3.46	0.177	12.42	0.002**
3	2.66	0.265	4.57	0.102	13.09	0.001**
4	2.87	0.239	4.44	0.109	13.41	0.001**
5	3.53	0.172	3.31	0.191	12.72	0.002**
6	3.30	0.192	3.53	0.171	12.64	0.002**
7	2.85	0.240	4.43	0.109	13.32	0.001**
8	3.53	0.171	3.39	0.183	12.83	0.002**
Mean	3.07	0.219	3.97	0.142	12.94	0.002**

2.4.2 Maternal investment in egg yolk antioxidants

Variation in yolk α-tocopherol concentration was relatively unaffected by over-winter feeding (w_i= 0.128, fat: β= -0.0001 ±0.0084, fat+VE: β= -0.0045 ±0.0138) (**Table 2.4 (a)**). In this analysis the best supported model received almost seven times more support (w_i= 0.872) by excluding treatment and just maintaining the effects of year (2009: β= -0.1345 ±0.0661, 2010: β= 0.1186 ±0.0673) and a lay date by clutch size interaction term (β= -0.0064 ±0.0021) (**Table 2.4 (a)**). Evidence of an effect of treatment increased (w= 0.316) when examining the total α-tocopherol content in the yolk (μg, calculated by multiplying the concentration values by the yolk mass) (**Table 2.4 (b)**). But this was primarily due to an interaction with clutch size (treatment × clutch size w= 0.243, treatment main effect w= 0.073), which demonstrated poor predictive power (fat × clutch size: β= -0.0201 ±0.0343, fat+VE × clutch size: β= -0.0203 ±0.0337). By comparison, support for the inclusion of year and the lay date by clutch size interaction remained high (w> 0.851, **Table 2.4 (b)**). These results suggest that deposition of vitamin E in eggs did not differ as a consequence of over-winter supplementation, but varied more as a result of annual and seasonal variation, such that α-tocopherol levels were lower in 2009, the same year clutch and egg masses were also reduced, and decreased as the season progressed, but to a greater extent in larger clutches.

By contrast, evidence of a difference in total carotenoid concentration between the treatment groups received a high level of support (**Table 2.4 (c)**). In an interaction with lay date, treatment had a selection probability of w= 1.000, where total carotenoid concentration was shown to increase more greatly across the season in the fat-fed treatment group compared to the fat+VE and control groups (**Figure 2.3**). *Post-hoc* comparisons of the slopes between treatment pairs revealed that the treatment effect was driven by fat provisioning differences (**Table 2.5 (a)**). Selection probabilities for year (2009: β= -0.002 ±0.042, 2010: β= -0.160 ±0.044) and a lay date by clutch size interaction (β= -0.003 ±0.001) were also high (w> 0.888) (**Table 2.4 (c)**).

Table 2.4. Confidence sets of ranked models for four separate analyses of yolk antioxidant composition, and selection probability of fixed effects (*w*). + denotes fixed effects included in each model. See §2.3.6 for description of terms and text for details of parameter estimates (β).

	Fixed effects											
Rank	Tr	Yr	LD	CS	Tr: LD	Tr: CS	LD: CS	*k*	Log-likelihood	AICc	ΔAICc	w_i
(a) α-Tocopherol concentration (μg / g yolk)												
1		+	+	+			+	9	-210.61	439.8	0.000	0.872
2	+	+	+	+			+	11	-210.39	443.7	0.110	0.128
***25*								*4*	*-224.34*	*456.8*	*16.974*	*0.000*
w	0.128	1.000	1.000	1.000			1.000					
(b) Yolk α-tocopherol content (μg)												
1		+	+	+			+	9	-205.37	429.4	0.000	0.609
2	+	+	+	+		+	+	13	-202.33	431.9	2.569	0.169
3		+	+	+				8	-208.53	433.6	4.192	0.075
4	+	+	+	+		+		12	-204.24	433.6	4.210	0.074
5	+	+	+	+			+	11	-205.35	433.6	4.251	0.073
***32*								*4*	*-219.50*	*447.2*	*17.824*	*0.000*
w	0.316	1.000	1.000	1.000		0.243	0.851					
(c) Total carotenoid concentration (μg / g yolk)												
1	+	+	+	+	+		+	13	-79.00	185.2	0.000	0.764
2	+	+	+	+	+	+	+	15	-78.62	188.9	3.638	0.124
3	+	+	+		+			11	-83.09	189.1	3.841	0.112
***35*								*4*	*-98.59*	*205.3*	*20.088*	*0.000*
w	1.000	1.000	1.000	0.888	1.000	0.124	0.888					
(d) Yolk total carotenoid content (μg)												
1	+	+	+	+	+		+	13	-82.4	192.7	0.000	0.377
2	+	+	+		+			11	-85.6	194.1	1.464	0.181
3		+	+					7	-90.1	194.7	1.992	0.139
4		+	+	+			+	9	-88.5	195.7	2.975	0.085
5	+	+	+	+	+			12	-85.6	196.2	3.525	0.065
6	+	+	+	+	+	+	+	15	-82.4	196.4	3.710	0.059
7		+	+	+				8	-90.1	196.8	4.071	0.049
8	+	+	+					9	-89.2	197.0	4.340	0.043
***28*								*4*	*-98.4*	*205.00*	*12.320*	*0.001*
w	0.726	1.000	1.000	0.636	0.683	0.059	0.522					

Tr, treatment (control, fat, fat+VE); Yr, year (2008, 2009, 2010); LD, lay date; CS, clutch size; :, interaction
**Intercept-only models (reported in *italics* with the unadjusted Akaike weight)

Each global model included all listed terms. Treatment × year did not feature in any confidence sets.
Candidate sets= 49 models. (a) n= 307, ER_{null} >1000, pseudo-R^2 = 0.120. (b) n= 299, ER_{null} >1000, pseudo-R^2 = 0.078. (c) n= 313, ER_{null} >1000, pseudo-R^2 = 0.251. (d) n= 305, ER_{null} 473.39, pseudo-R^2 = 0.206.

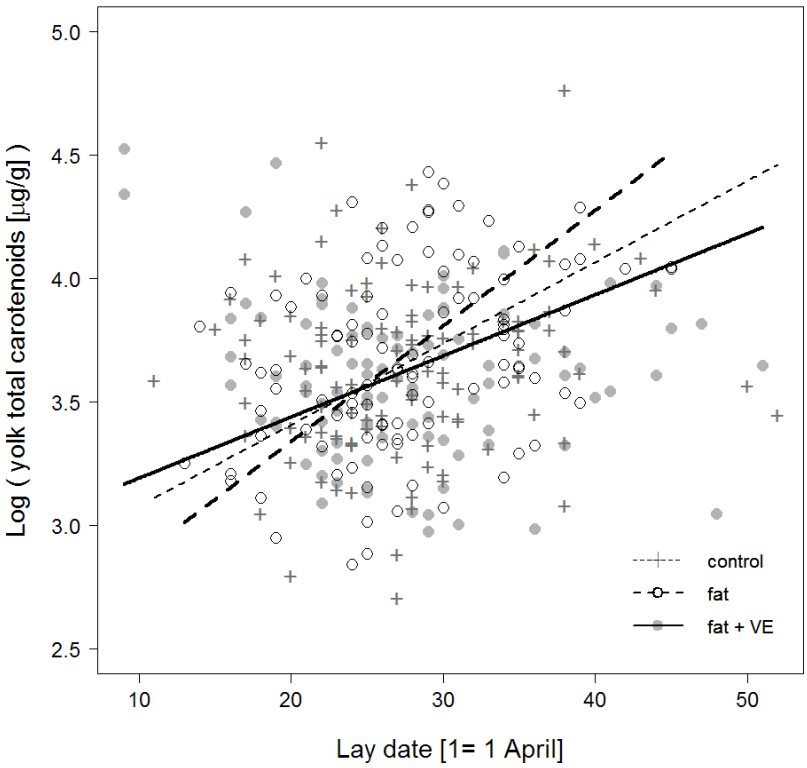

Figure 2.3. The relationship between yolk total carotenoid concentration and lay date differed between feeding treatment groups. Lines fitted using model averaged parameter estimates. See Tables 2.4 and 2.5 for statistical findings and text for details.

Table 2.5. Pairwise comparisons of the effect of feeding treatment on the relationship between (a) yolk total carotenoid concentration, or (b) yolk total carotenoid content, and lay date. Comparisons are made for all models in the confidence sets featuring the treatment × lay date interaction (see Table 2.4 (c) and (d)).

	control v fat		control v fat+VE		fat v fat+VE	
Rank	χ^2_2	*p*	χ^2_2	*p*	χ^2_2	*p*
(a) Total carotenoid concentration (µg / g yolk)						
1	6.81	0.033*	2.76	0.252	16.69	<0.001***
2	5.43	0.066	2.20	0.333	13.59	0.001**
3	5.98	0.050*	1.58	0.455	12.93	0.002**
Mean	6.07	0.050*	2.18	0.347	14.40	0.001**
(b) Yolk total carotenoid content (µg)						
1	4.89	0.087	1.64	0.441	11.23	0.004**
2	5.00	0.082	0.74	0.692	8.19	0.017*
5	5.04	0.080	0.79	0.674	8.24	0.016*
6	5.08	0.166	1.73	0.630	11.92	0.008**
Mean	5.00	0.104	1.22	0.609	9.90	0.011*

The result remained the same for yolk total carotenoid content (calculated as for α-tocopherol content) (**Table 2.4 (d)**). Such that, the response to lay date continued to differ between treatment groups (w= 0.683, fat × lay date: β= -0.010 ±0.008, fat+VE × lay date: β= -0.003 ±0.006), although a greater level of uncertainty increased the size of the confidence set and reduced the significance of the difference between control and fat treatment groups (**Table 2.5 (b)**). Both year and the lay date by clutch size interaction maintained strong selection probabilities (w> 0.522) (**Table 2.4(d)**).

In comparison to α-tocopherol in the yolk, total carotenoid levels increased through the season, to a greater extent in smaller clutches, and deposition was reduced in 2010. But interestingly, whilst the provisioning of fat-plus-vitamin E did not affect variation in total carotenoid levels through the season, it appeared that fat-provisioning alone allowed females laying later in the season to invest in greater than average level of carotenoids (**Figure 2.3**, **Table 2.5**).

2.4.3 Effects of feeding on egg yolk antioxidants at a population level

Using nests at which females were sampled (n= 126), further analysis was carried out, to test whether the treatment group effect on yolk total carotenoid concentration was the result of variation on phenotypic condition. In this instance, total carotenoid concentration in female feathers was additionally included in the model as a proxy for condition (mean sample number at each site per year= 4.67 ±0.58 SE). A high level of uncertainty resulted in a confidence set of eleven models (all w_i <0.21, candidate set= 137 models), with the selection probabilities of treatment (w= 0.949), lay date (w= 1.000) and year (w= 1.000) again receiving strong support. However yolk total carotenoid levels were not explained by female feather carotenoid concentration (w= 0.190, β< 0.001 ±< 0.001), and there was no evidence of a combined effect of treatment and female condition (treatment × feather carotenoid interaction: outside confidence set).

Finally, to test the hypothesis that over-winter feeding alters phenotypic quality at the population level, changes of variance in α-tocopherol and total carotenoid concentrations between treatment groups were examined using a Levene's test for homogeneity of variance. Variance did not significantly differ in response to feeding treatment for either α-tocopherol ($F_{2,304}$= 1.28, p= 0.30) or total carotenoid ($F_{2,310}$= 2.07, p= 0.13) concentrations in the yolk.

2.5 DISCUSSION

This chapter has revealed that the effects of over-winter feeding are carried-over to influence some aspects of maternal investment in egg production. Firstly, in woodlands provisioned with vitamin E, females benefited from improved egg quality, by allocating larger yolks into their eggs compared to females in woodlands provisioned with fat only (**Figure 2.2**). Secondly, females from fat-fed woodlands showed improved antioxidant deposition as the season progressed; such that total carotenoid levels in the yolk were increased to greater extent later in the laying period than in the other treatment groups (**Figure 2.3**). Although there was some evidence to suggest over-winter feeding influenced other aspects of egg production (**Tables 2.1** and **2.4**), its effect was always

poorly substantiated and marginal in comparison to the effects of temporal and seasonal variation.

2.5.1 Maternal investment into egg production

It is commonly acknowledged that egg size is relatively inflexible within clutches in comparison to between individuals (Christians 2002), and the blue tit is no exception (Nilsson & Svensson 1993b). Although there is some evidence to suggest dietary provisioning can influence egg size, the evidence is equivocal (Robb et al. 2008a) and in general egg size variation is predominantly better explained by seasonal variation (Perrins 1996). It is perhaps, therefore, of little surprise that there was not strong support for an effect of over-winter feeding on egg mass in this study.

Variation in the yolk component of eggs, as a function of egg size, reflects the absolute difference in the nutrient and energy content of eggs, and as such relative yolk mass is an important determinant of egg quality (Williams 1994). Therefore, by proportionally increasing the yolk content of their eggs, females in woodlands supplemented with vitamin E through the winter could potentially benefit their chicks with improved structural growth, increased nutrient reserves and superior health status (Williams 1994; Biard et al. 2005; Krist 2009; but see **Chapter 4**). Although relatively little experimental evidence is available to make accurate inferences as to the benefits of increasing relative yolk mass; Peach and Thomas (1986) have demonstrated that in Canada geese the remaining yolk sac acts as an energy reserve several days post-hatching and a source of nutrients for rapid growth, whilst Finkler and colleagues (1998) similarly surmise that greater yolk content may provide a nutritional supplement influencing post-hatching development in chickens. Indeed as well as providing the major energetic requirements for early growth and development, the yolk comprises a cocktail of essential micronutrients and maternally-derived compounds known to influence offspring fitness (Royle et al. 1999; Gasparini et al. 2001; Groothuis et al. 2005). In addition the benefits of large eggs for offspring growth and survival are often attributed to increased egg yolk resources (Williams 1994).

The adaptive value of proportionally increasing yolk mass appears well substantiated, but it remains to determine the mechanism by which this carry-over effect of winter

feeding has been brought about. As income breeders, blue tits do not call upon endogenous macronutrient reserves for reproduction (Jonsson 1997), and therefore the greater uptake of fat from supplements over-winter will not have been utilised for egg formation. Indeed, females from fat provisioned woodlands did not increase yolk resource allocation accordingly. However, benefits to egg quality arose through the addition of vitamin E to over-winter supplements. Yolk mass has been shown to be a function of food availability in the days leading up to laying in income breeders (Ardia et al. 2006). This suggests that females feeding on these supplements were in a better position to acquire the resources necessary for yolk production, and that the carry-over effects of winter vitamin E supply led to improved condition and foraging efficiency during the egg laying period, over a month after provisioning ceased.

2.5.2 Egg yolk antioxidant capacity

The provisioning of vitamin E (α-tocopherol) across the winter period appeared not to influence α-tocopherol concentration in the yolk (**Table 2.4**), and no correlation was found between vitamin E and carotenoid concentrations (cf. Biard et al. 2009; Møller et al. 2010). Furthermore, antioxidant levels responded differently to annual and seasonal variation. Vitamin E concentrations fell over the laying period in all treatment groups, whereas total carotenoid concentrations increased. However interestingly, females within fat-only supplemented woodlands appear to have benefited from a significantly greater increase in total carotenoids in the yolk as the laying period progressed, compared to the other groups (**Tables 2.4, 2.5, Figure 2.3**).

In birds, early breeders are seen to be of superior condition, affording their offspring with higher survival and recruitment prospects, and having greater reproductive success (Price et al. 1988). Therefore it stands to reason that females laying earlier in the season should produce more eggs and of better quality (Arnold et al. 1991; Ardia et al. 2006), and as such possess greater levels of antioxidants. However to counterbalance this, caterpillar supply, which provides the main food resource for breeding tits (Perrins 1979), increases in number and quality across the laying period (Arnold et al. 2010), and is thought to be responsible for seasonal increases in yolk vitamin E and carotenoid levels in the collared flycatcher (*Ficedula albicollis*) (Hargitai et al. 2009). Vitamin E plays a crucial role in the antioxidant defence system, and despite its potential seasonal

increase in availability, it is apparent that females still face physiological trade-offs in its allocation, either to protect their developing offspring or to maintain their own condition. The results here suggest that vitamin E is a limiting resource for female blue tits, and that females laying later in the season are required to invest a greater proportion of their vitamin E intake to self maintenance, irrespective of whether over-winter supplements were provided. By comparison, the antioxidant capacity of carotenoids is less well reputed (Costantini & Møller 2008), suggesting that females could potentially afford to invest a greater proportion of their intake to egg production, resulting in a positive seasonal effect as carotenoid availability increased. The improved capacity for carotenoid deposition, seen here to result from over-winter provisioning, suggests that these females have gained benefits to condition. The effects of over-winter provisioning have been carried-over, enabling them to take greater advantage of the seasonal change in carotenoid availability either by improved foraging efficiency or reduced necessity to utilise acquired carotenoids for self maintenance.

It seems counterintuitive, however, that birds provisioned with fat over-winter, but not fat-plus-vitamin, demonstrated a greater capacity for total carotenoid deposition as the season progressed (**Figure 2.3)**; particularly in light of the benefits seen to yolk proportionality of vitamin E-supplemented birds, but not fat-only supplemented birds. This indicates there may have been a change in the phenotypic structure of the population as a result of vitamin E provisioning, such that it allowed relatively low-quality individuals to enter the breeding population and have a negative influence at a population level. Nest box occupancy, however, was not seen to vary as a result of feeding; although it is noted that the abundance and utility of natural nesting sites is unknown and as such a true measure of breeding density is unavailable. There was also no apparent increase in variance in antioxidant levels resulting from over-winter vitamin E provisioning, which might have suggested that feeding allowed for relatively high and low quality birds to breed concurrently. In addition there was little indication that variation in maternal condition between treatment groups was responsible for differences in total carotenoid concentrations. Though female feather colouration has previously been found to be correlated to breeding success in blue tits (Hidalgo-Garcia 2006; Doutrelant et al. 2008), it is indicative of an individual's ability to resolve carotenoid-based trade-offs during moult but may not necessarily reflect breeding

condition and therefore may not be a reliable signal of a female's ability to invest in egg production (Biard et al. 2005). Therefore differential effects of maternal condition between treatment groups may have been better approximated using a measure of condition at the time of laying as oppose to feather carotenoid concentration. These findings suggest that feeding does not result in a change to the phenotypic structure of the population (but see **Chapter 3**), though this conclusion has made it more difficult to establish a mechanism driving the benefits of fat over fat-plus-vitamin E winter provisioning.

It is possible that the effects of over-winter vitamin E provisioning on yolk antioxidant levels have been masked, through the analysis of only the last-laid egg. In great tit populations, vitamin E concentration significantly decreases by 40% through the laying sequence, and carotenoid levels fall by 37% in rural populations, though not urban (Hõrak et al. 2002). Lesser black-blacked gulls (*Larus fuscus*) also show a marked reduction in levels of vitamin E and carotenoids with each successive egg laid (Royle et al. 1999; Royle et al. 2001; Blount et al. 2002); and in addition, supplementary feeding has previously been shown to have a reduced effect on egg size through the laying sequence in blue tits (Ramsay & Houston 1997). In tits, it is hypothesised that mothers place greater investment in first-laid eggs because they are more likely to produce viable offspring under instances of adaptive brood reduction (Hõrak et al. 2002). With this in mind, it is possible that the greatest effects of vitamin E provisioning were felt in earlier-laid, more valued eggs only, and thus by analysing only the last-laid eggs adjustments to antioxidant deposition may have been less easy to detect. Examination of laying order effects on antioxidant deposition in blue tit clutches would certainly be of benefit to gain a better understanding as to whether this is the case.

2.5.3 Mechanisms driving carry-over effects of winter feeding

In testing the mechanisms driving carry-over effects derived from over-winter feeding three potential hypotheses were presented; carry-over effects may be the product of (1) condition improvement, (2) micronutrient storage, or alternatively (3) a change in the phenotypic structure of the population.

Since α-tocopherol levels in the yolk were not strongly affected by α-tocopherol supplementation it appears unlikely that micronutrients are stored during winter for later use during egg production. But rather, it seems there is an indirect effect of over-winter provisioning on maternal condition. The uptake of additional energy may have fuelled metabolism during the harsh winter months, whilst the greater uptake of vitamin E is likely to have reduced oxidative stress and enhanced immune defences (Surai 2007). Supplemented females, therefore, may have entered the breeding season in better condition, either becoming more efficient foragers or able to invest a greater proportion of newly-acquired resources to egg production. This is best exemplified by the greater investment placed into yolk proportionality by females from vitamin E-fed woodlands.

The apparent improved total carotenoid allocation in yolks by females from fat-supplemented but not vitamin E-supplemented woodlands provides some support for the final hypothesis. However this was poorly substantiated, with no evidence that relatively poor individuals were breeding in the vitamin E treatment group or that phenotypic structure had been altered.

2.5.4 Conclusions

Treatment featured in all confidence sets with varying degrees of weighting and estimation precision. Given the nature of the experiment undertaken (whereby food was provisioned at a population level several weeks and months prior to the onset of breeding), the ability to detect a signal of over-winter feeding treatment gives firm indication that there are carry-over effects occurring. Robb et al. (2008b) showed blue tits supplemented with peanuts over-winter to have improved breeding success, whilst studies of migratory birds have also found similar effects inter-seasonal effects (Marra et al. 1998; Norris et al. 2004). Variation in natural food availability, differences in foraging and assimilation efficiency and competition at feeders will all have led to a great deal of variation in supplement uptake at the population level, and as such the carry-over effects of winter feeding on egg quality can be further substantiated at an individual level (see **Chapter 5**).

Although estimated as a valuable commodity in avian ecology, this is in fact the first study to test the effect of vitamin E supplementation on egg composition in a wild bird

population. The findings illustrate that provisioning vitamin E in winter food supplements leads to improved egg quality, and it is hypothesised that this is the result of an indirect effect on maternal condition. As popularity for garden birding feeding grows, this study highlights a potential benefit that over-winter feeding could have for wild birds in the subsequent breeding season; though it remains to be seen whether the benefits of over-winter provisioning to egg quality generate fitness consequences.

CHAPTER 3

The effects of over-winter feeding on phenotypic variation in breeding blue tits *Cyanistes caeruleus*

3.1 ABSTRACT

Resource limitation produces fundamental investment trade-offs between reproductive effort and subsequent survival. As a consequence, parents of superior condition are reported to provide better care, and produce offspring of higher quality which have improved survival prospects. Dietary antioxidants may be an important resource which underpin life history trade-offs, controlling levels of oxidative stress which might otherwise lead to disease and aging processes. Antioxidant supplementation has previously highlighted benefits for health and productivity in birds during the breeding season, however it is unknown whether micronutrient benefits might also be carried over from the preceding winter. In this chapter I have investigated the effects of energy and vitamin E-based over-winter supplements on blue tit (*Cyanistes caeruleus*) population structure and parental phenotypes during the breeding season. Mean concentrations of total carotenoids in the feathers of breeding birds were significantly lower within vitamin E supplemented woodlands, an indication that birds of inherently lower quality had recruited into the breeding population as a result of enhanced winter antioxidant availability. Neither feeding treatment nor feather carotenoid levels were strong predictors of plasma antioxidant concentrations, or body mass variation during the breeding season. However, males suffered lower levels of oxidative stress (plasma malondialdehyde; MDA) following vitamin E provisioning. These findings suggest that provisioning of antioxidant-rich supplements enhance over-winter survival, therefore influencing population structure and producing benefits for condition which are still distinguishable in the later stages of the brood-rearing period. In addition, it appears that males and females utilise resources differentially and it is hypothesised that benefits to male condition may be reflected in nestling provisioning performance. An investigation of brood provisioning activities during one breeding season illustrated greater variability in provisioning rates early in the brood-rearing period, when males take on the majority of feeding responsibility. These results highlight the impacts of garden bird feeding on wild bird health status, and provide further insight into the results presented in **Chapter 2**. It now remains to investigate if over-winter provisioning ultimately influences reproductive success.

3.2 INTRODUCTION

According to life-history theory, the benefits of increased parental investment into reproduction will be counterbalanced by costs to survival and future fecundity (Stearns 1989). Under conditions of limited resource availability, both males and females face a fundamental decision of how many resources to invest into current reproduction, and how much to retain for self-maintenance. As such, individuals of higher quality and condition during the breeding season can afford to allocate more time and resources to reproduction; thus benefiting from improved reproductive success (McNamara & Houston 1996; Hõrak et al. 2001).

For many bird species, the brood-rearing phase represents a time of intense energetic demand (Bryant & Tatner 1991). Elevated parental effort during this period has been shown to impose high costs on current condition and subsequent survival (Nur 1984a; Hõrak 1995), and it is hypothesised that oxidative stress may be a proximate mechanism responsible for these observed reproductive costs (Nilsson 2002; Alonso-Alvarez et al. 2004b; Wiersma et al. 2004). Oxidative stress arises when the production of harmful reactive oxygen species (ROS) exceeds an individual's capacity to neutralise them via the antioxidant system. ROS, comprising a collection of pro-oxidant molecules, are the highly reactive by-products of normal metabolic activity (Finkel & Holbrook 2000). As parental birds increase their metabolic rate to meet the demands of a growing brood (Drent & Daan 1980; Bryant & Tatner 1991), they risk a build-up of ROS and damaging repercussions for DNA, protein and lipid macromolecules (Finkel & Holbrook 2000). Indeed, increased reproductive effort, induced by experimental brood size manipulation, has previously been shown to produce a trade-off in oxidative protection in zebra finches (Wiersma et al. 2004), and proliferate weight loss and reduce survival prospects in blue tits (Nur 1984a).

The detrimental effects of oxidative stress are prevented through sufficient antioxidant protection. Dietary antioxidants, such as vitamin E (of which α-tocopherol is the major constituent) and carotenoids, function by breaking the chain reactions of lipid peroxidation, thereby neutralising the harmful effects of ROS (Sies & Stahl 1995). Synergistic relations also exist between these two groups, such that the recycling properties of many carotenoids allow for relatively low levels of vitamin E to still

provide high levels of protection (Surai 2007). However, since these valuable resources must be obtained through the diet, their availability is potentially limiting to breeding birds (Olson & Owens 1998). With food availability notably a critical determinant of performance during brood rearing, food supplementation studies have revealed benefits to parent provisioning activities, condition and survival, as well as nestling development and fledging prospects (Simons & Martin 1990; Cucco & Malacarne 1997; Davis et al. 2005). At this time supplemented food may relieve the pressures of prey location, or provide an energetic boost to improve the foraging efficiency of natural food items (Grieco 2002; Markman et al. 2002). But secondary to this, the provision of antioxidants specifically has highlighted improvements to antioxidant defences and provisioning performance during brood-rearing (Biard et al. 2005; Remeš et al. 2007). However, whilst many studies have observed and empirically tested the consequences of variable food availability, they have tended to target food at individuals and at a focused point of the breeding season only. But how ecological realistic this is, is uncertain, since food is normally more patchily distributed within the natural environment and birds are faced with foraging costs and competition for high quality resources. A prominent example of the wider ecological impacts of food availability is in the context of garden bird feeding, however little has been done to test this.

Food supplementation of wild bird populations occurs in great abundance in gardens, both in the UK and globally (O'Leary & Jones 2006; Robb et al. 2008b). This prolific and expanding phenomenon has been identified as a having potentially far reaching consequences for avian ecology (Robb et al. 2008a). The majority of garden bird feeding occurs during winter, where fat and energy gained at feeders is seen to be highly beneficial in fuelling over-winter survival (Jansson et al. 1981; Brittingham & Temple 1988; Gosler 1996). As such it has generally been assumed that energy is the primary currency mediating carry-over effects of winter food availability on subsequent reproduction (e.g. Norris et al. 2004; Robb et al. 2008b). However, the potential for micronutrients to generate carry-over effects is recently acknowledged, and could be particularly important for small passerines which, as income breeders, are not assumed to use endogenous reserves to fuel reproduction (Harrison et al. 2011).

It is predicted that antioxidants acquired at feeders may assist in combating a ROS build up, enabling birds to enter the breeding season in a superior condition and having suffered less debilitation due to parasites, diseases and oxidative damage during winter (Alonso-Alvarez et al. 2004a; Larcombe et al. 2008). Furthermore, micronutrients have the potential be stored in lipid-rich tissues, and therefore may be called upon during periods of increased demand (Negro et al. 2001; Surai 2007). As such, the effects of antioxidants gained though winter dietary provisioning are likely to be more substantial than energy only, with the potential to influence phenotypic variation in breeding bird populations. However, surprisingly the consequences of winter antioxidant availability on population structure and individual condition during the breeding season have not previously been investigated.

In this chapter, I have experimentally tested the effects of energy and antioxidant-based over-winter supplements on parental phenotype during the brood-rearing period in wild populations of blue tits (*Cyanistes caeruleus*). Blue tits exhibit carotenoid-based yellow breast plumage (Partali et al. 1987), which is hypothesised to be a reliable signal of individual quality at the time of feather growth, and therefore prior to winter feeding (reviewed in Møller et al. 2000). Using feather total carotenoid concentration as an indicator of 'inherent quality', the aims of the chapter were: (1) to establish whether winter feeding altered population structure, by influencing the quality of birds entering the breeding population, or increasing variation in parental condition within populations; (2) to determine whether feeding influenced individual condition or susceptibility to oxidative stress during brood-rearing, by increasing circulating antioxidants and body mass or reducing malondialdehyde (MDA) concentration in the plasma, a by-product of lipid peroxidation and commonly used biomarker of oxidative stress (Monaghan et al. 2009); and (3) to investigate the benefits of winter feeding on brood rearing capacity in an isolated analysis of parental provisioning rates and foraging efficiency. Finally, since males and females assume different roles prior to brood-rearing and are likely to utilise provisioned resources differentially due to greater male dominance at feeders (Hegner 1985; Robb et al. in press), it is anticipated that the effects of over-winter supplementation will manifest themselves differently in males and females at this late stage of the breeding season.

3.3 METHODS

3.3.1 Study sites, experimental design and supplementary feeding

The over-winter supplementary feeding experiment was conducted over three years from 2007 to 2009, and the carry-over effects on parental phenotype were measured during the subsequent breeding seasons, 2008 – 2010 respectively. The study took place in Cornwall, UK, across nine deciduous woodland sites. All sites and prescribed feeding treatments were as described in **Chapter 2 §2.3**. In brief, woodland sites, largely comprising oak (*Quercus* spp.), beech (*Fagus sylvatica*), sweet chestnut (*Castanea sativa*) and sycamore (*Acer pseudoplatanus*), were grouped into three triplets according to composition and common features. Within sites, nest boxes were evenly distributed at a density of *ca*. 4 boxes per hectare, giving a total of 346 boxes across all woodlands.

In the first year of the study, each site within a triplet was randomly allocated to one of three feeding treatments. These treatments were: *(1) no supplement* (hereafter 'control'), *(2) fat only* (to test for energy effects; hereafter 'fat'), and *(3) fat-plus-vitamin E* (to test for effects of energy plus antioxidants, hereafter 'fat+VE'). Treatments groups were then rotated within triplets across years, so that every site received all three treatments over the course of the study. Potential confounding effects of year have been prevented through the replication on treatment groups within years.

Supplementary food was provisioned through the winter only (14 December – 4 March 2007/08; 18 November – 11 March 2008/09 and 2009/10), leaving a gap of at least one month before laying commenced (8 April, 11 April, 15 April respectively). Squirrel-proof feeders were positioned 100m apart within the six fed sites each year (*ca*. 9 per site), and provisioned with a fresh 150g fat ball every 10 days. For the fat+VE treatment group, fat balls were supplemented with 10mg/ 100g α-tocopherol (≥96% DL-all-*rac*-α-Tocopherol (HPLC), Sigma-Aldrich Ltd., Dorset), a concentration equivalent to that occurring naturally in peanuts (Chun et al. 2005). Since α-tocopherol cannot be provisioned to wild birds without the use of a 'carrier' and fat is required for its absorption (Blount et al. 2002; Jeanes et al. 2004), the fat-plus-vitamin E treatment group provides an ecologically meaningful method of testing antioxidant effects. All

supplements were prepared and utilised by the target species as described in **Chapter 2 §2.3.2**.

3.3.2 Breeding parameters

All nest boxes occupied by blue tits were checked every other day to determine clutch completion date and the onset of incubation. Incubation length is typically 12 – 14 days in the blue tit (Perrins 1979), therefore from 11 days after clutch completion nests were re-visited every 1 – 2 days to determine hatch date (day 0). Due to logistical constraints, it was not possible to make daily visits to all sites; therefore hatching date was estimated according to the proportion of hatched eggs, female attendance and size of chicks. Brood size reduction is common in blue tits, particularly in the early nestling phase where dead chicks are removed from the nest by parents. Therefore, nests were visited *ca.* day 6 and day 12 of the nestling phase to monitor brood size changes, and brood size was recorded on the day of parental sampling.

Increased reproductive effort can lead to trade-offs in oxidative protection for both males and females (Wiersma et al. 2004). A measure of reproductive effort over the brood rearing period was therefore generated, as a means of controlling for variation in brood size as well at the stage of the nestling phase at which individuals were sampled. This was done using 'nestling-days' up until the point of parental sampling following Mayfield (1975), such that the number of nestlings in a nest each day between day 1 and the day of parental sampling were summed. For example, an adult with a brood size of 7 with no instances of chick mortality which was sampled at day 13 would have a reproductive effort of 91 (7 × 13). Since brood size was not recorded daily, in instances of chick mortality a mean brood size was drawn for the unknown days from the known values either side.

3.3.3 Parental sampling

Either one or both parents were sampled at a subset of nest boxes each year (total independent nests, n= 243; total individuals, n= 351). Adults were predominantly captured on the nest using spring traps (Amber Electronics Ltd, Daventry, UK) between day 8 – 17 of the nestling phase, to limit risk of desertion or premature fledging (mean

nestling age at sampling, 13.06 days ±3.30 SD, n=351; mean nestling age at fledging across study populations recorded in 2008, 20.20 days ±1.36 SD, n=93). Twelve individuals were opportunistically captured at day 5 – 7 during routine nest visits, but were not blood sampled to reduce time away from nest, and six individuals were captured 18 days post-hatching where nestlings were visibly under-developed and not at risk of early fledging. Adults were sexed according to the presence (female) or absence (male) of a brood patch, individually identified with a BTO aluminium leg ring, and aged (1 or >1 year) according to the coloration of the primary coverts following Svensson (1992). Body mass (±0.1g) was recorded using an electronic balance, and head-bill length (±0.05mm) measured twice with dial callipers then averaged (Redfern & Clark 2001).

Tissue samples were collected under Home Office license (PIL 30/8161). A feather sample was plucked from a standardized position on the yellow breast of each bird and stored in a sealed bag in the dark until later biochemical analysis (as described in **Chapter 2**). A blood sample (*ca.* 60 μl) was collected from the brachial vein into an EDTA-coated capillary tube (Bilbate Ltd., Daventry, UK), using venipuncture with a 27-gauge Microlance^{TM} needle (Fisher Scientific UK Ltd.). Blood samples were centrifuged (4min; 13,000 × g) and stored in a polystyrene cool box in the field (mean time stored in field ±SE: 386.8 ± 9.7 mins). When returned to the laboratory, plasma was removed for storage at -80°C until biochemical analysis. The length of time between blood sample collection and freezing did not influence levels of MDA (Pearson's: r= 0.026, n=171, p= 0.78) or α-tocopherol (r= -0.05, n=124, p= 0.48) in the plasma, but it did have a marginal effect on total carotenoid levels (r= -0.16, n=136, p= 0.06). However, preliminary model selection of main effects models revealed this not to be an important predictor term (95% confidence intervals around the parameter estimates included zero), and so it was excluded from further analyses.

3.3.4 Brood provisioning

Brood provisioning behaviour was monitored during the first breeding season only (2008) at two stages of the nestling phase – early (day 3 [± 1 day] post hatching, n= 61) and late (day 11 [± 1 day], n= 65). There was no difference in the average age of nestling at sampling for each stage between treatment groups (GLMs: p> 0.24), and age

variation did not significantly influence provisioning rates (Poisson GLMM with site random factor: $p > 0.08$) or foraging times (GLMM with site random factor: $p > 0.20$) at either stage of the nestling period. Feeding activity was filmed using Sony DCR – HC37E Handycams, mounted on tripods *ca.* 2 m from the tree base and at an angle perpendicular to the nest box. Following a 10 min settling period, 30 min observations were conducted at 0630 GMT or 0830 GMT, to optimise filming opportunities across days and sites. Filming time did not differ between treatments or nestling stages (binomial GLMs: $p > 0.15$, n=126). Prevailing weather conditions were recorded following filming, using three categories – sunny, raining or overcast – since this can influence brood provisioning activities (Radford et al. 2001). Provisioning rate and time spent foraging (hereafter referred to as 'foraging time') were extracted from video playbacks; where provisioning rate was defined as the number of visits per 30 min, and foraging time was defined as the mean interval between prey deliveries per 30 min. Adults could not be individually identified, and therefore were treated as a single unit.

3.3.5 Feather total carotenoid level determination

Levels of total carotenoids in the yellow breast feathers of parent birds were determined by mechanical extraction and spectrophotometry as described in **Chapter 2 §2.3.5**. Feather total carotenoid levels were successfully analysed for 335 individuals (female= 179, male= 156).

3.3.6 Biochemical analysis of plasma for antioxidants and oxidative stress

Antioxidants were extracted from blood plasma following methods previously described by Blount (2003). A 10µl aliquot of plasma was mixed with 10µl of 5% sodium chloride and 20µl ethanol, then vortexed for 20 seconds. 600µl of hexane was added and the mixture vortexed for a further 20 seconds, then centrifuged for 4 minutes (13,000 × g). The hexane phase was drawn off and total carotenoid concentration determined by spectrophotometry (spectrophotometer conditions as described in **Chapter 2 §2.3.5.1**). 500µl of hexane extract was dried and the residue re-dissolved in 150µl methanol for α-tocopherol determination by high-performance liquid chromatography (60µl injection volume; HPLC conditions and standards as described in **Chapter 2 §2.3.5.1**).

Plasma concentrations of malondialdehyde (MDA) were determined by HPLC, following Agarwal & Chase (2002). A 10µl aliquot of plasma was vortex mixed with 10µl of butylated hydroxytoluene (BHT) (0.05% w/v in 95% ethanol), 80µl of phosphoric acid (0.44 M) solution and 20µl of 2-thiobarbituric acid (TBA) (42 mM), then heated at 100°C for 1 hour in a dry bath incubator. After cooling on ice (5 min), 50µl of *n*-butanol was added and the mixture vortexed for 20s, then centrifuged for 3 minutes at 4°C (13,000 × g). The upper phase, containing the MDA-TBA adduct, was recovered and 20µl injected into an HPLC system (Dionex Corporation, California, USA) fitted with a Hewlett-Packard Hypersil 5µ ODS 100 x 4.6 mm column and a 5µ ODS guard column, maintained at 37°C. A mobile phase of methanol-buffer (40:60 v/v) was run at a flow rate of 1ml min^{-1}; using a buffer of 50mM potassium monobasic phosphate adjusted to pH 6.8 using 5M potassium hydroxide. Fluorescence detection (Dionex RF2000) was performed at 515 nm (excitation) and 553 nm (emission). Plasma MDA concentrations were calibrated using standards of 1,1,3,3-tetraethoxypropane (TEP) serially diluted with 40% ethanol in a parallel assay.

Due to limited sample volumes, plasma from 124, 136 and 172 individuals were successfully analysed for α-tocopherol, total carotenoids and MDA concentrations respectively.

3.3.7 Statistical Analysis

All statistical analyses were conducted using R version 2.12.2 (R Development Core Team 2011). To test the hypothesis that over-winter feeding alters population phenotypic structure, Levene's tests for homogeneity of variation were performed to compare the magnitude of relative variance between treatment groups for measures of parental condition and brood provisioning. Relative variation in condition measures were also compared between treatment groups within the two sexes and three breeding seasons.

3.3.7.1 Analysis of parental condition

A general linear mixed model (GLMM) was applied to feather total carotenoid concentration, to test the hypothesis that feeding affected the phenotypic structure of breeding populations. Feather carotenoid concentrations were correlated within

breeding pairs (Pearson's correlation: r= 0.40, n= 108, $p< 0.001$), therefore 'pair' was included in the random term, nested within site, to control for non-independence. GLMMs were also applied to plasma α-tocopherol, total carotenoid and MDA concentrations and to body mass, to test the influence of over-winter feeding and pre-feeding quality on parental phenotype during brood rearing. Here, a hierarchical nested random effects term was used – breeding pair nested within box identity nested within site – to control for non-independence of males and females within a pair, and temporal and spatial pseudoreplication. 39 individuals were sampled in repeated years, but did not provide enough power for inclusion in the random term. These analyses were performed in two stages, since treatment, sex and feather carotenoid covariate interactions were all of interest but resulted in overparameterisation of the full models.

GLMMs were conducted using 'lme' from the *nlme* package (Pinheiro et al. 2010). Sequential (type I) sums of squares (SS) were applied, which are more robust to unbalanced designed, with treatment fitted last as a three level factor (Hector et al. 2010). For all dependent variables, males and females were analysed together with sex included as a fixed factor. In addition annual variation was controlled for by the inclusion of year (2008, 2009 or 2010), and parental age (1 or >1 year) was fitted to account for differences between yearlings and older birds (Perrins & McCleery 1985; Delhey & Kempenaers 2006). For analysis of breeding condition measures, temporal variation was controlled using hatch date, and reproductive effort, as defined in §3.3.2 above, was included to account for effects of brood size and nestling stage (Nur 1984a; Wiersma et al. 2004). Skeletal body size was controlled for when analysing body mass by the inclusion of head – bill length. Time of capture was significantly correlated with increases in body mass (Spearman's rank: rs= 0.20, n=318, $p<0.001$), total carotenoid (rs= 0.190, n=136, $p= 0.027$) and MDA concentrations (rs= 0.178, n=171, $p= 0.021$). However, preliminary model selection of main effects models revealed this not to be an important predictor term (95% confidence intervals around the parameter estimates included zero), and so it was not included in further analyses to prevent over-fitting of models. All 2-way interactions involving treatment were included, to test for differences in main effects between treatment groups. Sex × reproductive effort and sex × age interaction terms were also fitted (where applicable) to account for differential sex effects. Overparameterization was avoided, since the number of estimated parameters

was less than *n*/3 in all cases (Crawley 2007). Following these initial analyses, models were re-fitted, including feather carotenoid concentration as a covariate, to estimate the influence of pre-feeding quality. For the second set of analyses only fixed effects within the original confidence sets were re-fitted, together with feather carotenoid concentration and its 2-way interactions.

Normality and heteroscedasticity of residuals from the global model were checked prior to model selection to test model fit. Concentrations of α-tocopherol in the plasma and total carotenoids in feathers and plasma were log-transformed, whilst MDA concentrations were square root-transformed to correct normality.

3.3.7.2 Analysis of parental provisioning behaviour

Provisioning rates were examined using a generalised linear mixed effect model (GzLMM) fitted with a Poisson error structure, using 'lmer' from the *lme4* package (Bates et al. 2011). Residuals were checked for overdispersion prior to model selection. Foraging time was analysed using a GLMM, and log-transformed to correct normality following examination of the residuals. In each analysis nest box nested within woodland site was included as the random term, to control for repeated measures and spatial replication. Treatment (control, fat, fat+VE) and nestling stage (early, late) were included as a fixed factors. Hatching date and brood size at filming were fitted as co-variates, since provisioning behaviour is associated with temporal variation in caterpillar abundance and is greater for larger broods (Nur 1984c; Naef-Daenzer & Keller 1999). In instances of brood mortality, the brood size was taken as the average between two known values (≤ 6 days apart), since brood size was not always recorded on the day of filming. Effects of weather were controlled for in both analyses. Provisioning rates differed between filming times (Poisson GLMM with site random factor: $\chi^2_2 = 10.91$, $p = 0.001$); therefore time of filming, and its interaction with nestling stage, were controlled for provisioning rate analysis only. There was no effect of time of filming on foraging time (GLMM with site random factor: $p = 0.83$).

3.3.7.3 Model selection and averaging

For analysis of feather carotenoid concentration difference between treatments, the best-fitting model was constructed by stepwise deletion of the least significant term until only significant terms ($p < 0.05$) remained in the model. To investigate the impacts of over-winter feeding on condition and provisioning performance, an information-theoretic approach was used for model selection, and multimodel inference used to estimate the parameters (Burnham & Anderson 2002; Johnson & Omland 2004; Symonds & Moussalli 2011), as described in **Chapter 2**. All possible models, given the explanatory variables described, were compared using the 'dredge' function implemented in the package *MuMIn* (Bartoń 2011). Models were ranked according to their relative AICc values, correcting for sample size bias since $n/k < 40$ in all cases (Burnham & Anderson 2002). AICc is a comparative measure of model fit and complexity, where the best fitting model has the lowest AICc value and all other models are ranked according to their difference in AICc from the top model (ΔAICc). Model averaging was applied across a confidence set of well supported models, where $\Delta AICc \leq 2.0$ (Burnham & Anderson 2002), and averaged parameter estimates (β) and standard errors (SE) generated along with selection probabilities of explanatory terms (*w*). A pseudo-R^2 value was generated for the top ranking model to estimate variance explained by the model and to evaluate model fit, following Nagelkerke (1991), as described in **Chapter 2**. *Post hoc* pairwise comparisons were carried out to assess between treatment group differences, using comparisons of the original ranked GLMM model with models in which treatment groups were paired. This was repeated for all models in the confidence set featuring the treatment effect or interaction of interest. Where appropriate, variance components analyses were carried out using top ranking models fitted with restricted maximum likelihood (REML), to estimate proportions of total variation explained at each level of the random term (Crawley 2007).

3.4 RESULTS

3.4.1 Quality of individuals entering the breeding population

To investigate the quality of individuals entering the breeding season, differences in feather total carotenoid concentrations between over-winter feeding treatments were

tested. Feeding treatment significantly influenced the quality of individuals within the breeding population (GLMM; χ^2_2= 8.52, p= 0.014), with individuals from fat+VE fed woodlands having significantly lower concentrations of carotenoids in their feathers compared to control and fat-fed birds (**Figure 3.1**). Feather carotenoids values also differed between years (GLMM; χ^2_2= 16.76, p< 0.001), and males were marginally brighter than females, though this was not significant (GLMM; χ^2_2= 3.23, p= 0.07). There was no significant interaction between treatment and sex (p= 0.418).

There was no evidence that over-winter feeding treatment resulted in greater variation in α-tocopherol (Levene's test; $F_{2,121}$= 0.28, p= 0.75) or total carotenoid (Levene's test; $F_{2,133}$= 2.06, p= 0.13) levels in the plasma of adults within fed populations. Furthermore the variance in body mass (Levene's test; $F_{2,347}$= 0.12, p= 0.88) and levels MDA in plasma (Levene's test; $F_{2,168}$= 1.15, p= 0.32) were not significantly different among treatment groups. These finding were consistent across both sexes and all years (Levene's test; all p> 0.16).

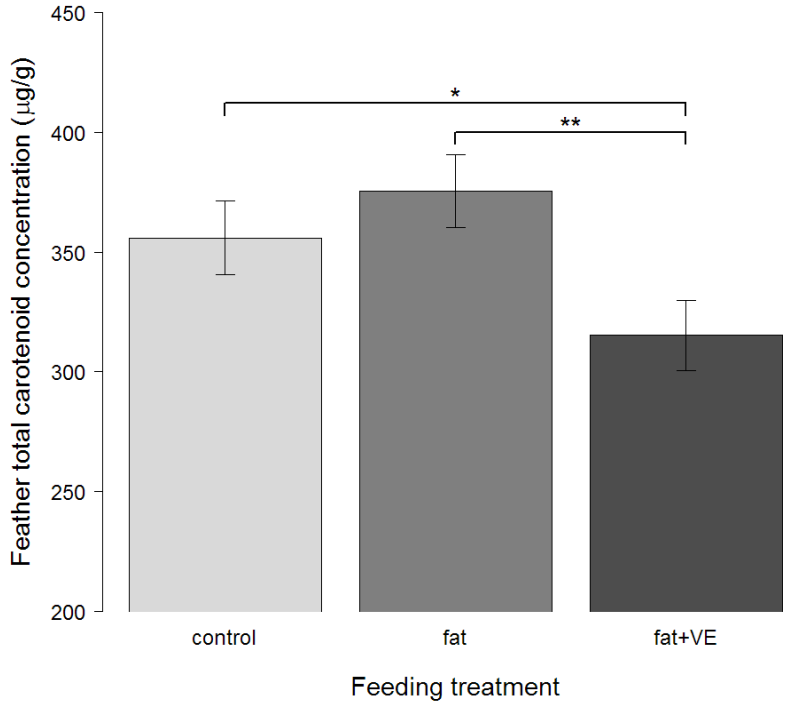

Figure 3.1. Total carotenoid concentrations in the feathers of breeding adults in relation to over-winter feeding treatment. Mean ± SE plotted using raw values. *Post-hoc* pairwise comparisons shown, where * = $p \leq 0.05$; ** = $p \leq 0.01$.

3.4.2 Parental phenotype during brood rearing

3.4.2.1 Antioxidant concentrations in the plasma

Concentrations of α-tocopherol in the plasma varied considerably amongst individuals (mean±SE: 22.95 ±1.04 μg/ ml, range: 3.30 – 70.91 μg/ ml), but were not influenced by over-winter feeding treatment (no models containing treatment or treatment-interactions within 2 ΔAIC confidence set) (**Table 3.1 (a)**). By comparison, differences in age and year were the best predictors of α-tocopherol variation (both *w*= 1.000; **Table 3.2**); older birds had lower levels of α-tocopherol (β= -0.274 ±0.090), and mean concentrations were greater in 2009 (2009: 28.67 ±1.35 μg/ ml, β= 0.672 ±0.119) and to a lesser extent in 2010 (2010: 20.77 ±1.89 μg/ ml, β= 0.279 ±0.131) than those in the first breeding season (2008: 13.26 ±0.89 μg/ ml). Although they were selected within the candidate set, there was little evidence that sex (*w*= 0.363) and hatching date (*w*= 0.463) influenced α-tocopherol concentration (**Table 3.2**) since 95% confidence intervals for the parameter estimates included zero. Furthermore, a variance components analysis revealed that 84.17% of variation could be attributed to inter-individual differences. Woodland site and nest box where responsible for a small amount of variation in α-tocopherol (7.30% and 8.53% respectively), but interestingly no variation was attributed to parents within the same breeding pairs (0.00%).

Males were found to have higher levels of total carotenoids in the plasma on average than females (males: 31.99 ±1.71 μg/ ml, β= 0.501 ±0.127; females: 21.36 ±1.08 μg/ ml) (*w*= 1.000; **Table 3.1 (b)** and **3.2**). In addition there was a relatively high level of support for a difference in male and female carotenoid concentrations in response to feeding treatment (treatment × sex interaction *w*= 0.633) (**Table 3.1 (b)** and **3.2**). Whilst females had similar total carotenoid concentrations between treatment groups (control: 22.46 ±2.20 μg/ ml; fat: 22.30 ±1.50 μg/ ml, β= -0.033 ±0.099; fat+VE: 18.71 ±1.67 μg/ ml, β= -0.091 ±0.121), males showed reduced levels in the fat group (control: 34.48 ±2.65 μg/ ml; fat: 27.80 ±2.41 μg/ ml, β= -0.093 ±0.150; fat+VE: 35.88 ±3.60 μg/ ml, β= 0.160 ±0.182). However high standard errors around the parameter estimates indicate that these treatment differences are not an accurate predictor of plasma total carotenoid concentration. As anticipated, increasing reproductive effort was correlated to a reduction in plasma carotenoid levels across both sexes (*w*= 1.000, β= -0.003 ±0.001), but this effect was not negated by over-winter supplementation (treatment ×

Table 3.1. Confidence sets of ranked models (in descending order) for analyses of parental phenotype measures. See § 3.3.7 for description of global models and modelling terms, Table 3.2 for relative variable importance and model fit, and text for parameter estimates (β).

Rank	Fixed effect	*k*	Log-likelihood	AICc	ΔAICc	w_i
(a)	α-tocopherol concentration (μg/ ml)					
1	age + year	8	-71.82	160.9	0.000	0.346
2	age + year + hatch dt	9	-70.83	161.2	0.347	0.291
3	age + year + sex	9	-71.25	162.1	1.193	0.191
4	age + year + hatch dt + sex	10	-70.13	162.3	1.398	0.172
812	*Intercept-only*	*5*	*-97.39*	*205.3*	*44.396*	*0.000*
(b)	Total carotenoid concentration (μg/ ml)					
1	(treat × sex) + effort + (sex × age)	13	-57.69	144.4	0.000	0.203
2	(treat × sex) + effort + age	12	-58.93	144.4	0.020	0.201
3	(treat × sex) + effort + age + year	14	-57.02	145.5	1.133	0.115
4	(treat × sex) + effort + (sex × age) + year	15	-55.75	145.5	1.140	0.115
5	effort + sex + age + year	10	-62.03	145.8	1.449	0.098
6	effort + (sex × age) + year	11	-60.89	145.9	1.542	0.094
7	effort + sex + age	8	-64.40	145.9	1.568	0.093
8	effort + (sex × age)	9	-63.37	146.2	1.804	0.082
812	*Intercept-only*	*5*	*-86.44*	*183.4*	*38.982*	*0.000*
(c)	MDA concentration (μg/ ml)					
1	(treat × sex) + year + hatch dt	13	-53.91	136.1	0.000	0.372
2	(treat × sex) + year	12	-55.44	136.9	0.714	0.261
3	(treat × sex) + year + (treat × hatch dt)	15	-52.05	137.2	1.063	0.219
4	(treat × sex) + year + hatch dt + age	14	-53.64	138.0	1.838	0.149
522	*Intercept-only*	*5*	*-124.74*	*259.8*	*123.699*	*0.000*
(d)	Body mass (g)					
1	head + year + effort + (sex × age)	12	-295.35	615.6	0.000	0.208
2	head + year + effort + (sex × age) + hatch dt	13	-294.69	616.5	0.838	0.137
3	head + year + effort + (sex × age) + (treat × year)	18	-289.21	616.5	0.859	0.135
4	head + year + effort + (sex × age) + treat	14	-293.71	616.7	1.062	0.122
5	head + year + effort + sex + age + year	11	-297.08	616.9	1.325	0.107
6	head + year + effort + (sex × age) + (sex × effort)	13	-294.95	617.0	1.369	0.105
7	head + year + effort + (sex × age) + treat + hatch dt	15	-292.89	617.2	1.590	0.094
8	head + year + effort + age	10	-298.30	617.3	1.634	0.092
2622	*Intercept-only*	*5*	*-351.67*	*713.5*	*97.895*	*0.000*

Model terms: treat, feeding treatment (control, fat, fat+VE); sex (female, male); age (1, >1 year); year (2008, 2009, 2010); hatch dt, hatch date [1= 1April]; effort, reproductive effort (as defined in text); head, head – bill length; (×), interaction term. Intercept-only models reported in *italics* with the unadjusted Akaike weight.

N. models in candidate sets= 583 (n= 124), 583 (n= 136), 583 (n= 172) and 1165 (n= 350) respectively.

reproductive effort interaction outside 2 ΔAIC confidence set, **Table 3.1 (b)** and **3.2**). Carotenoid concentrations were also found to be lower in older birds (w= 1.000, β= -0.134 ±0.106), with the difference more prominent for males (sex × age; w= 0.422, β= -0.093 ±0.137).

Furthermore, there was little evidence that α-tocopherol or total carotenoid levels in the plasma were affected by pre-feeding condition, or its interaction with treatment. For α-tocopherol, the confidence set remained almost indifferent after the inclusion of feather carotenoid concentration as a predictor term (w= 0.193, β<0.001 ±0.000) and this was consistent for all treatments (treatment × feather carotenoid interaction outside 2 ΔAIC confidence set). Whilst for total carotenoids, feather carotenoid concentration as a predictor, and all interactions, were outside the confidence set. Concentrations of α-tocopherol and total carotenoids in the plasma correlated positively and significantly with one another in 2009 (Pearson's: r= 0.43, n=59, p= 0.001) and 2010 (r= 0.58, n=39, p< 0.001), though not in 2008 (r= 0.03, n=26, p= 0.88).

Table 3.2. Relative importance of explanatory variables (Akaike weights, w) in analyses of feeding treatment effects on parental phenotype measures. * Denotes variables for which 95% confidence intervals for parameter estimates do not include zero. See Table 3.1 for confidence sets of ranks models and text for details of parameter estimates (β).

	Plasma α-tocopherol	Plasma carotenoids	MDA	Body mass
Treatment		0.633	1.000	0.351
Sex	0.363	1.000 *	1.000	0.908
Age	1.000 *	1.000	0.149	1.000
Year	1.000 *	0.422	1.000 *	1.000 *
Hatching date	0.463		0.739	0.231
Reproductive effort		1.000 *		1.000 *
Head – bill length	—	—	—	1.000 *
Treatment × sex		0.633	1.000 *	
Treatment × year				0.135
Treatment × hatch date			0.219	
Sex × age		0.422		0.801
Sex × reproductive effort				0.105
Pseudo-R^2	0.427	0.463	0.736	0.318

— denotes variable not included in global model. Each global model contained all listed terms, with the exception of head – bill length. A treatment × age interaction term did not feature in any confidence sets.

Plasma concentrations of MDA differed according to over-winter feeding treatment, though only for males (treatment × sex interaction; *w*= 1.000, **Table 3.1 (c)** and **3.2**) (**Figure 3.2**). Concentrations of MDA within female plasma were not statistically different between treatment groups (fat: β= -0.078 ±0.308; fat+VE: β= 0.199 ±0.446). Within the control group male MDA levels were similar to those of females (control: β= 0.099 ±0082), but interestingly males in the fat-fed group had significantly greater MDA concentrations than females (fat: β= 0.174 ±0.119). However, provisioning of vitamin E led to a significant reduction in levels of MDA in males compared to both control and fat-fed males, and equally supplemented females (fat+VE: β= -0.259 ±0.113). Treatment effects were consistent between years (treatment × year interaction outside 2 ΔAIC confidence set), despite dramatic variation in annual average levels (2008: 7.62 ±0.58 μg/ ml; 2009: 8.42 ±0.28 μg/ ml, β= 0.244 ±0.094; fat+VE: 3.97 ±0.14 μg/ ml, β= -0.579 ±0.098) (**Table 3.1 (c)** and **3.2**). Influences of hatching date and age (**Table 3.1 (c)** and **3.2**) were not well supported, with 95% confidence intervals including zero.

Given that male and female MDA concentrations differed in response to over-winter feeding, the correlation between feather carotenoid concentration and MDA levels was analysed separately for each sex with site as the random term (**Table 3.3**). For males, there was a high level of support for an effect of feather carotenoids on MDA which differed between years; whilst male MDA concentrations did not co-vary with feather carotenoid levels in 2009 (β= 0.004 ±0.001) and 2010 (β= 0.004 ±0.002), in the first year males with higher concentrations of carotenoids in their feathers had lower plasma levels of MDA (β= -0.004 ±0.003) (**Figure 3.3**). The same response to annual variation was seen in females; however large standard errors indicate that feather carotenoids are a relatively poor predictor of female susceptibility to oxidative stress (feather × year interaction: *w*= 0.217, β< 0.001 ± 0.001 for 2009 and 2010).

In all years MDA concentration was not significantly correlated to α-tocopherol concentration in the plasma (Pearson's: n=15, 51, 25, *p*> 0.453). In 2009 the correlation between MDA concentration and total carotenoid levels in the plasma was marginally significant (Pearson's: r= -0.26, n=52, *p*= 0.060), but this was not observed in 2008 and 2010 (n=15, 26, *p*> 0.38).

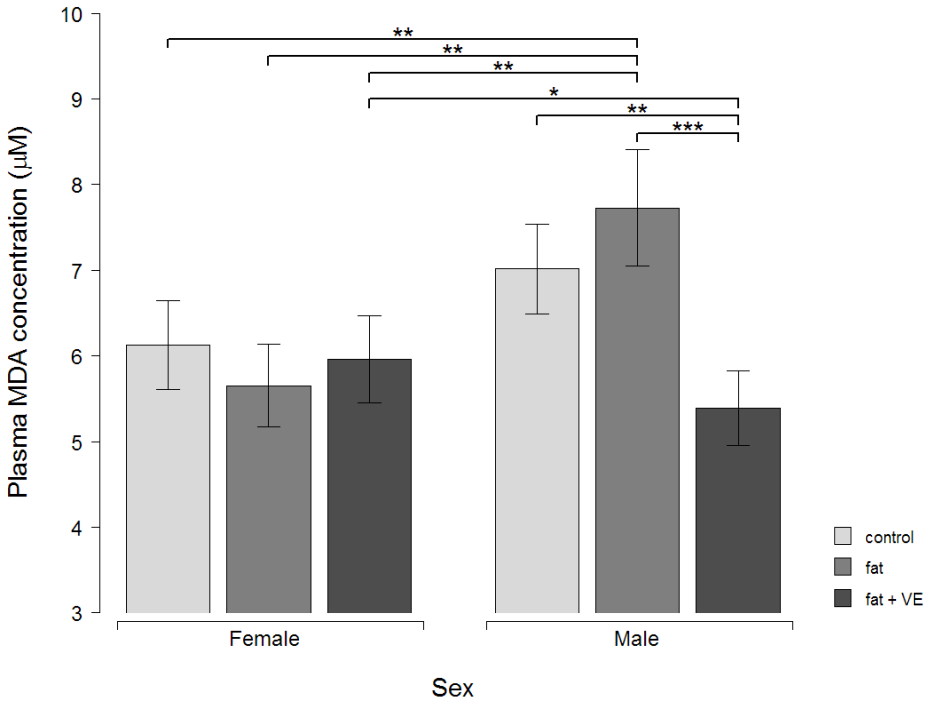

Figure 3.2. Malondialdehyde (MDA) concentrations in the plasma of parents, in relation to over-winter feeding treatment and sex. Mean ± SE plotted using raw values. *Post-hoc* pairwise comparisons shown, where * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$. Significance of pairwise comparisons was equivocal between all models within the confidence set. See Tables 3.1 and 3.2 for statistical details and text for parameter estimates (β).

Table 3.3. Relative importance of explanatory variables (Akaike weights, *w*) in analyses of the effect of feather carotenoid concentrations on female and male susceptibility to oxidative stress (plasma MDA concentration). * Denotes variables for which 95% confidence intervals for parameter estimates do not include zero. See Table text for details of parameter estimates (β).

	Female MDA	Male MDA
Feather carotenoids	0.330	1.000
Treatment		1.000
Age	0.577	0.133
Year	1.000	1.000 *
Hatching date	0.228	1.000
Feather carotenoids × treatment		0.360
Feather carotenoids × year	0.217	1.000 *
Feather carotenoids × hatch date		0.337
Pseudo-R^2	0.573	0.844

Candidate sets include 137 models, including listed terms. Feather carotenoids × age and treatment × hatching date interaction terms did not feature in confidence sets (female and male confidence sets include n= 7 and 5 models respectively).

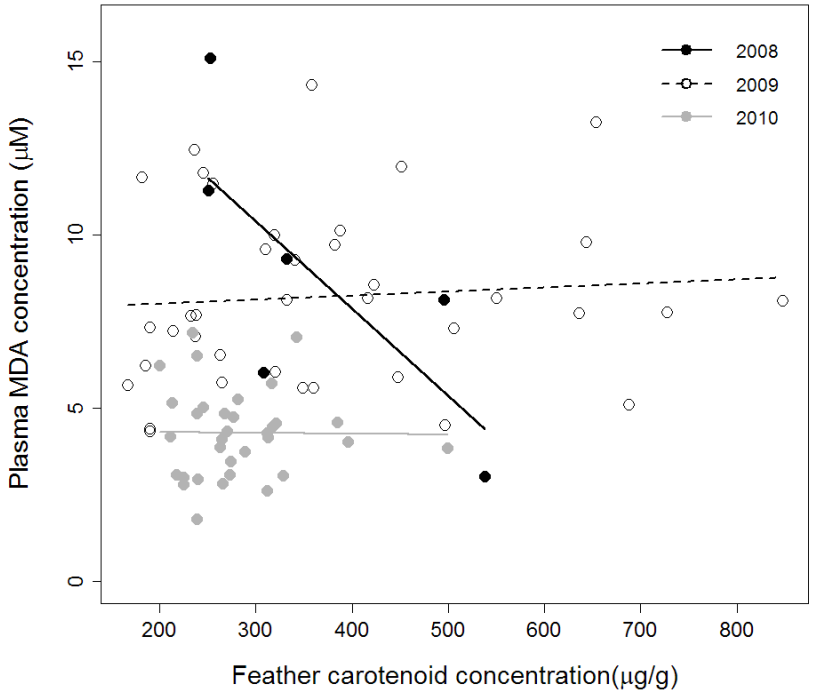

Figure 3.3. The linear relationship between male feather total carotenoid concentration and plasma MDA concentration differed between years. Plotted using raw values. P*ost-hoc* pairwise comparisons, 2008 v 2009: χ^2_2= 12.38, *p*= 0.002; 2008 v 2010: χ^2_2= 32.87, *p*< 0.001; 2009 v 2010 χ^2_2= 33.79 *p*< 0.001. Significance of pairwise comparisons were equivocal between all models within the confidence set; values presented are for the top-ranking model. See Table 3.3 for model summary findings.

3.4.2.3 Body mass

There was a moderate level of support for an effect of over-winter feeding on variation in adult body mass (*w*= 0.351, fat: β= 0.056 ±0.167; fat+VE: β= -0.028 ±0.098) (**Table 3.1 (d)** and **3.2**). However large standard errors and 95% confidence intervals crossing zero indicate that treatment was not an accurate predictor of body mass variation. The same was true of the treatment × year interaction term (*w*= 0.135; **Table 3.1 (d)** and **3.2**). The best predictors were differences in reproductive effort and year, and there was a high level of support for a sex-by-age effect (**Table 3.2**). Similar to its influence on total carotenoid concentrations, increases in reproductive effort led to a reduction in body mass (*w*= 1.000, β= -0.008 ± 0.001). An indication that this effect was more pronounced in females was not well supported (sex × reproductive effort interaction: *w*=

0.105, **Table 3.2**). Average body mass was greater in the first breeding season (2008: 10.62 ±0.10) compared to 2009 (10.29 ± 0.05, β= -0.291 ±0.148) and 2010 (10.20 ±0.05, β= -0.544 ±0.141), and the heaviest individuals were older males (sex × age interaction: *w*= 0.801, β= 0.170 ±0.133) (**Table 3.1 (d)** and **3.2**).

In a separate analysis, the probability of feather carotenoid levels explaining differences in body mass was poorly estimated (*w*= 0.162, β<0.001 ±0.000) with it only featuring in lower ranking models, and did not differ in relation to treatment and other fixed effects (all interactions outside confidence set). Further, differences in body mass were not correlated to variation in α-tocopherol, total carotenoid or MDA concentrations in the plasma in any year (Pearson's: *p*> 0.132).

3.4.3 Parental provisioning rates

Over-winter feeding treatment did not affect brood provisioning rates or time spent foraging (treatment and interaction terms outside confidence sets, **Table 3.4**). Provisioning rate was increased later in the brood rearing period (β= 0.844 ±0.119, **Figure 3.4**), and was positively correlated with brood size (β= 0.139 ±0.023) but decreased with hatching date (β= -0.022 ±0.007) (**Table 3.4 (a)** and **3.5**). Within re-sampled nests, provisioning rates were significantly correlated between nestling stages (Spearman's rank: rs= 0.37, n= 53, p= 0.007). Foraging time responded in the opposite manner, reducing later in the brood rearing period (β= -0.430 ±0.084) and in response to brood size (β= -0.073 ± 0.023), but increasing across the season (β= 0.018 ±0.007) (**Table 3.4 (b)** and **3.5**).

Whilst there was no absolute difference in brood provisioning behaviour between treatments, the magnitude of variation in provisioning rates of young nestlings was significantly influenced by over-winter feeding (Levene's test; $F_{2,58}$= 4.09, *p*= 0.022). *Post-hoc* comparisons with Bonferroni corrections reveal that VE-supplementation led to greater provisioning rate variation compared to the control group (Levene's test: $F_{1,37}$= 10.52, *p*= 0.008). Though supplementation of fat also increased variance, this was not significantly different from the other treatment groups (Levene's tests: *p*> 0.16). There was no difference in the levels of variation within treatment groups later in the brood-rearing period (Levene's test: *p*= 0.88), and variation in time spend foraging

within populations did not differ in response to feeding at either stage (Levene's test: $p > 0.38$).

To investigate the relationship between parental condition and brood-rearing activity provisioning data from the 2008 breeding season was reanalysed, using a reduced subset to include either mean parental feather carotenoid concentration (n= 68), or plasma MDA concentration (n= 31) as a covariate, plus 2-way interactions with treatment and nestling stage. Poorly supported model terms were removed from the global model to prevent overparameterization. Model averaging produced similar confidence sets as the original analysis (**Table 3.5**), with the likelihood of feather carotenoid levels predicting variation in provisioning rate or foraging time low and poorly estimated ($w < 0.262$, $\beta < 0.000 \pm 0.000$ **Table 3.5 II**). Similarly plasma MDA concentration was a poor predictor of foraging time ($w = 0.320$, $\beta = -0.017 \pm 0.032$), and did not influence rates of provisioning (**Table 3.5 III**). There was no evidence that these effects differed according to treatment of nestling stage (all interaction terms outside confidence set).

Table 3.4. Confidence sets of ranked models (in descending order) for analyses of brood provisioning in 2008. See § 3.3.7 for description of global models and modelling terms, Table 3.5 for relative variable importance and model fit, and text for parameter estimates (β).

Rank	Fixed effect	*k*	Log-likelihood	AICc	ΔAICc	w_i
(a)	Provisioning rate (visits per 30 min)					
1	brood size + hatch dt + (stage × TOD) + weather	10	-138.60	299.1	0.000	0.424
2	brood size + hatch dt + (stage × TOD)	8	-141.09	299.4	0.302	0.365
3	brood size + hatch dt + stage + weather	8	-141.64	300.5	1.392	0.211
250	*Intercept-only*	*3*	*-236.21*	*478.6*	*179.514*	*0.000*
(b)	Mean foraging time					
1	brood size + hatch dt + stage	7	-81.52	178.0	0.000	0.671
2	brood size + hatch dt + stage + weather	9	-79.93	179.4	1.426	0.329
58	*Intercept-only*	*4*	*-99.57*	*207.5*	*29.481*	*0.000*

Model terms: hatch dt, hatch date [1= 1April]; stage, nestling stage; TOD, time of day (0630, 0830 GMT); weather (overcast, rain, sun); (×), interaction term. Treatment outside confidence set. Intercept-only models reported in *italics* with the unadjusted Akaike weight. N. models in candidate sets= 274 and 70 (n= 136).

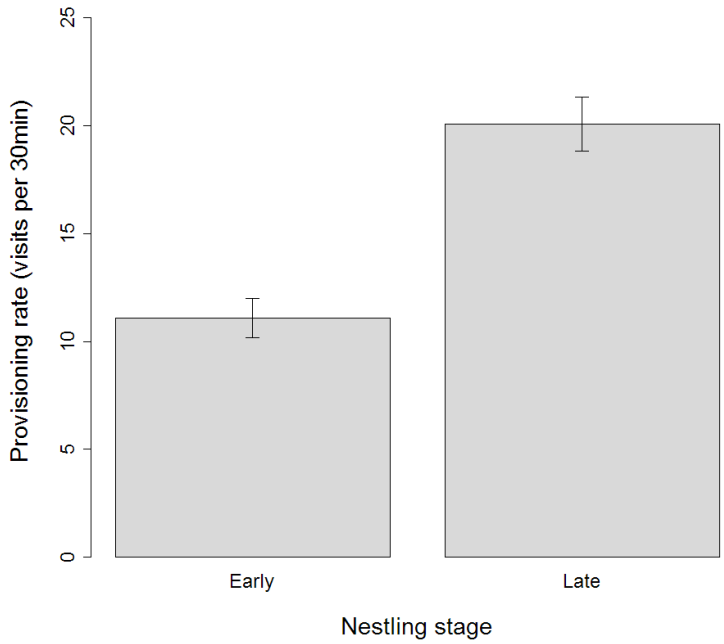

Figure 3.4. Provisioning rate differences between early (*ca.* day 3) and late (*ca.* day 11) stages of the brood-rearing period. Mean ± SE plotted using raw values.

Table 3.5. Relative importance of explanatory variables (Akaike weights, *w*) in analyses brood provisioning behaviour in 2008. * Denotes variables for which 95% confidence intervals for parameter estimates do not include zero. — Denotes variables not in the global model. See Table 3.3 for (I) confidence sets of text for details of parameter estimates (β).

	(a) Provisioning rate			(b) Foraging time		
	I [a]	II [b]	III [c]	I [a]	II [b]	III [c]
Feather carotenoids	—	0.254	—	—	0.262	—
Plasma MDA	—	—		—	—	0.320
Nestling stage	1.000 *	1.000 *	1.000 *	1.000 *	1.000 *	1.000 *
Hatching date	1.000 *	1.000 *	0.610	1.000 *	0.790	0.749
Brood size	1.000 *	1.000 *	1.000 *	1.000 *	1.000 *	0.445
Time of day	0.789	0.148	—	—	—	—
Weather conditions	0.635	0.444	—	0.329		—
Nestling stage × time of day	0.789	0.148	—	—	—	—
Pseudo-R^2	0.811	0.889	0.961	0.314	0.379	0.899

[a] Table 3.3 corresponding values; all available provisioning data analysed (n=136).
[b] Dataset reduced to include mean parental feather carotenoid concentration as covariate (n= 68)
[c] Dataset reduced to include mean parental plasma MDA concentration as covariate (n= 31)
Treatment outside confidence set.

3.5 DISCUSSION

As predicted, over-winter vitamin E supplementation resulted in a change in the phenotypic structure of breeding population, with respect to the concentrations of total carotenoids in the feathers (**Figure 3.1**). However neither winter provisioning nor feather carotenoid levels were good predictors of circulating antioxidant concentrations or body mass variation during the brood-rearing period. Interestingly, whilst susceptibility to oxidative stress appeared similar between females, males from vitamin E supplemented woodlands showed a reduction in plasma MDA concentrations (**Figure 3.2**). This may be a reflection of their differing reproductive roles, and a capacity for males to store micronutrients acquired at winter feeders and mobilise them during the breeding season. Brood provisioning rates were more variable within vitamin E supplemented woodlands early in the nestling phase, though not when nestlings reached day 11; and there was little evidence that provisioning and foraging behaviour were

influenced by feather carotenoid or plasma oxidative stress levels, though this was for a much reduced sample in one year only. These findings suggest that the supplementation of vitamin E over-winter both alters population phenotypic structure and may enable males (though not females) to combat oxidative stress more successfully in the later stages of the breeding season. The mechanisms determining these results and their further implications are discussed below.

3.5.1 Vitamin E provisioning and population phenotypic structure

Carotenoids encompass a large group of biologically-active pigments which, in addition to functioning as part of the antioxidant defence system, play a primary role in producing sexually-selected colouration (Stahl & Sies 2005). In the blue tit, both males and females have carotenoid-derived yellow breast plumage (Partali et al. 1987), and males are often described as the brighter sex (Figuerola et al. 1999), though variation in feather carotenoid concentrations between the sexes was not significant in the present study. Since carotenoids have limited environmental availability, physiological trade-offs exist in their allocation, and it is commonly hypothesised that carotenoid-based signals provide a reliable indicator of individual quality (Olson & Owens 1998; Møller et al. 2000). Variation in habitat quality, foraging efficiency, body condition and the speed of feather growth during the moulting period may all interact to influence the concentration of carotenoids allocated to plumage colouration (Slagsvold & Lifjeld 1985; Hill & Montgomerie 1994; McGraw & Hill 2000; Serra et al. 2007; Ferns & Hinsley 2008).

The current findings show that the inherent quality of birds was lower in the vitamin E treatment group (**Figure 3.1**). Since moult is completed annually between the breeding season and the beginning of winter (Perrins 1979), this indicates that over-winter provisioning of vitamin E has changed the phenotypic structure of the population by enabling relatively low quality individuals to breed several weeks and months after feeding finished. Though variation in habitat, and consequently access to sufficient carotenoid resources, is known to influence colouration (e.g. Slagsvold & Lifjeld 1985; Ferns & Hinsley 2008), the present result is unlikely to be confounded by woodland site differences since it stands true across replicate sites and years; further highlighting its significance. If feather carotenoid concentration is indeed a reflection of foraging

efficiency, or health and body condition at the time of feather growth (Hill & Montgomerie 1994; McGraw & Hill 2000; Mougeot et al. 2010), this phenotype would be carried into the winter season. Under normal conditions of food abundance, one would predict that individuals of lower phenotypic quality would have limited over-winter survival prospects (e.g. Nilsson & Svensson 1996). The change in foraging opportunities, natural food availability, energetic strain and prevailing debilitation due to higher levels of oxidative stress during the winter period would all offset the likelihood of recovery and therefore survival. Indeed, condition-dependent survival in relation to feather colouration has been shown in great tits (*Parus major*), where reduced yellow hue in breast feathers indicated higher levels of blood parasite infection, resulting in a reduced probability of surviving from one breeding season to the next (Hõrak et al. 2001). However in the present study, access to feeders appears to have alleviated some of the pressures of winter survival. It would seem that availability of supplementary food has compensated for the poor foraging efficiency or condition of those individuals with lower levels of carotenoid deposits in their feathers.

The influence of foraging efficiency and condition on carotenoid allocation are certainly not mutually exclusive. The ability to acquire dietary-exclusive resources, such as carotenoids and vitamin E, will be reflected in an individual's ability to combat a build-up of ROS (Catoni et al. 2008); whilst deterioration in body condition via increased parasitism, disease and elevated oxidative stress, will undoubtedly inhibit foraging. Therefore either one of these mechanisms, or a combination of the two, may potentially alter the trade-off between allocating carotenoids to colouration or antioxidant defences. However, as Møller et al. (2000) stipulate, carotenoid-based signalling is unlikely to have evolved on the basis of foraging efficiency alone. If colouration was simply a reflection of foraging ability, independent of condition, the provision of fat-only supplements would have sufficed to meet the energetic costs of winter survival (Gosler 1996). Similarly, if feather carotenoid levels indicate speed of moult, and subsequently poorer structural and thermoregulatory properties (Nilsson & Svensson 1996; Dawson et al. 2000), individuals with access to fat feeders would have a lower risk of starvation overnight resulting from increased thermoregulation costs (Gosler 1996). Therefore according to these hypotheses, provisioning of fat would assist poor foragers to survive and enter the breeding population. However there was no evidence that individuals

from fat-supplemented woodlands had lower levels of feather carotenoids than control birds, as displayed in vitamin E supplemented sites (**Figure 3.1**).

Numerous studies have linked condition indices with the intensity of carotenoid-based signals in avian species. For example, Harper (1999) found that individuals with greater parasite loads produced duller feathers in a range of species, including the blue tit. More recently Mougeot et al. (2010) have shown that variation in circulating antioxidant levels and susceptibility to oxidative stress influenced carotenoid-based ornamentation in the red grouse (*Lagopus lagopus scoticus*); and Peters et al. (2011) similarly conclude that the blue tit's structural-coloured blue crown is indicative of stress levels during moult. It seems likely that similar mechanisms are responsible for variation in carotenoid allocation to yellow breast plumage in the current study, and that greater vitamin E uptake subsequently has helped to improve condition or alleviate oxidative stress of lower quality individuals sufficiently enough to improve over-winter survival. It is therefore surmised that low quality birds have gained condition benefits to condition as a result of greater antioxidant uptake over winter. This hypothesis compliments that drawn in **Chapter 2**, given evidence that vitamin E provisioning enabled females to increase resource allocation into egg production.

3.5.2 Vitamin E provisioning and parental condition

Neither treatment nor feather carotenoid concentrations were strong predictors of circulating α-tocopherol and carotenoid levels, or body mass in both males and females during the brood-rearing period (**Table 3.2**). Therefore, although populations provisioned with vitamin E over-winter have been shown to include individuals of inherently lower quality, this was not reflected by a difference in condition during the subsequent breeding season (**Table 3.1**). In addition, it appears unlikely that α-tocopherol taken up from supplementary food was stored for use in the breeding season (Negro et al. 2001; McGraw & Toomey 2010), since α-tocopherol concentrations in the plasma were not affected by α-tocopherol provisioning. Furthermore, there was no indication that antioxidant levels or body mass were more variable within vitamin E fed populations, which might have indicated that individuals of relatively low and high quality were breeding concurrently.

Within the nine blue tit populations studied, it appears that parental antioxidant levels during the brood-rearing period reflect a necessity to resolve current trade-offs, rather than an influence by previous condition or feeding treatment. However, despite being of poorer condition on average prior to winter feeding, it seems that individuals within vitamin E supplemented woodlands have regained a level of health status in line with birds from control and fat-supplemented woodlands. This gives further weight to the hypothesis that individuals achieve benefits to condition from over-winter supplementation of vitamin E, and provides empirical evidence that carry-over effects may be derived from micronutrients for the first time.

Whilst carotenoid-based colouration has been seen to indicate parental condition during the breeding season in tits (Senar et al. 2002; Hidalgo-Garcia 2006; Doutrelant et al. 2008), this is not always the case (present study; **Chapter 2**; Hõrak et al. 2004; Biard et al. 2005). By comparison, brood size differences are known to represent a notable variation in the costs of reproduction between individuals (Nur 1984a; Wiersma et al. 2004), with larger brood requiring greater food resources and therefore greater foraging effort and efficiency, resulting in detrimental effects on condition (Perrins 1979). Indeed, reproductive effort at the time of sampling was a strong predictor of both total carotenoid concentration in the plasma and body mass (**Table 3.2**). In addition, α-tocopherol levels and body mass were strongly influenced by annual variation (**Table 3.2**). Biard et al. (2005) stipulate that high variation in antioxidant availability, or a difference in the requirements for carotenoid allocation between seasons, result in carotenoid-based colouration being a poor determinant of reproductive investment. The present findings appear to reiterate this.

3.5.3 Vitamin E provisioning and susceptibility to oxidative stress

Over-winter feeding was found to affect susceptibility to oxidative stress during the breeding season and, as predicted, the response to provisioning differed according to parental sex. Interestingly, whilst levels of lipid peroxidation were comparable between females, males responded differentially to treatment. Males from vitamin E supplemented woodlands benefited from a significant reduction in concentrations of MDA in the plasma compared to those from control or fat-fed woodlands (**Figure 3.2**). Breast feather carotenoid concentration was not a good predictor oxidative stress

susceptibility during the breeding season, irrespective of over-winter feeding. The only indication of a correlative relationship was evidence of a reduction in plasma MDA concentration in males with higher feather carotenoid concentrations during the first breeding season, 2008 (**Figure 3.3**) However given the relationship was absent in the following years and based on a relatively small sample size by comparison (n: 2008= 10, 2009= 38, 2010= 33), it should perhaps be judged with caution.

The differential effect of feeding on oxidative stress between males and females may be explained by three mechanisms highlighted in the predictions. Firstly, variation in the susceptibility of males and females to oxidative stress during the brood-rearing may reflect their different reproductive roles earlier in the season. Female blue tits adopt the majority of the reproductive work load from nest-building, egg laying and incubation, through to nestling provisioning (Perrins 1979). Therefore, one might anticipate that when plasma was collected in the latter half of the brood-rearing period, *ca*. 13 days post-hatching, the influence of over-winter provisioning may be difficult to distinguish. Since egg quality maternal effects are seen to play an importance role in determining offspring condition and survival (Biard et al. 2005; McGraw et al. 2005), perhaps females have placed greater investment into egg production at the expense of their condition later in the season. Indeed, females from vitamin E supplemented woodlands have previously been shown to invest more heavily in egg production (**Chapter 2**). Males also undergo a succession of stressful events, including territorial defence, mate-guarding and nestling provisioning (Perrins 1979), and consequently demonstrate similar levels of MDA in the plasma as females in the control group. However, the reduction in MDA levels in vitamin E fed males may suggest that because they have not made a substantial direct investment into offspring quality (c.f. egg production) until brood provisioning, the benefits of vitamin E supplementation for condition still prevail at this later stage.

Secondly there is perhaps stronger evidence that male and female differential oxidative stress responses are the result of variation in supplement uptake between the sexes. Males have been described as being more dominant on bird feeders than females (Hegner 1985), and a recent study also indicates greater uptake of winter supplementary food by males than females (Robb et al. in press). If this is the case, males potentially

utilised winter supplements to a great extent than females, therefore gaining greater condition-based benefits and able to resolve physiological trade-offs more effectively during brood-rearing (but see **Chapter 5**). Furthermore, plasma MDA concentrations in males were lower than in females in the vitamin E treatment group, but higher for the fat supplemented group (**Figure 3.2**). High uptake of polyunsaturated fat could render birds more susceptible to oxidative stress, and therefore whilst vitamin E supplemented individuals have means to combat this and appear to have improved in condition as a result, the opposite is true of fat supplementation.

Thirdly, it was hypothesised that micronutrient carry-over effects may result from their capacity to be stored in significant quantities within lipid-rich tissues. As such, the improvements seen in male oxidative status may be a reflection of their increased capacity to combat ROS via the antioxidant system, having mobilised vitamin E from reserves acquired at feeders in the winter. This is an intriguing concept, suggesting that blue tits may be utilising capital reserves for reproductive investment in addition to resources acquired daily.

One possible further explanation is that males from vitamin E supplemented woodlands have adopted a lower workload during brood-rearing and as such face reduced oxidative challenge compared to other males or their female counterparts. Johnsen et al. (2005) found that females mated to males of experimentally increasing attractiveness increased nestling provisioning rates, and less attractive males provisioned at a greater rate than attractive males. Females may perceive vitamin E supplemented males to be more attractive, following an improvement in condition gained through increased antioxidant uptake over-winter (Helfenstein et al. 2008). In which case they may allocate greater investment into their offspring, and as a consequence their male partner may concurrently reduce provisioning activities. However it is difficult to ascertain whether differential allocation is occurring from this study, or indeed whether vitamin E supplemented males appear more attractive.

3.5.4 Vitamin E provisioning and brood-rearing activities

One would predict that vitamin E supplemented males, having been less debilitated by oxidative damage, would invest greater time and energy into brood-rearing activities.

Indeed Remeš et al. (2007) found that carotenoid supplementation during laying led to improved male provisioning rates, whilst females invested more carotenoids into eggs. The results of the brood-provisioning investigation indicate that even though provision activities were similar at a population level for all treatments (**Tables 3.4** and **3.5**), there was greater variation in provisioning activities in the early stage of nestling phase within vitamin E supplemented woodlands. At this time the female is still required to brood the offspring, and therefore the male takes on the majority of the provisioning responsibility (Perrins 1979). Therefore, although it was not possible to directly gauge male and female activity, more variation at this early stage might be indicative of greater variability in the work rates of males within the vitamin E supplemented treatment group. Since foraging time was not similarly affected by treatment, it does not appear that individuals were taking time seeking out higher quality prey items (Grieco 2002), but instead were likely to be provisioning similar food but at differing rates. The apparent greater variation in the vitamin E supplemented group may be the result of variable foraging efficiencies, if vitamin E supplementation did indeed allow for poorer foragers to enter the breeding population, or be a reflection of improved male oxidative status. It would however suggest that individuals of variable quality, and potentially males more specifically, are breeding concurrently within the same population.

Later in the nestling phase provisioning rates were significantly greater (**Figure 3.4**) as one would predict, however there was no evidence that this was influenced by feeding. Furthermore, parental feather carotenoid concentrations and oxidative stress susceptibility were not strong predictors of brood-rearing activities at either stage. However these findings were based on only one year of results, for which male and female values have been averaged. Since colouration, oxidative trade-offs and provisioning efficiency are all known to vary differential between the sexes (e.g. Senar et al. 2002), this analysis would inevitability be more powerful and revealing if parental sex was identified. However given that small sample sizes were used (**Table 3.5**) and total provisioning rates were measured, rather than males and females separately, this analysis was most appropriate with the data available. However it should perhaps be judged with caution.

3.5.5 Conclusions

In **Chapter 2** it was hypothesised that indirect effects on maternal condition lead to improved egg quality in woodlands provisioned with vitamin E over winter. The findings presented in this chapter further support this hypothesis. The provision of vitamin E appears to have helped to alleviate physiological trade-offs, such that individuals of poor condition in the lead up to winter have improved survival and reach condition thresholds necessary to reproduce during the subsequent breeding season. As a consequence the phenotypic structure of the population has been altered.

It has further been inferred that benefits to condition are visible at different stages of the reproductive period according to parental sex. Whilst vitamin E provisioning has enabled females to invest more resources into egg production (**Chapter 2**), males are less susceptible to oxidative trade-offs during the brood-rearing period and as such may be able to place greater investment in nestling provisioning. Although this was not revealed during the provisioning activity investigation, a further, more detailed study of male provisioning would be of benefit. Furthermore, it is hypothesised that this apparent improvement in male oxidative status may reflect a direct investment of vitamin E acquired at winter feeders into the antioxidant defence system during the brood-rearing period. Therefore in addition to being used to maintain body condition over-winter, micronutrients could also be stored and called upon during this period of intense metabolic stress. This suggestion that blue tits, classically defined as 'income breeders', may utilise capital reserves during reproduction is certainly worthy of further investigation.

Finally, there is little indication that vitamin E-fed populations included birds which varied more greatly in individual quality as a result of feeding, since the magnitudes of variation in breeding condition measures were consistent among treatment groups. In addition, as reported in **Chapter 2**, breeding density was not increased in vitamin E supplemented woodlands, although this has not been accurately quantified. Therefore despite the observation that breeding condition was independent of individual capacity to allocate carotenoid during moult and winter feeding treatment, it still remains that provisioning of vitamin E has resulted in a population of inherently poorer quality individuals on average. As such, it is with great interest that **Chapter 4** goes on to

investigate whether inherent quality, over-winter supplementation or breeding condition ultimately influence reproductive success.

CHAPTER 4

Ecological traps: the possible implications of over-winter feeding on breeding success in wild bird populations

4.1 ABSTRACT

In a resource limited environment, supplementary food presents a super abundant and heavily utilised feeding opportunity for garden birds. With provisioning most prevalent during the winter months, this huge perturbation of food availability has the potential to influence survival and reproductive success within wild bird populations. However, the ecological implications are poorly understood. Energy supply is a key determinant of winter survival, however it is predicted that antioxidants will be of greater importance in mediating the carry-over effects of winter food in income breeding birds. Using a three year, landscape-scale study, the effects of over-winter provisioning of fat and vitamin E on breeding performance in blue tits (*Cyanistes caeruleus*) were investigated. Whilst there was evidence that vitamin E supplementation influenced hatching success and nestling plasma carotenoid concentrations, the most concerning finding was a reduction in fledging success across both supplemented treatment groups. These novel observations suggest that fed birds are making an unsustainable investment in offspring number, and that winter feeders may act as an ecological trap. With garden bird feeding promoted as a method for conserving declining wild bird populations, these new insights suggest much more needs to be done to fully understand its impacts.

4.2 INTRODUCTION

Food limitation is a key issue in avian ecology; influencing individual life-history traits and regulating population sizes and community structures (Newton 1980). For many species, both in the UK and globally, supplementary food provides an enormous additional feeding opportunity, with the capacity to alleviate trade-offs caused by limited resources. Recent estimates suggest almost half of UK householders provision bird food (Davies et al. 2009), and in excess of 500,000 tonnes of commercial bird food purchased across the UK and US each year (O'Leary & Jones 2006). This has the potential to affect virtually every aspect of bird ecology, from daily survival to social behaviour (reviewed by Jones & Reynolds 2008; Robb et al. 2008a). Despite this, there remains a relatively poor understanding of the measureable impact this is having on wild bird populations.

Previous supplementary feeding studies have focused on the influence of food availability during different stages of the breeding season, revealing numerous effects on avian breeding parameters; such as: advanced laying (Nager et al. 1997); greater egg quality (Blount et al. 2002); increased brood size and parental provisioning capacity (Wiehn & Korpimaki 1997; Markman et al. 2002); and improved fitness and survival rates of both offspring and parent birds (Biard et al. 2005; Davis et al. 2005; de Ayala et al. 2006). However, provisioning of garden bird food occurs in greatest abundance outside of the breeding season, where its potential ecological impacts on breeding performance have received less attention (but see Robb et al. 2008b). Over-winter feeding is expected to improve the survival prospects of wild birds at a time of low natural food availability and increased energetic requirement (Jansson et al. 1981; Newton 1998; Chamberlain et al. 2005). But in addition, recent findings suggest that the benefits of increased winter food availability may be carried-over to influence reproductive investment and breeding success (Robb et al. 2008b; **Chapter 2**).

It is hypothesised that carry-over effects resulting from over-winter supplementation may be due to improvements in 'condition' gained at feeders (Robb et al. 2008b; **Chapters 2** and **3**). Macronutrients, and particularly energy, gained from food supplements may be an important determinant of over-winter survival (Koivula et al. 1995). However, although additional energy might fuel metabolism and enable

individuals to maintain a higher body mass during the provisioning period (Gosler 1996), small passerines are classically considered to be income breeders relying on daily dietary intake, not stored macronutrients, to fuel breeding requirements (Drent & Daan 1980). Therefore, it is anticipated that micronutrients gained from supplementary food are more likely to have a sustainable influence on breeding activity.

Popular bird foods, particularly nut and seed mixes, are rich in lipophilic antioxidants. Vitamin E and carotenoids, for example, play a fundamental role in the antioxidant defence system, by limiting the propagation of harmful reactive oxygen species (ROS) and therefore reducing oxidative stress (Catoni et al. 2008; Monaghan et al. 2009). ROS are the natural by-products of normal metabolic activity, however if accumulated they may cause serious molecular damage to protein, lipid and DNA macromolecules (Finkel & Holbrook 2000). To provide protection, vitamin E functions as a chain-breaking antioxidant, neutralising unstable ROS and reducing lipid peroxidation (Burton 1994). Carotenoids on the other hand, are biologically active pigments primarily responsible for signalling colouration (Møller et al. 2000). But in addition they are effective quenchers of ROS and offer synergistic protection and recycling of vitamin E, therefore also playing an important role as part of the antioxidant defence system (Sies & Stahl 1995; Surai 2007). As lipophilic molecules, both vitamin E and carotenoids have the potential to be stored for long periods in lipid-rich tissues, such as subcutaneous fat and the liver (Negro et al. 2001; McGraw & Toomey 2010). However, these valuable resources cannot be synthesised endogenously (Goodwin 1984), and therefore due to variation in their environmental availability and foraging efficiencies amongst individuals they are hypothesised to be a limiting resource in avian ecology (Olson & Owens 1998). This may be particularly acute over-winter, when insectivorous birds such as the blue tit (*Cyanistes caeruleus*) feed predominantly on relatively antioxidant-poor invertebrates, such as spiders (Betts 1955; Arnold et al. 2010).

Over-winter supplementation of α-tocopherol, the most ubiquitous and bioactive form of vitamin E in nature, has been shown to influence the condition and egg production abilities of income breeding blue tits, despite provisioning stopping several weeks before the start of the breeding season. For example supplementation has enabled

relatively poorer quality individuals to breed (**Chapter 3**), increased maternal resource allocation into egg production (**Chapter 2**) and improved paternal oxidative status in the later stages of the breeding cycle (**Chapter 3**). Consequently, it is anticipated that over-winter feeding will also lead to an improvement in reproductive output. This may be manifested through advancements in the timing of breeding (Martin 1987); improvements to hatching success (Nilsson & Smith 1988); the production of higher quality offspring, for example exhibiting improved growth, immune defences or sexual signalling (Biard et al. 2005; McGraw et al. 2005) or an increase in the numbers of nestlings fledged (Robb et al. 2008b).

Here, I investigate the influence of over-winter provisioning of energy and antioxidant-rich supplements on breeding productivity in wild blue tit populations over three consecutive breeding seasons. The aims were to determine whether over-winter feeding affected: (1) the timing of egg laying; (2) the proportion of eggs hatched; (3) nestling phenotype, including measures of body mass and plasma concentrations of α-tocopherol, total carotenoids and malondialdehyde (MDA; a by-product of lipid peroxidation); and (4) fledging success. In addition, in light of the findings in **Chapter 2** and **3**, the following were also tested (a) the influence of egg yolk antioxidants on nestling antioxidant status; (b) the impacts of yellow breast feather carotenoid concentration of both females and males, indicative of 'inherent parental quality' at the time of feather growth (Møller et al. 2000), on reproductive output; and (c) the relevance of male oxidative status (MDA) to nestling condition and fledging prospects.

4.3 METHODS

4.3.1 Study sites, experimental design and supplementary feeding

The over-winter supplementary feeding experiment was conducted over three years from 2007 to 2009, and the carry-over effects on breeding performance were measured during the subsequent breeding seasons, 2008 – 2010 respectively. The study took place in Cornwall, UK, across nine deciduous woodland sites. All sites and prescribed feeding treatments were as described in **Chapters 2** and **3**. In brief, woodland sites, largely comprising oak (*Quercus* spp.), beech (*Fagus sylvatica*), sweet chestnut

(*Castanea sativa*) and sycamore (*Acer pseudoplatanus*), were grouped into three triplets according to composition and common features. Within sites, nest boxes were evenly distributed at a density of *ca*. 4 boxes per hectare, giving a total of 346 boxes across all woodlands.

In the first year of the study, each site within a triplet was randomly allocated to one of three feeding treatments. These treatments were: *(1) no supplement* (hereafter 'control'), *(2) fat only* (to test for energy effects; hereafter 'fat'), and *(3) fat-plus-vitamin E* (to test for effects of energy plus antioxidants, hereafter 'fat+VE'). Treatments groups were then rotated within triplets across years, so that every site received all three treatments over the course of the study. Potential confounding effects of year have been prevented through the replication on treatment groups within years.

Supplementary food was provisioned through the winter only (14 December – 4 March 2007/08; 18 November – 11 March 2008/09 and 2009/10), leaving a gap of at least one month before laying commenced (8 April, 11 April, 15 April respectively). Squirrel-proof feeders were positioned 100m apart within the six fed sites each year (*ca*. 9 per site), and provisioned with a fresh 150g fat ball every 10 days. For the fat+VE treatment group, fat balls were supplemented with 10mg/ 100g α-tocopherol (≥96% DL-all-*rac*-α-Tocopherol (HPLC), Sigma-Aldrich Ltd., Dorset), a concentration equivalent to that occurring naturally in peanuts (Chun et al. 2005). Since α-tocopherol cannot be provisioned to wild birds without the use of a 'carrier' and fat is required for its absorption (Blount et al. 2002; Jeanes et al. 2004), the fat-plus-vitamin E treatment group provides an ecologically meaningful method of testing antioxidant effects. All supplements were prepared and utilised by the target species as described in **Chapter 2 §2.3.2**.

4.3.2 Breeding parameters

Nest boxes were inspected regularly from April to June to monitor breeding performance. Lay date and clutch size were determined as described in **Chapter 2**, and one egg per clutch removed for yolk antioxidant level determination (see **§2.3.3** for details of egg collection). Hatching date was determined as described in **Chapter 3.** Brood size was recorded *ca.* 6 and 12 days after hatching during nestling sampling visits

(see below), and on the day of parental sampling in a subset of nests (reported in **Chapter 3**). All nests were re-visited after day 20 to determine fledging numbers. Hatching success was defined as the proportion of a clutch which hatched; whereby number hatched was calculated as the number of eggs incubated at clutch completion (thus accounting for eggs removed, reported in **Chapter 2**) minus number of unhatched eggs (counted at day 6). Fledging success was defined as the proportion of hatched eggs which fledged; whereby number fledged was calculated as brood size at last visit (≥ day 12) minus number of dead chicks found during fledging checks. An alternative measure of fledging success, using the proportion of incubated eggs which fledged was also investigated.

4.3.3 Nestling sampling

Nestling growth was measured for a subset of three offspring in each brood (survivorship permitting) between two time points *ca*. 6 days apart (mean *n* days between measuring ±SE: 6.04 ±0.01, *n*= 821, brood *n*= 306). On *ca.* day 6 of the nestling phase (mean nestling age ±SE: 6.11 ± 0.02, *n*= 1053, brood *n*= 358), the largest and smallest chicks by mass, plus a random third individual were identified and individually marked using black nail vanish on claws and black marker pen on the body. For marked individuals, body mass (±0.1g) was recorded using an electronic balance and head – bill length (±0.05mm) measured twice with dial callipers then averaged (Redfern & Clark 2001).

Broods were re-visited *ca*. day 12 for growth and blood sampling of marked individuals (survivorship permitting) and morphometrics of unmarked individuals (mean nestling age ±SE: 12.19 ±0.02, *n*= 1706, brood *n*= 319). Mass and head – bill length were recorded for all individuals in the nest using methods described above. Blood samples (*ca.* 60-100 µl) were collected from the brachial vein of marked individuals under Home Office license (PIL 30/8161), using the same protocol described for adult blood sampling in **Chapter 3 §3.3.3**. In the instance of mortality of marked nestling, blood samples were collected from unmarked individuals to give a total of 3 blood samples per nest. Blood samples were centrifuged (4min; 13,000 × g) and stored in a polystyrene cool box in the field (mean time stored in field ±SE: 353.3 ± 5.9 mins). When returned to the laboratory plasma was removed for storage at -80°C until

biochemical analysis. The length of time between blood sample collection and freezing did not influence levels of MDA in the plasma (Pearson's: r= 0.025, n=774, *p*= 0.493). However, it did have a significant effect on total carotenoid (r= -0.083, n=572, *p*= 0.045), and α-tocopherol concentrations (r = -0.187, n=572, *p*< 0.001). Therefore this was controlled for during further statistical analyses of antioxidant levels.

4.3.4 Biochemical analysis of eggs, adult feathers and adult and nestling plasma

4.3.4.1 Determination of antioxidant levels in egg yolk

Levels of total carotenoids and α-tocopherol in egg yolk were quantified using high-performance liquid chromatography (HPLC) as described in **Chapter 2 §2.3.4**.

4.3.4.2 Determination of carotenoid concentration in parental feathers

Levels of total carotenoids in the yellow breast feathers of parent birds were determined by mechanical extraction and spectrophotometry as described in **Chapters 2** and **3**.

4.3.4.3 Antioxidant and oxidative stress level determination in plasma

Concentrations of total carotenoids and α-tocopherol in nestling plasma were extracted and quantified as described for parental plasma in **Chapter 3 §3.3.5**. Total carotenoid concentrations was determined by spectrophotometry (as described in **Chapter 2 §2.3.4**), and α-tocopherol concentrations quantified by HPLC (40 – 60 µl injection volume; HPLC conditions and standards as described in **Chapter 2 §2.3.4**). Malondialdehyde (MDA) concentrations in the plasma of male parent birds were also quantified by HPLC as described in **Chapter 3**. Protocol was repeated for analysis of MDA levels in nestling plasma (10 µl plasma volume; see**§3.3.5**). Due to limited sample volumes, sample numbers of nestling condition measures vary between assays. Plasma from 572, 577 and 771 individuals were successfully analysed for α-tocopherol, total carotenoids and MDA concentrations respectively, representing 265-300 broods per analysis. Refer to **Chapter 3** for details of adult sample numbers.

4.3.5 Statistical Analysis

All statistical analyses were conducted using R version 2.12.2 (R Development Core Team 2011). To test the hypothesis that over-winter feeding effects breeding

productivity, a GLMM was applied to an analysis of lay date, and binomial GzLMMs were used to investigate hatching and fledging successes having checked for residual overdispersion. Nest box identity nested within woodland site was specified as the random term, to control for temporal and special pseudoreplication. Nestling phenotypic measures (body mass day 6 and day 12, and plasma α-tocopherol, total carotenoid and MDA concentrations) were examined using GLMMs, with brood identity added to the random term, nested within box identity, to control for non-independence of nestlings within broods. Clutches and broods anticipated to have been abandoned as a result of sampling activities were conservatively excluded from analyses where appropriate. Normality and heteroscedasticity of residuals from nestling phenotype models were checked prior to model selection to test model fit. Plasma concentrations of α-tocopherol and total carotenoids were log-transformed, whilst MDA concentrations were square root-transformed to correct normality.

4.3.5.1 Fixed effects

In all analyses, treatment was fitted as a three level factor, year was included as a factor accounting for annual differences in breeding conditions (Svensson & Nilsson 1995) and seasonal variation was controlled for by the inclusion of either lay date or hatching date as a covariate (Norris 1993). Since nestling body mass and survival are reduced in larger broods (Nur 1984b), brood size on the day of sampling was included as a covariate in analyses of nestling phenotype. Also, nestling age at sampling was controlled for in nestling analyses (day 6 ± 1 day or day 12 ± 1 day), since this was positively correlated to all aspects of phenotype measured (GLMMs with site random factor; $p< 0.001$). Body mass variation was investigated independently for nestlings at day 6 and day 12, due to differences in survival and sampling effort, and unreliability of measuring growth from only two points along the growth curve. At both time points, skeletal body size was controlled for when analyzing body mass by the inclusion of head-bill length. For antioxidant analysis, the length of time between blood sampling and freezing (minutes) was included for reasons detailed above **(§4.3.3**). Only models including head-bill length or 'time until freezing' were compared in analysis of body mass and antioxidant concentrations respectively, and therefore their results have not been presented in **Tables 4.3** and **4.4** for ease of comparison. In addition, all two-way

interactions involving treatment were fitted in all models to test for differences in main effects between treatment groups.

Given that adult birds were not caught at all nests and were sampled in the later stages of the breeding season, measures of parental phenotype were not initially included in models to avoid the dismissal of large numbers of data points and avoid sampling bias of surviving clutches. However, given the notable variations in parental feather carotenoid concentrations (**Figure 3.1**) and in male MDA concentrations (**Figure 3.2**) between treatment groups presented in **Chapter 3**, the potential influence of these factors on breeding performance and nestling phenotype was of interest. Therefore, all models were re-run on reduced sample sizes to include either female feather carotenoid concentration, male feather carotenoid concentration or male MDA concentration where appropriate. Although female and male feather carotenoid concentrations were positively correlated within pairs (**Chapter 3**), it was anticipated that this measure of inherent quality might display differential effects between males and female warranting their separate analysis. Modeling sample sizes were also improved by the separate analysis, since both partners were not sampled in every nest. Feather carotenoid concentrations were positively-skewed, and therefore were log-transformed prior to inclusion as fixed effects. Two-way interactions between the parental phenotype measure of interest and treatment were also included.

4.3.5.2 Model selection and averaging

An information-theoretic approach was used for model selection (Burnham & Anderson 2002; Johnson & Omland 2004; Symonds & Moussalli 2011), as described in **Chapters 2** and **3**. All possible models, given the explanatory variables described, were compared using the 'dredge' function implemented in the package *MuMIn* (Bartoń 2011). Models were ranked according to their relative AICc values, correcting for sample size bias since $n/k < 40$, with the exception of the analysis of nestling mass on day 12 where models were compared using AIC since $n/k = 68.24$ (Burnham & Anderson 2002). AIC(c) provides a comparative measure of model fit and complexity, where the best fitting model has the lowest value and all other models are ranked according to their difference from the top model (ΔAIC(c)). Model averaging was applied across a confidence set of well supported models, where $\Delta AIC(c) \leq 2.0$ (Burnham & Anderson

2002). Averaged parameter estimates (β) and standard errors (SE) were generated along with selection probabilities of explanatory terms (*w*). All parameter estimates presented are those directly produced by modal averaging, and are therefore based on transformed data in instances specified above. A pseudo-R^2 value was calculated for the top ranking model to estimate variance explained by the model and to evaluate model fit, following Nagelkerke (Nagelkerke 1991), as described in **Chapters 2**. *Post hoc* pairwise comparisons were carried out to assess between treatment group differences, using comparisons of the original ranked GLMM or GzLMM model with models in which treatment groups were paired. This was repeated for all models in the confidence set featuring the treatment effect or interaction of interest.

Additional statistical results for clutch abandonment (binomial GzLMM), fledging numbers (Poisson GzLMM) and antioxidant correlations between egg yolk and nestling plasma (GLMM) described in text have been derived by stepwise elimination of least significant terms, after controlling for appropriate fixed effects terms described above. Furthermore, correlations between nestling phenotypic measures were assessed using Pearson's product-moment correlation following the same data-transformations applied during mixed modeling.

4.4 RESULTS

A total of 476 nest boxes were occupied by blue tits from 2008 to 2010, but occupancy did not differ significantly between treatments or years (statistics reported in **Chapter 2**; **Table 4.1**). Late breeding attempts have been excluded from further analysis, since lay dates and hatching successes were unknown (n= 9; first egg laid >7 days later than others breeders within same woodland). A total of 159 nests were abandoned at various stages of the breeding season, and across analyses some of these nests have been removed if suspected to be related to sampling activity (total n= 49; independent of treatment; binomial GLM p= 0.225). In addition, broods which were not monitored throughout were excluded from fledging success analysis, due to inaccurate knowledge of brood size changes (n= 19). Sample sizes for each analysis are reported throughout.

Table 4.1. Summaries of breeding parameters by over-winter feeding treatment and year (mean ±SD) between 2008 – 2010. Overall results per treatment group are also highlighted

	CONTROL				FAT				FAT-PLUS-VITAMIN E			
	2008	2009	2010	Control total / mean	2008	2009	2010	Fat total / mean	2008	2009	2010	Fat+VE total / mean
No. boxes occupied	45	45	57	147	51	54	58	163	51	51	64	166
Lay date [1= 1 April]	27.6 (8.2)	24.3 (6.1)	29.6 (6.9)	27.3 (7.4)	26.4 (6.9)	24.6 (7.2)	29.3 (5.6)	26.8 (6.8)	26.0 (7.5)	25.4 (6.9)	31.7 (8.2)	28.0 (8.1)
(a) Indicators of hatching success												
No. broods hatched	38	40	43	121	41	48	52	141	41	45	44	130
Hatching success (%)	79.0 (34.3)	81.0 (27.7)	84.7 (27.9)	81.6 (30.0)	80.8 (34.5)	82.6 (31.7)	84.8 (25.3)	82.8 (30.2)	88.9 (23.3)	85.2 (26.6)	79.4 (36.1)	84.2 (36.1)
No. hatched per nest	5.5 (2.7)	6.2 (2.5)	6.0 (2.4)	5.9 (2.5)	5.6 (2.9)	5.7 (2.5)	6.4 (2.4)	5.9 (2.6)	6.8 (2.4)	5.8 (2.3)	5.3 (2.6)	5.9 (2.5)
(b) Indicators of fledging success												
No. broods fledged	30	34	35	99	30	36	44	110	30	41	34	105
Fledging success (%)	61.7 (38.2)	72.5 (30.5)	75.3 (36.0)	69.9 (35.3)	62.9 (39.4)	60.0 (37.4)	64.3 (36.1)	62.4 (37.2)	44.6 (34.4)	72.7 (30.1)	70.1 (37.3)	63.2 (35.9)
No. fledged per nest	3.8 (2.5)	4.8 (2.5)	4.8 (2.6)	4.5 (2.5)	4.1 (2.8)	3.8 (2.6)	4.0 (2.5)	4.0 (2.6)	3.1 (2.4)	4.6 (2.2)	4.4 (2.5)	4.0 (2.4)
(c) Indicators of nestling condition												
α-Tocopherol (μg/ml)	16.2 (4.0)	18.4 (6.5)	12.0 (5.5)	15.8 (6.1)	15.1 (2.8)	16.4 (8.6)	13.6 (5.0)	15.1 (6.3)	15.2 (4.2)	17.7 (6.5)	10.5 (5.7)	14.9 (6.4)
Total carotenoid (μg/ml)	55.6 (18.2)	41.3 (11.4)	35.1 (9.8)	44.0 (15.7)	62.2 (16.0)	36.6 (12.2)	36.2 (9.5)	44.3 (17.4)	50.8 (9.8)	43.6 (12.9)	31.1 (8.9)	41.9 (13.3)
MDA (μg/ml)	10.1 (2.8)	10.0 (2.8)	4.4 (1.0)	8.0 (3.6)	10.5 (3.1)	10.0 (2.9)	4.5 (0.8)	7.9 (3.7)	11.3 (5.2)	9.4 (3.5)	4.2 (0.8)	8.2 (4.6)

4.4.1 Clutch initiation date

Lay dates did not differ between feeding treatment groups, but were strongly predicted by between year differences (**Tables 4.1 and 4.2 (a, i)**). The top model, featuring year only, received a very high level of support (unadjusted w_i= 0.993) compared to the alternatives within the candidate set which included treatment (w_i> 0.007), and *post-hoc* pairwise tests revealed significant differences in lay dates amongst all years (χ^2_1> 6.901, p< 0.009). Inclusion of female feather carotenoid concentration as a fixed effect improved model fit (pseudo-r^2= 0.220), and this was also found to influence laying dates (w= 1.000; log-transformed β= -0.414 ± 0.130) (**Table 4.2 (a, ii)**). Females with greater feather carotenoid deposits had earlier laying dates (**Figure 4.1**), irrespective of over-winter feeding (treatment × feather carotenoid interaction outside Δ2 AIC set, **Table 4.2 (a, ii)**). There was no significant difference in the spread of lay dates between treatment groups (Levene's test; $F_{2,464}$= 1.48, p= 0.23).

Table 4.2. Confidence sets for lay date and hatching success. (a) Lay date analysed (i) for all clutches (n= 467), and (ii) repeated for clutches where female feather carotenoid concentration was known (n= 179). (b) Hatching success analysed for complete clutches (n= 418). See §4.3.5 for description of global models and modelling terms, and text for further details.

	Rank	Fixed effect	*k*	Log-likelihood	AICc	ΔAICc	w_i	Pseudo-R^2
(a)	Lay date							
(i)	1	year	6	-452.7	917.5	0.000	1.000	0.109
	4	*Intercept-only*	*4*	*-477.4*	*962.9*	*45.387*	*0.000*	
(ii)	1	year + ♀ feather carotenoids	7	-171.1	356.8	0.000	0.705	0.209
	2	year + ♀ feather carotenoids + ♀ age	8	-170.9	358.6	1.745	0.295	
	68	*Intercept-only*	*4*	*-187.0*	*382.2*	*29.673*	*0.000*	
(b)	Hatching success							
	1	(treatment × year) + (treatment × lay date)	14	-533.0	1095.0	0.000	1.000	0.117
	12	*Intercept-only*	*3*	*-557.0*	*1120.1*	*25.061*	*0.000*	

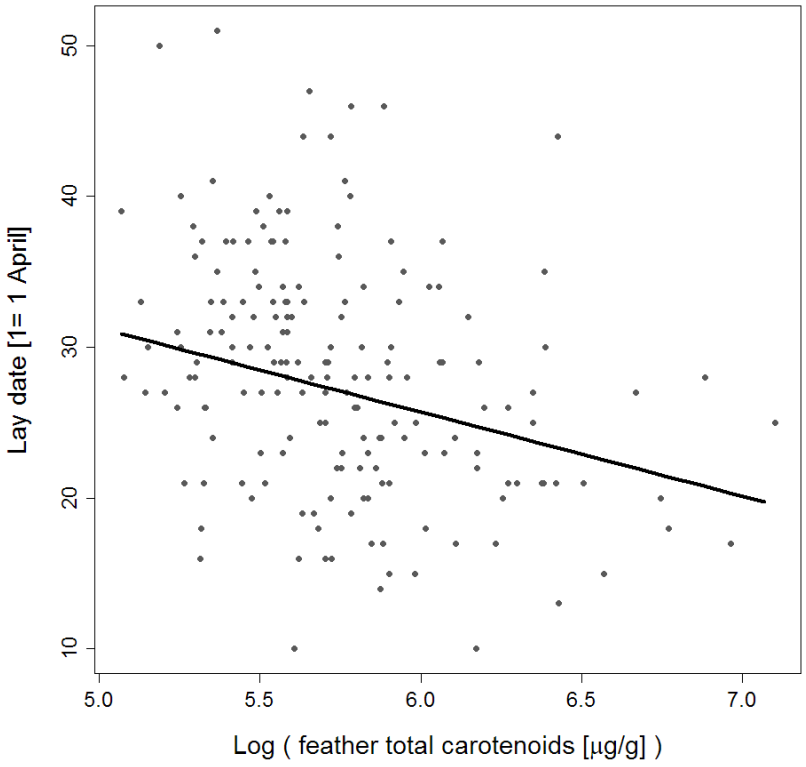

Figure 4.1. Lay dates in relation to female feather total carotenoid concentrations from 2008 – 2010. Feather carotenoid concentrations have been logged to correct for positive skew. Plotted using raw values, line fitted from top model with random effects excluded. See text for details.

4.4.2 Hatching success

Across years, 51 nests were abandoned during egg laying and incubation, excluding clutches potentially affected by sampling. The propensity for clutch abandonment was not influenced by over-winter feeding (binomial GzLMM: χ^2_2 = 1.88, p= 0.391), and was also independent of year (χ^2_2 = 4.54, p= 0.103). But it was seen to increase in later laid clutches (χ^2_2 = 10.33, p= 0.001).

Hatching success, defined as the proportion of eggs incubated which hatched, was significantly influenced by over-winter feeding treatment during the first breeding season only, 2008 (*Post-hoc* pairwise comparisons: $\chi^2_1 > 5.54$, $p < 0.019$) (**Table 4.1**, **Figure 4.2**). Only one model was supported within the 2 ΔAIC confidence set (unadjusted $w_i = 0.805$; n= 418; **Table 4.2 (b)**). This also showed a decrease in hatching success over the season (β= -0.103 ± 0.002), though to a lesser extent in fat (β= 0.072 ± 0.030) and fat+VE fed woodlands (β= 0.109 ± 0.029). Inclusion of either female (n= 177) or male (n= 153) feather carotenoid concentrations resulted in poorly fitted confidence sets (r^2= 0.043 and 0.015), both including 6 equally supported models of which one was the null. Confidence sets are not presented, due to their unreliability in drawing further inference.

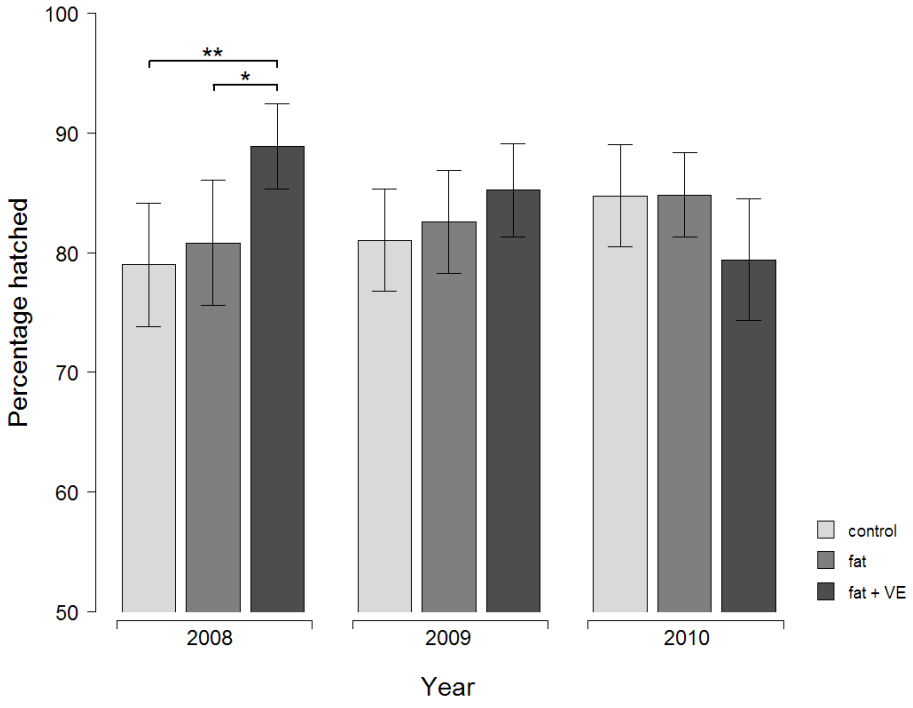

Figure 4.2. The percentage of eggs which hatched from incubated clutches in relation to over-winter feeding treatment differed between years (mean ± SE from raw values). *Post-hoc* pairwise comparisons shown, where * = $p \leq 0.05$; ** = $p \leq 0.01$. See Table 4.2 for model summary and text for details.

4.4.3 Nestling phenotype

4.4.3.1 Body mass, plasma antioxidants and oxidative stress

A total of 2498 chicks were known to have hatched during the 2008 – 2010 breeding seasons. Of the individuals measured on day 6 (± 1 day) post-hatching (n= 1053), there was moderate support for an effect of treatment on variation in body mass at respective ages (*w*= 0.634, **Tables 4.3 (a)** and **4.4**). However large standard errors and confidence intervals around parameter estimates indicate this is not an accurate predictor (fat × age: β= -0.113 ± 0.126; fat+VE × age: β= -0.063 ± 0.085). Variation in nestling mass on *ca.* day 6 was best predicted by differences between years (2009: β= -0.294 ± 0.068; 2010: β= -0.557 ± 0.070), brood sizes (β= -0.050 ± 0.013), and hatching dates (β= 0.027 ± 0.005), having controlled for head-bill length (*w*= 1.000, β= 1.023 ±0.016) (**Table 4.4**). By day 12, differential effects of winter feeding treatment were more distinguishable and showed significant differences between years (n= 1706; *w*= 1.000; **Figure 4.3**). At this later stage, brood size continued to be a good predictor of body mass differences (β= -0.126 ± 0.053), as did nestling age at measurement (β= -0.216 ± 0.081), and again head-bill length was controlled for (β= 1.039 ± 0.024) (**Tables 4.3 (b)** and **4.4**).

Concentrations of α-tocopherol and total carotenoids in nestling plasma correlated positively and significantly across years (Pearson's; r= 0.591, n= 573, $p< 0.001$). Similarly, heavier chicks had significantly higher concentrations of antioxidants in the plasma (Pearson's; α-tocopherol: r= 0.110, n= 575, $p= 0.008$; total carotenoids: r= 0.332, n= 582, $p< 0.001$). MDA concentration, however, was negatively correlated to total carotenoid concentration in 2009 only (r = -0.136, n= 237, $p= 0.036$), and there were also no correlations between MDA levels and α-tocopherol concentration or body mass in any year ($p> 0.134$).

α-Tocopherol concentration in nestling plasma was not affected by over-winter feeding treatment (**Tables 4.3 (c)** and **4.4**). However, total carotenoid concentrations differed seasonally between treatment groups (**Tables 4.3 (d)** and **4.4**). Whilst nestlings from control and fat-fed woodlands showed a very marginal change in plasma carotenoids through the season (control: β= -0.022 ± 0.020; fat: β= 0.036 ± 0.025), concentrations from nestlings in fat+VE woodlands were positively correlated with hatching date (β= 0.089 ± 0.025) (**Figure 4.4**). *Post-hoc* pairwise tests shown a significant difference in

Table 4.3. Confidence sets of ranked models (in descending order) for analyses of nestling phenotype. See §4.3.5 for description of global models and modelling terms, Table 4.4 for relative variable importance and model fit, and text for parameter estimates (β).

Rank	Fixed effect	*k*	Log-likelihood	AIC / AICc	ΔAIC / ΔAICc	w_i
(a) Nestling mass day 6						
1	BS + YR + HD + (treat × age)	15	-841.59	1713.6	0.000	0.274
2	BS + YR + HD	10	-846.89	1714.0	0.363	0.228
3	BS + YR + HD + age	11	-846.38	1715.0	1.370	0.138
4	BS + YR + (treat × HD) + (treat × age)	17	-840.28	1715.1	1.509	0.129
5	BS + YR + HD + treat	12	-845.49	1715.3	1.638	0.121
6	BS + (treat × YR) + HD + (treat × age)	19	-838.35	1715.4	1.802	0.111
179	*Intercept-only*	*5*	*-1767.97*	*3546.0*	*1832.371*	*0.000*
(b) Nestling mass day 12						
1	(treat × YR) + (treat × HD) + (treat × BS) + age	21	-1894.55	3831.1	0.000	0.411
2	(treat × YR) + (treat × HD) + BS + age	19	-1897.07	3832.1	1.043	0.244
3	(treat × YR) + HD + (treat × BS) + age	19	-1897.36	3832.7	1.636	0.181
4	(treat × YR) + (treat × HD) + (treat × BS) + (treat × age)	23	-1893.47	3832.9	1.843	0.164
179	*Intercept-only*	*5*	*-2553.62*	*5117.2*	*1286.146*	*0.000*
(c) Plasma α-tocopherol concentration						
1	year + HD + BS	10	-706.98	1434.4	0.000	0.508
2	year + HD	9	-708.73	1435.8	1.432	0.248
3	year + HD + BS + age	11	-706.67	1435.8	1.467	0.244
98	*Intercept-only*	*5*	*-744.40*	*1498.9*	*64.554*	*0.000*
(d) Plasma total carotenoid concentration						
1	(treat × HD) + (treat × YR) + age	18	-788.93	1615.1	0.000	0.539
2	(treat × HD) + YR + age	14	-793.33	1615.4	0.316	0.461
69	*Incept-only*	*5*	*-187.0*	*382.2*	*29.673*	*0.000*
(e) Plasma MDA concentration						
1	year + HD + BS	10	-288.21	596.7	0.000	0.466
2	year + HD + BS + treat	12	-286.61	597.6	0.935	0.292
3	year + HD + age	9	-289.89	598.0	1.306	0.242
87	*Intercept-only*	*5*	*-449.74*	*909.6*	*312.847*	*0.000*

Model terms: treat, feeding treatment (control, fat, fat+VE); YR, year (2008, 2009, 2010); HD, hatch date [1= 1April]; BS, brood size at sampling; age, nestling age at sampling (days post-hatching); (×), interaction term. Head-bill length controlled for in all mass models and blood sample 'time until freezing' controlled for in all α-tocopherol and total carotenoid models – thought both are not listed for easy of model comparison. Intercept-only models reported in *italics* with the unadjusted Akaike weight.

fat+VE group nestling total carotenoid concentration compared to those from both the control (χ^2_2 = 12.86, p= 0.002) and the fat-fed woodlands (χ^2_2 = 6.29, p= 0.043), but no difference between control and fat groups (χ^2_2 = 3.23, p= 0.199). Pairwise significance was consistent across both models in the confidence set (**Table 4.3 (d)**). There was little support for an effect of winter feeding treatment on nestling plasma MDA concentration (w= 0.292) (**Tables 4.3 (e)** and **4.4**). Variation in both nestling plasma antioxidants and MDA levels were best explained by annual differences (see **Table 4.1**) and seasonal variation (α-tocopherol: β= 0.038 ±0.009; MDA: β= -0.009 ± 0.003), with brood size and age at sampling being of lesser importance (**Table 4.4**).

Table 4.4. Relative importance of explanatory variables (Akaike weights, *w*) in analyses of feeding treatment effects on nestling phenotype measures. * Denotes variables for which 95% confidence intervals for parameter estimates do not include zero. See Table 4.3 for confidence sets of ranks models and text for details of parameter estimates (β).

	Body mass		Plasma α-tocopherol	Plasma carotenoids	MDA
	Day 6	Day 12			
Treatment	0.634	1.000		1.000 *	0.292
Year	1.000 *	1.000	1.000 *	1.000 *	1.000 *
Hatching date	1.000 *	1.000	1.000 *	1.000	1.000 *
Brood size	1.000 *	1.000 *	0.752		0.758
Nestling age	0.651	1.000 *	0.244	1.000	1.000
Treatment × year	0.111	1.000 *		0.539	
Treatment × hatch date	0.129	0.819		1.000 *	
Treatment × brood size		0.756			
Treatment × age	0.513	0.164			
Pseudo-R^2	0.858	0.567	0.132	0.246	0.497

Each global model contained all listed terms, and in addition head – bill length was controlled for in body mass analyses and blood sample 'time until freezing' was controlled for in α-tocopherol and total carotenoid analyses, but have not been included in the table.

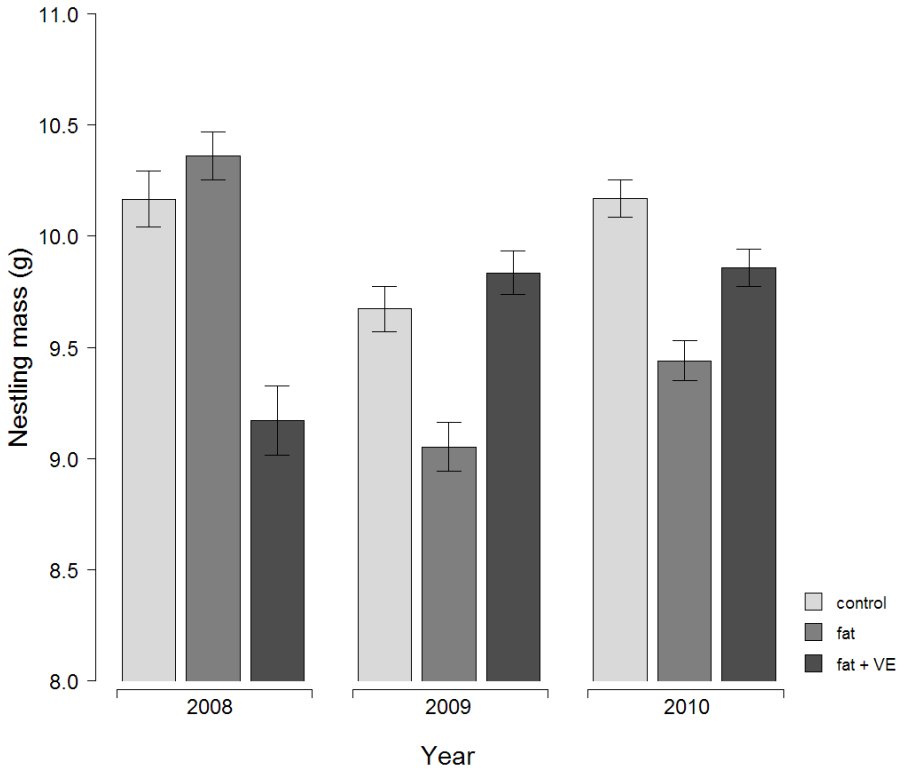

Figure 4.3. The mass of nestling on day 12 post-hatching in relation to over-winter feeding treatment differed between years (mean ± SE from raw values). *Post-hoc* pairwise comparisons reveal a significant difference between the fat+VE and both other treatment groups in 2008 ($p < 0.002$), and also between the fat group in 2008 compared to the following years ($p < 0.001$). See Tables 4.3 and 4.4 for model summaries and text for details.

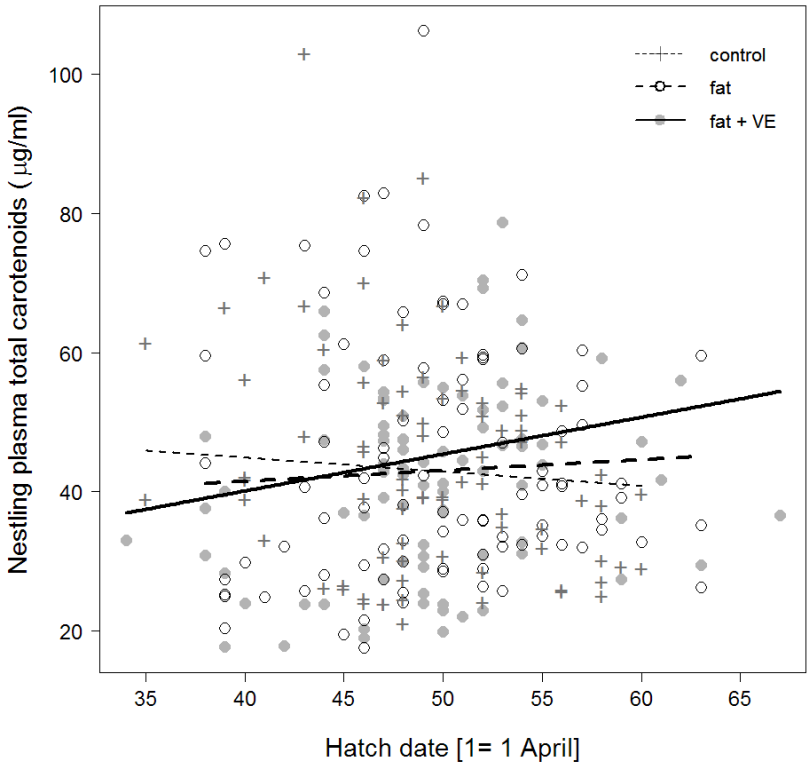

Figure 4.4. The relationship between total carotenoid concentration in nestling plasma and hatch date differed between treatment groups. Plotted using brood means. See Tables 4.4 and 4.5 for statistical findings and text for details of parameter estimates (β).

4.4.3.2 The influence of parental phenotype on nestling condition

Given that adult birds were not caught at all nests, models for nestling masses, and plasma concentrations of antioxidants and MDA were re-run with reduced sample sizes to include either female or male feather carotenoid concentration testing for effects of parental phenotype on offspring condition measures. In each instance there was only a low level of support for an effect of parental inherent quality, and no indication that this varied between treatments (feather carotenoid × treatment interaction always outside 2 ΔAIC set). Specifically, female carotenoid concentration had selection probabilities of *w*= 0.275, 0.317, 0.000 (n= 282 – 409) and males *w*= 0.230, 0.167, 0.115 (n= 262 – 371) for explaining variation in nestlings plasma α-tocopherol, total carotenoid and MDA concentrations respectively, with large standard errors around parameter

estimates and confidence intervals spanning zero. Similarly, neither male nor female feather carotenoids featured in confidence sets for nestling mass at day 6 (n= 454 or 518) or day 12 (n= 860 or 906). Effects of other explanatory variables described (§4.4.3.1) remained consistent across repeated models.

Given that male MDA concentration was seen to vary in relation to over-winter feeding in **Chapter 2**, the potential impact of this on offspring phenotype was also investigated. Again, however, there was little or no support for the inclusion of male MDA concentration in models explaining body mass (*w*= 0.000, n= 443), or concentrations of α-tocopherol (*w*= 0.000, n= 146), total carotenoids (*w*= 0.185, n= 145) and MDA (*w*= 0.000, n= 196) in nestlings 12 days after hatching.

In addition no correlations were found between concentrations of total carotenoid (GLMM: χ^2_1 = 1.45, n= 577, *p*= 0.228) or α-tocopherol (GLMM: χ^2_1 = 0.83, n= 572, *p*= 0.363) in nestling plasma and the concentrations observed in the yolk of eggs sampled from the same nest.

4.4.4 Fledging success

Fledging success, defined as the proportion of hatched chicks which fledged, was significantly reduced in both fed treatment groups during 2008 – 2010 (n= 351, **Table 4.5 (a)**, **Figure 4.5**). The average proportion of chicks which fledged was also significantly lower in 2008 (mean ±SE: 56.2 ± 0.04%) than 2009 (67.9 ±0.03%) and 2010 (69.6 ±0.03%) (2008 pairwise *post-hocs*: χ^2_1 > 6.71, *p*< 0.010; 2009 v 2010 *p*= 0.18 NS). However despite notable variation in the effect of treatment among years (**Table 4.1**) the treatment × year interaction term was not included within the confidence set. Again only one model was supported within the 2 ΔAIC set, which also showed that broods hatched later in the season fledged a greater proportion of nestlings (β= 0.037 ±0.017) (**Table 4.5 (a))**. When included as explanatory variables, neither male (n= 145, *w*= 0.280) nor female (n= 160, *w*= 0.375) feather total carotenoid concentrations were accurate predictors of fledging success variation, and male MDA concentration was not included in the confidence set (n= 77, *w*= 0.000).

Table 4.5. Confidence sets for fledging success. (a) Analysis of proportion of hatched chicks which fledged per nest (n= 351); and (b) an analysis of the proportion of all laid eggs which fledged per nest (n= 405). See §4.3.5 for description of global models and modelling terms, and text for further details.

Rank	Fixed effect	*k*	Log-likelihood	AICc	ΔAICc	w_i	Pseudo-R^2
(a) Fledging success of hatched eggs							
1	treat + year + hatch date	8	-495.6	1007.6	0.000	1.000	0.107
13	*Intercept-only*	*3*	*-514.4*	*1034.8*	*27.236*	*0.000*	
(b) Fledging success of laid eggs							
1	(treatment × year)	11	-636.6	1295.8	0.000	0.324	0.137
2	treatment + year	7	-641.2	1296.6	0.836	0.213	
3	(treatment × year) + lay date	12	-636.0	1296.7	0.922	0.204	
4	treatment + year + lay date	8	-640.6	1297.5	1.720	0.137	
5	(treatment × lay date) + year	10	-638.6	1297.7	1.945	0.122	
13	*Intercept-only*	*3*	*-665.3*	*1336.7*	*40.884*	*0.000*	

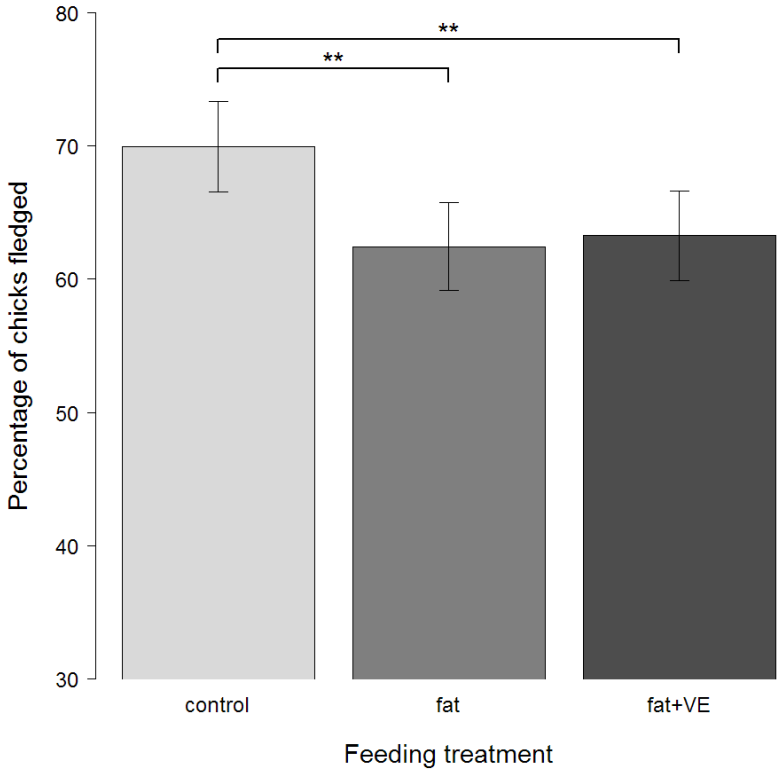

Figure 4.5. Mean percentage of chicks which fledged per nest during 2008-2010 in relation to over-winter feeding treatment. Mean ± SE plotted using raw values. *Post-hoc* pairwise comparisons shown, where ** = $p \leq 0.01$. See Table 4.5 for model summary and text for details.

However in an alternative analysis, using the proportion of laid eggs which fledged as the response variable, over-winter feeding was no longer an accurate predictor of fledging success. In this analysis, model selection uncertainty increased to produce a large confidence set (**Table 4.5 (b)**), and despite featuring in all models (w= 1.000) standard errors around treatment parameter estimates were high (fat: β= -0.282 ± 0.504; fat+VE: β= -0.412 ± 0.499) and confidence intervals spanned zero. Again, when included as an explanatory variable, female feather carotenoid concentration was a poor predictor of laid egg fledging success variation (w= 0.426, β= 0.181 ± 0.528; feather carotenoids × treatment: w= 0.237), and there was no influence of male phenotype

(MDA and feather carotenoid, w= 0.000). The only accurate predictor of laid egg fledging success, for which confidence intervals did not include zero, was year (w= 1.000; 2009: β= 0.834 ± 0.378; 2010: β= 0.571 ± 0.377).

A total of 480, 514 and 465 chicks fledged from control, fat and fat+VE treatment groups respectively during 2008 – 2010, and despite proportional differences in fledging success, the mean number of nestlings fledged per brood did not differ significantly among treatment groups (Poisson GzLMM: χ^2_2 = 4.03, p= 0.113, see **Table 4.1**), but was marginally, but non-significantly, influenced by annual variation (χ^2_2 = 5.92, p= 0.052). Again, measures of parental phenotype did not significantly influence numbers of chicks fledged (p> 0.14; 2-way interactions with treatment, p>0.23).

4.5 DISCUSSION

In previous chapters, over-winter provisioning of α-tocopherol has been suggested to influence population phenotypic structure, maternal egg investment and paternal oxidative status (**Chapters 2** and **3**). It was hypothesised that this would also culminate in an improvement in productivity. Whilst there was evidence of an improvement in hatching success resulting from vitamin E provisioning in one year (**Figure 4.2**), individuals breeding in both fat and vitamin E supplemented woodlands in fact went on to show a significant reduction in nestling phase productivity, fledging 7.1% fewer hatched chicks on average than unfed birds across three years (**Figure 4.5**). This suggests that initial investments made by fed birds were not sustainable post-hatching. However, assessment of the effects of parental phenotypes did not elucidate this. Fledging success and numbers were not significantly influenced by feather carotenoid or paternal MDA concentrations.

Furthermore, laying date did not differ in response to supplementation, though females with higher feather carotenoid concentrations were able to lay earlier (**Figure 4.1**). There was evidence that nestling mass *ca.* 12 days after hatching responded differently to treatment in 2008 only; such that nestlings within vitamin E supplemented woodlands were shown to be of lower average body mass, having controlled for brood size and age

differences (**Figure 4.3**). Interestingly, plasma concentrations of total carotenoids showed a seasonal increase in the vitamin E supplemented woodlands (**Figure 4.4**), whilst concentration of α-tocopherol and susceptibility to oxidative damage were not different between treatment groups (**Tables 4.3** and **4.4**). In the following sections I will interpret the possible mechanisms underlying these results and discuss their applied ecological relevance.

4.5.1 Clutch initiation date

The timing of breeding is a notable determinant of reproductive success (Nilsson 2000; Naef-Daenzer et al. 2001), with supplementary feeding often seen to advance reproduction (Martin 1987; Robb et al. 2008a). Furthermore, in migratory bird species wintering habitat quality has been shown in influence timing of arrival at breeding grounds (Marra et al. 1998; Norris et al. 2004), and similarly winter provisioning has previously advanced laying dates in resident blue tit populations (Robb et al. 2008b). Females who lay earlier may benefit from greater prey availability for raising nestlings and an increased probability of offspring recruitment (Verhulst & Tinbergen 1991; Noordwijk et al. 1995). Since antioxidant benefits of winter supplementary food are hypothesised to alleviate oxidative stress, one might therefore predict that individuals with access to antioxidant-rich foods would enter the breeding season in superior condition, and reach thresholds necessary to breed earlier (Perrins 1965; Svensson & Nilsson 1995; Robb et al. 2008b). However, in the present study, no effects of winter provisioning of either energetic or micronutrient-based supplements on clutch initiation dates were apparent (**Table 4.2**). By comparison, the findings presented here suggest that females with higher concentrations of feather carotenoids lay earlier than their conspecifics irrespective of supplementation (**Figure 4.1**).

Carotenoid-based feather colouration is hypothesised to be a reliable signal of individual quality during moult (Møller et al. 2000), and the yellow breast plumage of blue tits has previously been shown to be indicative of reproductive capacity and brood-rearing capabilities (Senar et al. 2002; Hidalgo-Garcia 2006; Doutrelant et al. 2008). However, feather carotenoid concentrations are reported not have influenced resource allocation to eggs (**Chapter 2**) or parental phenotype later in the breeding season (**Chapter 3**) within these blue tit populations, potentially due to seasonal variation in

antioxidant availability and allocation demands (Biard et al. 2005). However it would seem that for females, inherent quality does in part influence breeding traits (**Figure 4.1**). It is perhaps surprising that there was no support for differential effect of inherent quality between treatment groups on laying dates, given that in **Chapter 3** a reduction in average feather carotenoid levels was reported in vitamin E-supplemented birds (**Figure 3.1**). This suggests that over-winter feeding is of little importance in comparison to variation in natural food abundance, as indicated by between year differences. But in addition, it is possible that at a population level, the influence of supplementary feeding on the timing of breeding may be difficult to detect (see **Chapter 5**).

4.5.2 Hatching success and nestling phenotype

Embryo development, hatching success and early nestling survival may all benefit from increased resource allocation into eggs (Perrins 1996; Blount et al. 2000; Royle et al. 2001). Furthermore, hatching success has been shown to increase as a result of food supplementation during incubation (Nilsson & Smith 1988), and nestling survival has been attributed to the provisioning of carotenoids specifically prior to egg laying (McGraw et al. 2005). Indeed as predicted, hatching success was improved following vitamin E provisioning, which likewise influenced egg resource allocation in **Chapter 2**. However this was inconsistent across years and only significant during the 2008 breeding season (**Figure 4.2**). Annual variation has been reported across numerous aspects of avian ecology; including population size responses to winter food uptake (Kallander 1981), egg composition and number (Arnold et al. 1991; Nager et al. 1997) and offspring survival (Norris 1993). In particular it is acknowledged that supplementary food is only one factor influencing breeding performance and survival, and that if food availability and quality thresholds are met by natural resources, the role of supplementation becomes diluted or redundant (Svensson & Nilsson 1995; Nager et al. 1997). Interestingly, 2008 was a relatively mild but wet winter compared to the subsequent years, and supplement uptake was considerably lower (see **Chapter 2 §2.3.2**). Although also followed by a wetter than average breeding season, it is surprising that improvements in hatching success should be seen in this first year. It may be possible that the effects of winter supplementation were carried-over from previous years to influence performance in 2009 and 2010 (Grieco et al. 2002), thus

making most recent treatment effects more difficult to detect. However given that other breeding parameters have responded equally to treatment across years this is perhaps unlikely. With a sample size of n= 3 for each treatment group in any given year, it should perhaps also not be discounted that this result could have been derived by chance.

Improvements to egg quality and parental condition in response to pre-laying antioxidant supplementation have previously been found to influence nestling phenotype (Biard et al. 2005; McGraw et al. 2005). Although not shown explicitly in **Chapter 3**, it was predicted that the benefits seen in male oxidative status as a result of winter vitamin E availability might allow for an increase in nestling provisioning effort. Similarly, it was expected that improvements in parental body condition and health status generated via increased uptake of antioxidants at feeders would allow for an increase in the quantity and quality of food allocated to offspring (Grieco 2002), therefore resulting in nestlings of superior condition. Nestling phenotype, however, was not strongly influenced by over-winter supplementation of parental birds in this study (**Tables 4.3** and **4.4**). Nestling mass was similar across all treatment groups in the early nestling phase. Since males assume a larger proportion of the provisioning responsibility until *ca.* day 6 of the nestling phase due to female brooding activities (Perrins 1979), this suggests that male brood-rearing capacity may not have been influenced by supplementation. The subsequent reduction in comparative nestling mass at day 12 in response to vitamin E provisioning shown in 2008 (**Figure 4.3**) gives an indication of an incapacity of parents to sustain a higher workload across the brood-rearing period, particularly given the earlier reported increased hatching success during the same breeding season.

Neither plasma concentrations of α-tocopherol or MDA in nestlings were influenced by parental feeding treatment (**Tables 4.3** and **4.4**). Furthermore, plasma antioxidant concentrations were not correlated with those seen in an egg collected from the same clutch. However, contrary to this a seasonal increase in nestling plasma concentrations of total carotenoids was found within the vitamin E treatment group (**Figure 4.4**). Blue tits predominantly feed nestlings a diet of antioxidant-rich caterpillars (Perrins 1991), however as the season progresses their abundance is diminished and late nestlings may

suffer a poorer diet in terms of antioxidant content (Arnold et al. 2010). This suggests that vitamin E supplemented individuals may be provisioning nestlings with higher quality prey items later in the season. Alternatively, as eluded to in **Chapter 3**, they might be able to cache antioxidant reserves within lipid-rich tissues over-winter, liberating them during provisioning to relieve physiological trade-offs when caterpillars become scarce (McGraw & Toomey 2010; Metzger & Bairlein 2011). Early nutrition can have a profound effect on survival and fitness-related traits in adulthood, with nestling carotenoid uptake influencing sexually-selected colouration and antioxidant assimilation capacity in adult birds for example (Hõrak et al. 2000; Blount et al. 2003). Therefore nestlings fledging later from vitamin E supplemented woodlands may be expected to benefit from improved immune function, brighter carotenoid-based plumage or increased post-fledging survival (Hõrak et al. 2000). However, this awaits further study.

Parental feather carotenoid levels were poor indicators of hatching success and nestling phenotypes. It is noted that feather sampling occurred later in the season, and therefore the analyses reported are likely to disproportionally represent individuals with greater hatching successes, making an effect more difficult to detect. However, with the exception of laying dates, feather carotenoid values have failed to influence a number of breeding parameters later in the season (see also **Chapters 2 and 3**). Therefore it would appear that carotenoid-based feather colouration might not always be a reliable indicator of reproductive capacity (Biard et al. 2005; Hidalgo-Garcia 2006; Doutrelant et al. 2008).

4.5.3 Fledging success

It was predicted that supplementary feeding would increase nestling survival until fledging (Hõrak et al. 2000; Robb et al. 2008a). However the results presented here indicate that supplemented birds made an unsustainable investment in offspring number, and as such, they were unable to fledge the same proportion of hatched chicks as control birds (**Figure 4.5**). Although over-winter provisioning has previously been found to improve fledging success during the subsequent breeding season in blue tits (Robb et al. 2008b), concern has also been raised that winter feeders might act as an ecological trap by encouraging birds to settle in areas which have insufficient natural resources once

supplementary food is removed (Jones & Reynolds 2008; Robb et al. 2008a). This seems a plausible explanation for the present findings, particularly since reductions in fledging success where seen across both treatment groups. Although feeding was stopped at least one month before laying commenced, pairs are likely to have already begun forming territories at this time (Perrins 1979). As such it is possible they made poorly informed decisions about nesting locations, with regard to local food availability. Nest box occupancy was not influenced by feeding treatments, and therefore it does not appear that population densities have been altered in addition, although pairs using natural nesting sites are not accounted for (Jansson et al. 1981).

In **Chapter 3** it was reported that population phenotypic structure was altered in response to supplementary feeding; vitamin E fed populations consisted of individuals with significantly lower feather carotenoid concentrations (**Figure 3.1**). Although this was not seen to influence parental phenotype measures during brood-rearing, and has had a negligible effect on nestling phenotype also, it would appear that apparent benefits to condition gained from supplementary feeding are not carried-over into the final stages of the breeding season. As a result, nestlings from supplemented birds showed increased mortality. Indeed, reduced nestling mass is known to decrease fledging and later survival in blue tits (Nur 1984b), therefore the earlier reported reduction in body mass may have contributed to reduced fledging success. It would seem that parents from supplemented woodlands have made poor investment decisions, having produced more chicks than they were able to sustain. However, in the context of total reproductive output supplementation made little difference, with both the proportion of eggs laid which fledged and actual fledging numbers similar across treatment groups.

4.5.4 Ecological relevance

As urban land cover expands, garden bird feeding has been promoted as a valuable method of conservation for declining wild bird populations (e.g. BTO 2009; RSPB 2009). However, this advice is based on very little empirical evidence of its true ecological impact. The findings presented here suggest that provisioning supplementary food in winter, and antioxidants in particular, may indeed produce some of the benefits hoped for; such as higher hatching success and improved nestling plasma carotenoids, in addition to other benefits already reported in **Chapters 2 and 3**. However, feeding

may not ultimately impact upon reproductive output, in terms of the actual numbers of nestlings fledging at the end of the breeding season. But perhaps more concerning, it appears that winter provisioning may give birds false cues as to natural food availability and encourage them to make an unsustainable investment in nestling numbers, thereby acting as an ecological trap with detrimental consequences for fledgling success.

These findings are based on three years of results, and draw attention to the notably impact annual variation has in determining how supplementary feeding influences breeding performance. Both hatching success and consequently nestling body mass differed in response to feeding only during 2008, and across all measures of performance and nestling phenotype examined, prevailing differences were the result of between year variations. Annual variation in both breeding performance and responses to supplementation are commonly reported (Kallander 1981; Arnold et al. 1991; Norris 1993; Svensson & Nilsson 1995; Nager et al. 1997), however many studies of food provisioning will often only report on one year of work (Robb et al. 2008b). Perhaps above all, these finding highlight a necessity to pay closer attention to the ecological impacts garden bird feeding might be having on wild bird populations, and entertain the possibly that that these effects may not all be beneficial.

CHAPTER 5

Individual variation in feeder use: revealing the mechanisms which mediate carry-over effects of winter food supply

5.1 ABSTRACT

Carry-over effects arise when ecological conditions experienced during winter influence reproductive success in the subsequent breeding season. Energy is traditionally viewed as the major currency driving carry-over effects. However other potential mechanisms, such as antioxidant availability could be important. This may be particularly true for income breeders, which do not use endogenous macronutrient reserves to fuel reproduction. Using a supplementary feeding study, I have investigated the effects of fat and fat-plus-vitamin E uptake as mechanisms influencing the carry-over effects of winter feeding conditions in blue tits (*Cyanistes caeruleus*). By provisioning supplements which were isotopically distinct from natural food sources, it was possible to use stable nitrogen isotope signatures ($\delta^{15}N$) in claws to infer individual uptake of supplementary food during winter. Considerable variation existed in the use of provisioned food, with individuals of lowest expected over-winter survival taking greatest advance of this additional resource; juveniles and females with lower concentrations of feather carotenoids. This indication of a lack of competitive exclusion at feeders suggests these may not be limited resources for all individuals. Furthermore, increases in supplement uptake were correlated to earlier laying, despite a previous suggestion that timing of breeding was not influenced by over-winter feeding at a population level in **Chapter 4**. It was particularly interesting to note that this relationship was independent of treatment group, and that blue tits, classically described as income breeders had benefitted from over-winter energetic gains. Females using vitamin E supplements additionally benefitted from improved carotenoid investment into eggs, empirically showing for the first time that carry-over effects can also be fuelled by antioxidant uptake. With garden bird feeding a popular and growing phenomenon, these findings provide new insight into the ways in which large-scale, diffuse supplementary feeding can affect wild bird populations.

5.2 INTRODUCTION

Winter food availability is an important short-term determinant of survival (Jansson et al. 1981; Kallander 1981; Perdeck et al. 2000; Siriwardena et al. 2007). But analogous to this, the decisions made during one season may impact upon events at a later time or place (Fretwell 1972). It is increasingly recognised, therefore, that in addition to influencing survival, winter feeding conditions could affect subsequent life-history events such as reproduction (reviewed in Harrison et al. 2011).

Carry-over effects, where an individual's previous ability to resolve trade-offs can affect future fitness (Harrison et al. 2011), have commonly been demonstrated in migratory birds. For example, it is evident that wintering habitat choices can affect both body condition and reproductive success on the breeding grounds (Marra et al. 1998; Bearhop et al. 2004b; Norris et al. 2004; Bearhop et al. 2005). Since these species are predominantly capital breeders, investing stored energy reserves built-up during the preceding months into reproduction (Drent & Daan 1980), it has often been assumed that carry-over effects are the result of energetic, and specifically fat, gains (e.g. Inger et al. 2008). Indeed, macronutrients, and energy supply in particular, can be key determinants of over-winter survival in birds (Koivula et al. 1995). However, the possible carry-over effects of winter energy supply on subsequent reproduction have not been subject to investigation (but see **Chapters 2 – 4**). Furthermore, it seems highly unlikely that energy is the only currency driving carry-over effects (Harrison et al. 2011), especially in species classically referred to as 'income breeders' such as the blue tit *Cyanistes caeruleus* (Drent & Daan 1980; Robb et al. 2008b).

Income breeders are thought not to rely on endogenous macronutrient reserves during breeding, but adjust food intake to meet the energetic demands of reproduction (Drent & Daan 1980; Jonsson 1997). As such, winter energy supply can only influence future breeding performance indirectly through its benefits to condition. It is thought that micronutrients, and antioxidants in particular, may also be important mediators of carry-over effects, particularly for income breeders (Harrison et al. 2011; **Chapters 2 - 4**). Vitamin E, for example, plays a vital role in immune defence and disease prevention (Halliwell & Gutteridge 2007; Catoni et al. 2008). Functioning as a chain-breaking antioxidant, it mitigates against lipid peroxidation and thereby prevents the propagation

of reactive oxygen species (ROS) (Sies & Stahl 1995). Similarly, carotenoids are effective ROS quenchers, in addition their role in signalling colouration (Møller et al. 2000). The importance of carotenoids as effective antioxidants is under debate (Costantini & Møller 2008), but their capacity to provide synergistic protection and recycling of vitamin E still affords them a valued position in avian antioxidant defence systems (Sies & Stahl 1995; Surai 2007), As a result antioxidants have the potential to to control levels of oxidative stress, and concomitant serious lipophilic damage of proteins and DNA macromolecules (Burton 1994; Brigelius-Flohe & Traber 1999). However, vitamin E and carotenoids cannot be synthesized endogenously and must be obtained through feeding alone (Goodwin 1984). As such, antioxidant supply can be limiting, and result in physiological trade-offs in their allocation (Olson & Owens 1998; Catoni et al. 2008).

With a greater access to antioxidant resources during winter, specific aspects of immune defences could be enhanced, enabling birds to enter the breeding season in a superior condition and having suffered less debilitation due to parasites, diseases and oxidative damage. But furthermore, as lipophilic molecules of low weight, there is potential for high quantities of antioxidants to be stored in lipid-rich tissues in all bird species (Negro et al. 2001; Metzger & Bairlein 2011). Therefore in addition to having an indirect effect on reproduction through condition-based benefits, antioxidant might also be used as direct capital investment in the breeding season. Whilst previous studies have investigated the effects of energy and antioxidant supplementation during the breeding season (Ramsay & Houston 1997; Blount et al. 2002), none have focused on the potential carry-over effects that winter feeding conditions might have on productivity (but see **Chapters 2 – 4**).

Garden birds have access to a wide abundance of supplementary food resources, (Davies et al. 2009) which can prove critical to over-winter survival (Kallander 1981), when natural foraging opportunities are reduced (Newton 1980). However, natural food availability, access to and competition at feeders and differences in individual foraging or assimilation efficiency may all lead to intra-population variation in the uptake of provisioned food. Recent evidence suggests that over-winter dietary provisioning can influence subsequent reproductive traits at a population level; including egg

composition, parental oxidative status and hatching and fledging success rates (**Chapters 2 – 4**). However, a level of uncertainty in drawing accurate predictions about the mechanistic effects of over-winter feeding has been highlighted (e.g. **Chapter 2**), and it is anticipated that by assessing the impacts of supplementary feeding solely at a population level (e.g. Chapters 2 - 4; Robb et al. 2008b) the true magnitude of its effects may become masked (Catoni et al. 2008).

Since environmental factors and individual differences in behaviour may generate considerable variation within a population, the understanding of how carry-over effects operate may be improved by accounting for intra-population variations. Using stable isotope analysis the proportion of supplementary food in the diet of individuals can be determined (Davis et al. 2005). Stable isotope ratios expressed in body tissues reflect the diet of individuals at the time of tissue synthesis, and since avian claws are metabolically inert and have a slow growth rate they can be used to infer diet up to 5 months prior to sampling (Bearhop et al. 2003; Inger & Bearhop 2008). As such, stable isotope analysis of claw samples can provide a direct link between the reliance of individual birds on supplemented food in the winter and their reproductive performance during the following spring.

Through the application of stable isotope analysis, the aims of this chapter are to (1) investigate the causes of intra-population variation in supplement use; and (2) to reveal the impacts of this variation on subsequent breeding performance in populations of blue tits provisioned with either fat or fat-plus-vitamin E (fat+VE, α-tocopherol) supplements through the winter period only. By fuelling metabolism and enabling birds to maintain a higher body mass, increased fat uptake is predicted to enhance over-winter condition and possibly influence spring breeding as a result. Whereas uptake of fat+VE is predicted to enhance condition through a reduction in oxidative damage in winter, and have an additional direct effect on productivity through its capacity to be stored.

5.3 METHODS

5.3.1 Study sites and experimental design

The stable isotope experiment was conducted within supplemented deciduous woodland sites during the winter of 2008 – 2009 and the carry-over effects measured in the subsequent 2009 breeding season, as part of a larger study (reported in **Chapters 2 – 4**). A subset of four sites were selected for inclusion in the stable isotope study; namely Kennall Vale, Devichoys, Trevarno and Trelissick (see **Appendix 1**). These sites were paired within predefined triplet groups according to vegetative composition, and randomly assigned to one of two supplementary feeding treatments (as described in **Chapter 2 §2.3**). Feeding treatments included: *(1) fat only* (to test for energy effects; hereafter 'fat'), and *(2) fat-plus-vitamin E* (to test for effects of energy plus antioxidants, hereafter 'fat+VE').

Supplementary food was provisioned through the winter, from 18 November 2008 – 11 March 2009, leaving an interval of at least one month before laying commenced (11 April). Fresh 150g fat balls where provisioned at squirrel-proof feeders, positioned 100m apart (*ca.* 9 per site), every 10 days. For the fat+VE treatment group, fat balls were supplemented with 10mg/ 100g α-tocopherol (≥96% DL-all-*rac*-α-tocopherol (HPLC), Sigma-Aldrich Ltd., Dorset), a concentration equivalent to that occurring naturally in peanuts, a popular garden bird food (Chun et al. 2005). Since α-tocopherol cannot be provisioned without the use of a 'carrier' and fat is required for its absorption (Blount et al. 2002; Jeanes et al. 2004), the fat+VE treatment provides an ecologically meaningful method of testing antioxidant effects. Supplementary food was prepared as described in **Chapter 2 §2.3.2**.

5.3.2 Isotopic labelling of supplementary food

An isotopically distinct marker was added to supplementary food during the final 3 weeks of the provisioning period. A mix of ^{15}N-enriched alanine (L-alanine, 98% ^{15}N; CK Gas Products Ltd, Hampshire, UK) and DL-alanine (Sigma-Aldrich Ltd., Dorset) was prepared at a ratio of 7.5g / kg DL-alanine. This was added to viscose fat at a concentration of 5g/ 100g fat as part of regular food preparation methods described in

Chapter 2 §2.3.2, and mixed repeatedly to allow for an even distribution throughout the food. The fat was then weighed and allowed to cool until solidified as individual fat balls as previously described (**Chapter 2 §2.3.2**). Average consumption of food supplemented during the whole provisioning period was 3.08 ± 0.94 kg (mean per site ± SD), and for the period during which the ^{15}N-enriched food was provisioned it was 1.17 ± 0.16 kg.

During the period of ^{15}N-enriched food provisioning, natural food items were also sampled at all sites for comparison, following Parid dietary patterns presented in Betts (1955). These included arachnids, other invertebrates and beech seeds (*Fagus* spp.), which were stored at -18^{o}C until stable isotope analysis.

5.3.3 Measurement of breeding parameters and adult sampling

Within sites, nest boxes were evenly distributed at a density of *ca*. 4 boxes per hectare. These were monitored every 1 – 3 days from April to June to investigate the effects of supplementary food uptake on breeding performance. Lay date and clutch size were determined as described in **Chapter 2**, and one egg per clutch removed for yolk antioxidant level determination (see **§2.3.3** for details of egg collection). Hatching date was determined as described in **Chapter 3**. Hatching success was defined as the proportion of a clutch which hatched; and fledging success defined as the proportion of hatched eggs which fledged. These were calculated as described in **Chapter 4**.

Either one or both parents were captured at a subset of nest boxes using spring traps (Amber Electronics Ltd, Daventry, UK) between day 5 – 18 of the nestling phase (total independent nests, n= 38; total individuals, n= 51). Sex and age were recorded, and yellow breast feathers sampled as described in **Chapters 2 and 3**. Yellow carotenoid-based plumage colouration exhibited in blue tits is hypothesised to be a reliable signal of condition at the time of feather growth, and therefore prior to winter feeding (Møller et al. 2000). As such feather carotenoid concentrations have been used as an indicator of pre-feeding condition in this study. One claw sample (*ca.* 3mm) per individual was taken using scissors and stored at room temperature until stable isotope analysis. Claws and feather samples were collected under Home Office license (PIL 30/8161).

5.3.4 Biochemical assays

Levels of α-tocopherol and total carotenoids in egg yolk were quantified using high-performance liquid chromatography (HPLC) as described in **Chapter 2 §2.3.4**. Levels of total carotenoids in the yellow breast feathers were determined by mechanical extraction and spectrophotometry as described in **Chapters 2 and 3**.

5.3.5 Stable isotope analysis

Whole claw samples where weighed into tin capsules. Dietary samples were first dried at 80^{o}C for *ca.* two days, ground into a powder using a pestle and mortar, and then weighed (0.7 mg) into the tin capsules (excepted fat, 3.0 mg not dried) . Stable isotope analysis was conducted at the Scottish Universities Environmental Research Centre (SUERC) in East Kilbride by continuous-flow isotope ratio mass spectrometry (CF-IRMS), using a Thermo Fisher Scientific (Bremen, Germany) Delta Plus XP IRMS with Costech ECS 4010 elemental analyzer (Costech, Milan, Italy). Nitrogen stable isotope ratios are expressed as δ^{15}N values in parts per thousand (‰), and correspond to the ^{15}N/^{14}N isotope ratio. Replicate analyses of laboratory standard gelatine and alanine reference samples yielded standard deviations better than 0·23 ‰ for δ^{15}N.

The enriched food had a δ^{15}N value far exceeding blue tit's natural food sources (**Table 5.1**), and therefore allowing for the use of stable isotope signatures of individual claw samples to infer individual level differences in supplement uptake.

Table 5.1. Mean nitrogen isotopic values for natural food sources compared to enriched supplementary food

Food source	δ^{15}N	SD
Arachnids	4.86	2.21
Other invertebrates	0.91	1.43
Seeds	-2.14	0.80
Supplementary fat balls	207.96	84.81

5.3.6 Statistical analyses

All statistical analyses were conducted using R version 2.12.2 (R Development Core Team 2011). In all analyses claw $\delta^{15}N$ values were used as a proxy for supplementary food uptake, fitted as either a dependent variable or a fixed effect, and were log-transformed to correct for positive-skew. The influences of sex, age, pre-feeding condition (feather total carotenoid concentration), treatment type and woodland sites on levels of supplement uptake were examined using a general linear mixed model (GLMM) fitted using 'lme' from the *nlme* package (Pinheiro et al. 2010). Woodland site was specified as a random effect; sex, age and treatment as two level fixed factors; and feather carotenoid concentration as a covariate following log-transformation to correct for positive-skew. All 2-way interactions were also fitted, and normality and heteroscedasticity of model residuals check prior to model simplification. The influence of supplementary food use on breeding performance was examined using uptake per pair as an explanatory variable, controlling for feeding treatment and lay date and including supplement uptake 2-way interactions. GLMMs were applied to lay date and clutch size data, and binomial generalized linear mixed models (GzLMM) fitted to hatching and fledging success having checked for overdispersion using 'lmer' from the *lme4* package (Bates et al. 2011). Site was specified as the random effect in all cases, and data were filtered as described in **Chapters 2 and 4**. Uptake per pair was calculated as the mean claw $\delta^{15}N$ value for the male and female within a breeding pair; for nests in which both individuals were not sampled, a value for either the male or female was used. Variation in yolk antioxidant concentrations were examined using female $\delta^{15}N$ values only, again controlling for treatment and lay date.

The significance of fixed terms was calculated using maximum likelihood (ML) and χ^2 likelihood ratio tests, with the minimum adequate model (MAM) obtained by stepwise deletion of the least significant terms until only significant terms ($p< 0.05$) remained in the model. The significance of woodland site was accessed by comparing the MAM which included the site random variable to a GLMM fitted with a dummy random term following (Crawley 2007). *Post hoc* pairwise comparisons were carried out by comparing the original GLMM minimum model with models in which factor groups were paired.

5.4 RESULTS

There was a large degree of variation in levels of supplementary food uptake amongst the 49 blue tit individuals sampled (mean claw $\delta^{15}N$ value ±SD = 7.47 ± 6.37‰; range = 1.44 – 32.00‰). Upon consumption and assimilation, isotopic ratios are automatically shifted up the trophic scale. For small passerines, the shift in $\delta^{15}N$ signature between food sources and keratin-rich tissues such as claw, referred to as the trophic enrichment factor (TEF; Inger & Bearhop 2008), is estimated at +3.70 ± 0.21 ‰ (Hobson & Bairlein 2003; Pearson et al. 2003). Comparison of claw and natural food $\delta^{15}N$ values suggests, therefore, that whilst some individuals relied heavily on supplements, others with $\delta^{15}N$ values close to or below the mean fed predominantly on invertebrates and seeds (**Table 5.1**). Variation also existed within breeding pairs, with no significant correlation between 13 pairs of males and females sampled from the same nests (Pearson's correlation: r= 0.23, n=13, *p*= 0.45).

5.4.1 The causes of variation in uptake

Mean values of $\delta^{15}N$ in claws were significantly different between woodland sites (**Figure 5.1, Table 5.2**), and this was independent of whether energy or antioxidant-based supplements were provisioned (**Table 5.2**). Supplement uptake was greater in yearling birds than individuals aged 1 year or greater, however this was only within fat+VE fed sites. Furthermore, males and females significantly differed in their uptake of supplemented food depending upon the concentration of carotenoids in the feathers, such that uptake was negatively correlated with female feather carotenoid concentration, but positively correlated within males (**Figure 5.2, Table 5.2**).

Table 5.2. The causes of variation in supplementary food uptake (claw $\delta^{15}N$ values). All main effects and significant interactions from GLMM minimum adequate model presented. NS, denotes non-significant effects.

Variable	χ^2_1	*p*	Effect
Fixed effects:			
Sex	0.13	0.717	NS
Log (feather carotenoids)	0.00	0.960	NS
Age	0.55	0.460	NS
Treatment	1.05	0.305	NS
Sex × log (feather carotenoids)	4.70	0.030 *	♂= positive correlation ♀= negative correlation
Age × treatment	5.27	0.022 *	Yearlings higher in fat+VE group only (p= 0.024), other *post-hoc* comparisons NS.
Random effects:			
Site	14.76	<0.001 ***	All *post-hoc* p< 0.001, except KV v TV: p= 0.029 and DV v TV: p= 0.78 NS.

Site codes: DV, Devichoys; KV, Kennall Vale; TL, Trelissick; TV, Trevarno.

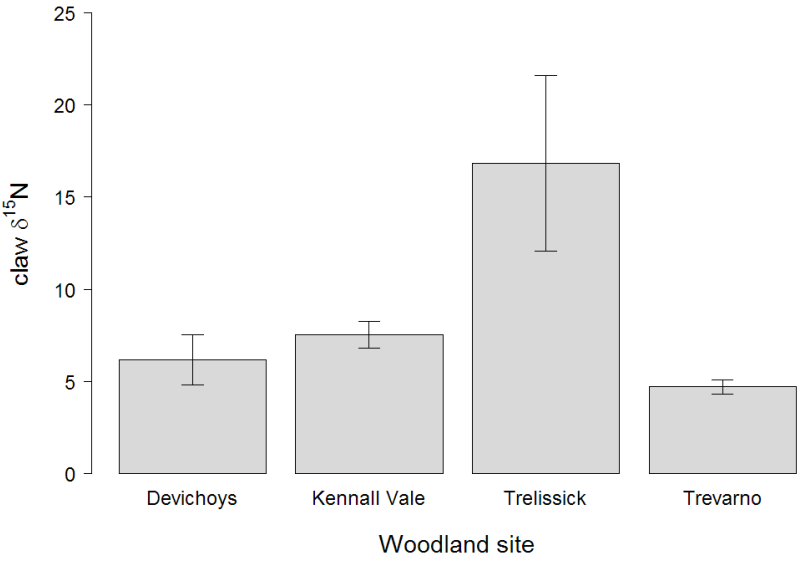

Figure 5.1. Variation in supplement uptake (claw $\delta^{15}N$ value) between woodlands. See Table 2 for statistical findings and text for further details. See Appendix 1 for woodland descriptions.

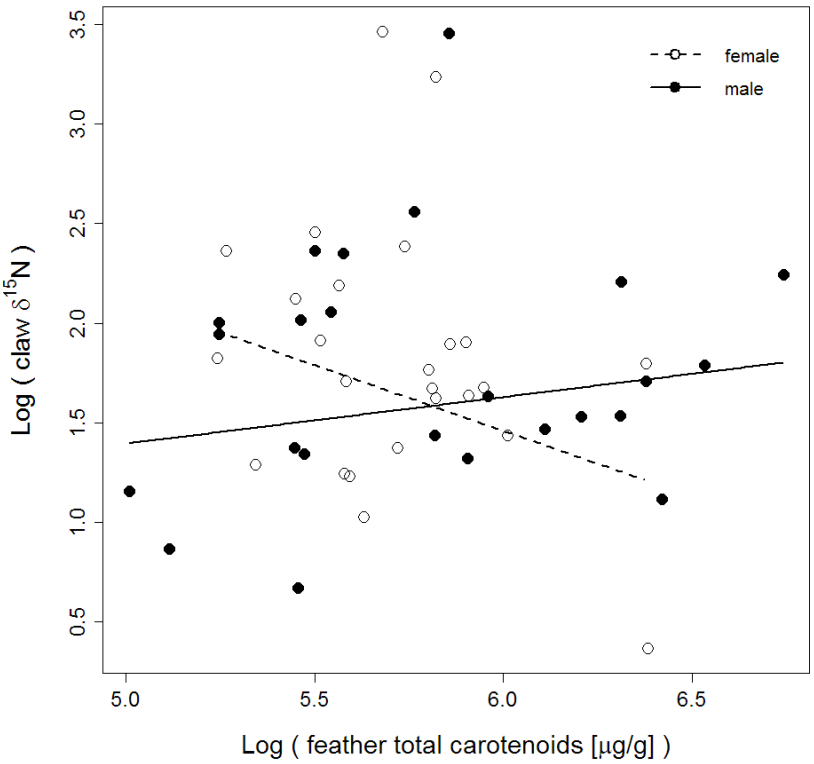

Figure 5.2. The linear relationship between feather total carotenoid concentration and claw δ^{15}N value differed between sexes. Lines plotted using statistical estimates. See Table 2 for statistical findings and text for further details.

5.4.2 The effects of variation in uptake on breeding performance

Pairs with higher mean claw δ^{15}N values had significantly earlier lay dates (n= 36, GLMM; χ^2_1 = 6.46, p= 0.011; **Figure 5.3**), and this was irrespective of the whether fat or antioxidant-based supplements were consumed (log(δ^{15}N) × treatment interaction; χ^2_1 = 0.64, p= 0.64). The relationship between yolk total carotenoid concentrations and female claw δ^{15}N value did differ according to treatment (n= 17, GLMM: log(δ^{15}N) × treatment interaction, χ^2_1 = 4.93, p= 0.026, **Figure 5.4**), but there was no effect of uptake levels on yolk α-tocopherol concentrations (GLMM: log(δ^{15}N) × treatment interaction, χ^2_1 = 1.07, p= 0.300). Variation in relative supplement uptake levels per pair did not significantly affect clutch size (GLMM: χ^2_1 = 0.92, p= 0.338), hatching

success (binomial GzLMM: χ^2_1 = 0.10, p= 0.754) or fledging success (binomial GzLMM: χ^2_1 = 0.48, p= 0.489), and this was also consistent across treatment groups (log(δ^{15}N) × treatment interactions, $p \geq 0.33$).

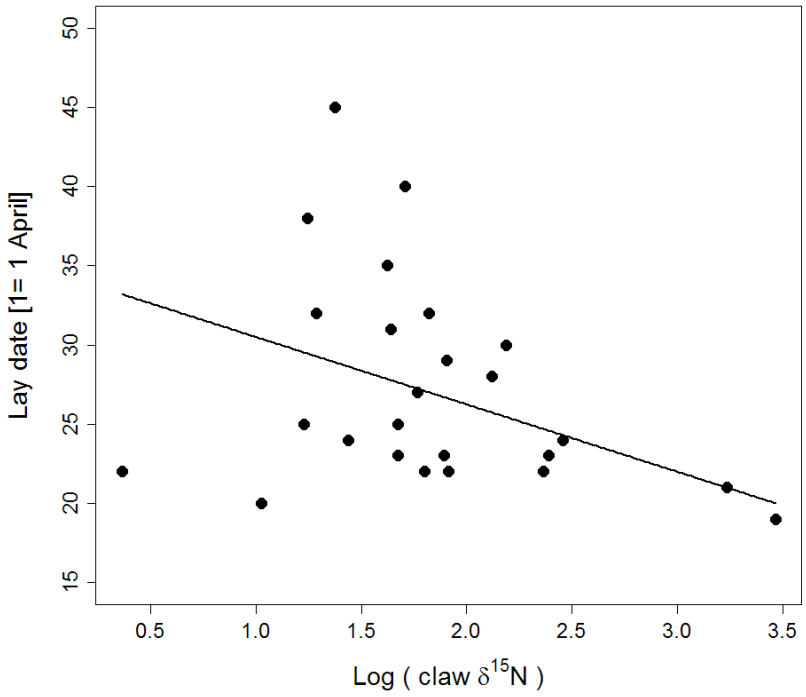

Figure 5.3. The linear relationship between mean claw δ^{15}N value per pair and lay date. Line plotted using statistical estimates. See text for statistical findings.

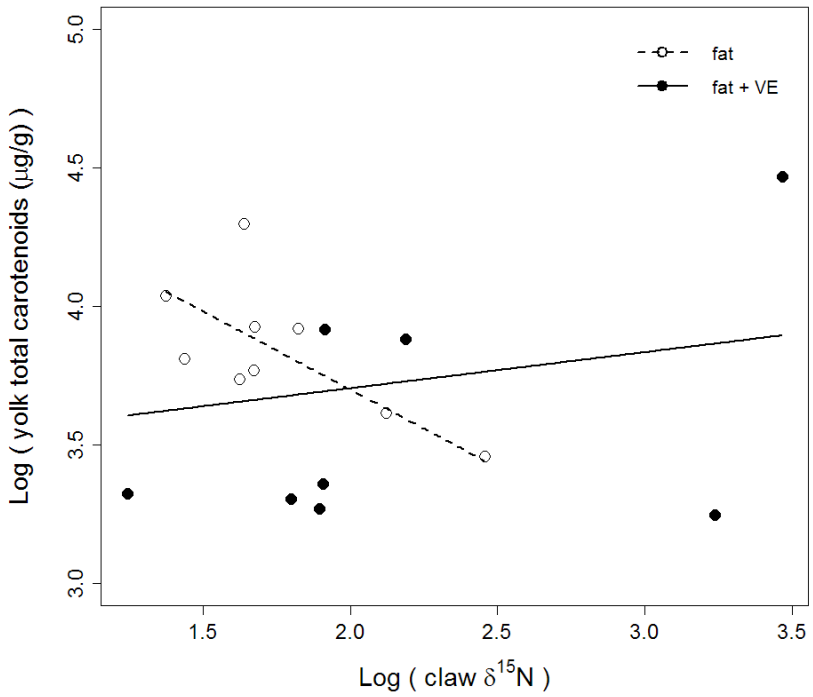

Figure 5.4. The linear relationship between female claw $\delta^{15}N$ value and yolk total carotenoid concentration differed between feeding treatments. Lines plotted using statistical estimates. See text for statistical findings.

5.5 DISCUSSION

As predicted, these findings demonstrate that considerable variation does exist in the uptake of winter supplementary food resources. In particular, there were significant differences in the uptake of supplements between woodland sites independent of feeding treatment (**Figure 5.1**), undoubtedly a response to variations in natural food availability and population densities. In addition, it appears that pre-feeding condition influences supplement use differentially between the sexes (**Figure 5.2**), and that yearlings may rely on supplementary food to a greater extent than older birds (**Table 5.2**). As predicted, individual differences in the use of winter supplements did influence breeding performance, most notably through advancements in laying (**Figure 5.3**).

Furthermore higher antioxidant uptake led to an increase in female allocation of carotenoids into egg yolk, whilst females using only fat-based supplements showed a reduction in carotenoid deposition in response to greater uptake (**Figure 5.3**).

5.5.1 The causes of variation in uptake of winter supplementary food

As anticipated the present findings demonstrate that a complex array of environmental factors and individual differences influenced the use of supplementary food (**Table 5.1**). Natural variation in habitat quality, both spatial and temporal, can have a marked effect on breeding performance and will inevitability dictate supplementary food use also (Kallander 1981; Svensson & Nilsson 1995; Lambrechts et al. 2004). Indeed, variation in the uptake of provisioned food was strongly influenced by inter-woodland differences in this study (**Figure 5.1**). The almost two-fold increase in average use of supplements in Trelissick, for example, may reflect poor natural food availability and a greater reliance on the additional food, or a lower population density and therefore reduced competition for feeder use.

Considerable variation in uptake between individuals within populations was also evident. Provisioning of isotopically-labelled food took place towards the end of the winter period, at a time when pairs would be initiating breeding territory formation, and so is unlikely to have coincided with a large degree of immigration (Perrins 1979). Therefore variation in uptake is most likely to reflect differences in access to feeders, social dominance and natural foraging opportunities and abilities. During the winter months, tits assemble into loosely organised flocks to optimise location of foraging sites and predator detection (Perrins 1979; Ekman 1989). Within social groups, clear dominance hierarchies have been described, with males and older birds generally seen to be the most dominant at feeders (Arcese & Smith 1985; Korsten et al. 2007). Carotenoid-based colouration may also signal competitive abilities (Pryke & Andersson 2003).

Interestingly, the patterns of supplement uptake described here somewhat contradict those predicted by social dominance hierarchies and resource competition theory (**Table 5.1**); females of lower feather carotenoid concentration exhibited the highest uptake

(**Figure 5.2**) and yearlings utilised provisioned food to a greater extent than older birds, though only in antioxidant provisioned sites (**Table 5.1**). Although **Figure 5.1** similarly points to a more gradual increase in male uptake with increasing feather carotenoid levels; these findings generally suggest that competitive exclusion of subdominant individuals was low Therefore, it is stipulated that either natural food availability was not limiting or it was not the preferred feeding resource, at least during the period of isotopic labelling. Reduced allocation of carotenoids into feather colouration may be indicative of poor foraging ability or diminished health (Hill & Montgomerie 1994; McGraw & Hill 2000; Møller et al. 2000), and juvenile birds are typically forced onto poorer foraging sites and show reduced winter survival (Jansson et al. 1981; Gustafsson 1988). To this end, individuals expected to exhibit reduced over-winter survival under natural feeding conditions have taken advantage of feeders to the greatest extent. This is an interesting result, and provides greater insight into the mechanisms generating changes in breeding population structure exhibited in **Chapter 3** (**Figure 3.1**). Further investigation of individual foraging patterns during periods of severe winter conditions and increased natural resource competition would undoubtedly prove beneficial.

5.5.2 The effects of variation in uptake on breeding performance

Like many other small passerines blue tits are considered to be income breeders, adjusting food intake concurrently with reproduction to acquire necessary resource (Drent & Daan 1980). However, the results presented here give clear indication that food uptake outside of the breeding season also factors in determining reproductive performance. Lay date is an important determinant of breeding success in temperate species such as the blue tit (Nilsson 2000). Laying earlier benefits both parents and their offspring; improving growth and survival through close synchronisation with peak caterpillar availability, and improving recruitment rates as a result of earlier fledging (Verhulst & Tinbergen 1991; Noordwijk et al. 1995). Although investigating over-winter feeding effects on lay date at a population level did not indicate a difference between treatment groups in **Chapter 4** (**Table 4.2**), when taking variation in supplement uptake into account, using mean $\delta^{15}N$ values per breeding pair, the carry-over effects of winter provisioning become much more apparent (**Figure 5.2**).

Furthermore, there were no differential effects of fat and fat-plus-vitamin E supplements on clutch initiation dates. It seems that uptake of additional energy up to a month before egg laying commences may assist income breeding blue tits to reach thresholds necessary to begin laying earlier. It is unlikely that these resources are used directly as expected in capital breeding migrants (Inger et al. 2008). Indeed, in another income breeder, the American Redstart (*Setophaga ruticilla*), Langin and colleagues (2006) have shown that resources gained on breeding grounds are used to form eggs rather than endogenous reserves acquired during winter. But more definitively, this result (**Figure 5.2**) accounts for both male and female uptake, and has not been biased by assortative mating in terms of supplement use (no correlation in uptake within pairs) or by the dominance of high quality individuals at feeders. Consequently, it is hypothesised that additional energy in winter helps individuals to maintain a higher body mass, fuelling metabolism and alleviating the need to catabolise stored nutrient reserves. As a result, greater use of over-winter supplementary food seems to have enabled birds to enter the breeding season in superior body 'condition' and better able to acquire resources necessary to reproduce.

Antioxidant provisioning appears to have benefited females over and above fat alone, with only vitamin E fed females showing greater carotenoid investment into egg yolk with increasing supplement uptake (**Figure 5.3**). Perhaps more evident though, is the apparent reduction in carotenoid investment with greater winter uptake within the fat only fed treatment group. Increased carotenoid deposition in the yolk may have a profound effect on offspring phenotype and survival (Biard et al. 2005), however there may be physiological trade-offs in its allocation (McGraw et al. 2005; Catoni et al. 2008). Although additional energy has enabled birds to breed earlier this has been counterbalanced by production of poorer quality eggs, in terms of their antioxidant capacity. Fat-supplemented females appear to either allocate more carotenoids away from egg production and into self-maintenance, potentially compensating by increased oxidative stress induced by early laying, or have a poorer ability to acquire these resources. Furthermore, no correlation was seen between levels of uptake of vitamin E supplements and concentrations of α-tocopherol in the yolk. This suggests that females are not utilising stored reserves during egg production as predicted; or, perhaps more correctly, they are not using α-tocopherol acquired from supplementary food directly.

Whether this has allowed for females to store more carotenoids in lipid-rich tissue in winter, to be utilised during reproduction is uncertain, but an interesting possibility.

Beyond egg laying, variation in supplementary food uptake does not appear to have influenced clutch size or hatching and fledging success in this study. This may be due to a number of reasons. For instance, clutch size may be independent of dietary provisioning, as has often be documented in supplementary feeding studies (reviewed in Robb et al. 2008a; and see **Chapter 2**). Or the carry-over effects of winter food use might not stretch beyond the early stages of the breeding season. However, given that population level effects on hatching and fledging success were reported in **Chapter 4**, it appears there could be an alternative explanation as to why effects on individual variation have not been detected. Samples sizes are comparable to other similar studies (e.g. Davis et al. 2005; Robb et al. in press), but are perhaps still too small to infer significance if the effect sizes of these breeding parameters are also small. Since isotopic labelling was introduced at the end of the winter period, claw $\delta^{15}N$ values only provide a snap shot of supplement uptake over a much larger timeframe. Further consideration of feeder use over this extended period, combined with an analysis of the total contribution of supplemented food to the diet using mixing models (Parnell et al. 2010) may help to pick up additional effects of variation in feeder use.

5.5.3 Conclusions

Stable isotope analysis has proven to be a valuable method of accessing the carry-over effects supplementary feeding. Firstly, it has highlighted the extent of the variation in supplement use which exists both within and between populations; secondly, it has demonstrated the complex nature by which environmental factors and individual differences might interact to influence supplementary food use; and finally, it has revealed effects of feeding on individual variation in laying dates which had become diluted at a population level (see **Chapter 4**).

As income breeders, blue tits are expected to rely on resources acquired on a daily basis to fuel reproduction. Whilst this might be true, these findings give clear indication of the influences both energy and antioxidant uptake outside of the breeding season can have on reproductive performance. It is hypothesised that additional energy might

assist in fuelling metabolism, enabling birds to enter the breeding season in superior 'condition' and reach thresholds necessary to reproduce earlier. Furthermore, it is anticipated that the addition of vitamin E may help to improve antioxidant defences, assisting in oxidative stress reduction and self maintenance, thereby allowing individuals to divert more resource into reproduction during the breeding season, as illustrated by increased allocation of carotenoids into eggs. It remains uncertain whether tits utilise stored antioxidant reserves acquired during winter as direct investment during reproduction. From these findings, it is evident that the effects of winter resource availability can be carried-over to influence subsequent breeding performance, and that accountability of inter-individual differences is of considerable importance when evaluating the influences of food supplementation within populations or across treatment groups.

CHAPTER 6

General discussion

Food supply is expected to play a key role in regulating bird populations; and a huge body of work now exists examining the survival and fitness consequences of variable food availability. Much of this research has focused on how resource limitations during the breeding season influence reproduction (e.g. Martin 1987). However another equally important point for consideration is: how does food supply during the winter influence subsequent reproductive success? For many small passerines, both in the UK and globally, winter food availability is substantial improved through the provision of supplementary food in gardens. For example, it is estimated that there is enough commercially-bought bird food in British gardens to support 30 million great tits without a need to use additional natural resources (Robb et al. 2008b). This is clearly an enormous resource; and it continues to grow as bird feeding is actively encouraged as a means of conserving declining population sizes (e.g. British Trust for Ornithology 2009). However, until now very little has been done to empirical test the ecological impacts this may be having on wild bird populations.

The primary aim of this thesis, therefore, was too investigate the effects of over-winter supplementary feeding on health and productivity during the breeding season. In the following pages I summarise the key finding of my work, discussing them within the context of garden bird feeding, and providing further insights as to their broader ecological implications.

6.1 ARE THE EFFECTS OF WINTER FOOD AVAILABILITY CARRIED-OVER INTO THE BREEDING SEASON?

Carry-over effects – where events in one season influence an individual's performance at a later time or place – have been well described in migratory birds (Harrison et al. 2011). But until recently they had received little attention in resident populations. When Robb et al. (2008b) demonstrated that winter food availability does influence

subsequent breeding success for the first time, it became clear that over-winter supplementary feeding can indeed produce carry-over effects. However, the mechanisms by which these carry-over effects were being mediated remained uncertain. It has traditionally been assumed that energy is limiting (Stearns 1989), and as such there has been a general assumption that carry-over effects are a consequence of increased fat and energy consumption during the winter months. But equally, micronutrients, in particular antioxidants such as vitamin E, may play an important role (Harrison et al. 2011).

6.1.1 Generating ecologically meaningful answers

Throughout this thesis I have compared, for the first time, these two candidate mechanisms; asking the question: are carry-over effects mediated by energy and/or antioxidant supply during winter? In order to provide answers, I have been very careful in my attempt to replicate natural responses to supplementary feeding, in order to draw meaningful conclusions about the true ecological impacts of garden bird feeding. This therefore led me to implement a large landscape-scale investigation across multiple blue tit populations, and across three consecutive years. I have expressly aimed to mimic the diffuse nature by which supplementary food is providing in gardens. In doing so, I have provisioned vitamin E in the form of α-tocopherol, a potent antioxidant thought to play a valuable role in avian ecology (Costantini 2008), and supplemented it at levels equivocal to those found in peanuts, a popular garden bird food (British Trust for Ornithology 2006). To account for the impacts of feeding on health and productivity in the breeding season I have predominantly taken a population level approach (**Chapters 2 – 4**). However, it is acknowledged that responses to supplementary feeding may be highly variable between individuals within a population, and therefore I have investigated this also in **Chapter 5**.

6.1.2 Summary of key findings

It was anticipated that the carry-over effects of winter feeding may be mediated through benefits to egg production, or through benefits to parental health status. In **Chapter 2**, maternal reproductive investment in egg production was investigated. Females were found to increase resource allocation in response to vitamin E supplementation,

allocating proportionally larger yolks into their eggs compared to females was fat supplemented-woodlands (**Figure 2.1**), suggesting vitamin E had an indirect influence of maternal condition thereby enabling females to invest more newly-acquired resources into egg productions, and away from self-maintenance. Many of the other parameters investigated: clutch size, clutch and egg mass, and yolk antioxidant deposition; remained largely unaffected in response to over-winter feeding.

Blue tits exhibit yellow carotenoid-based breast feather colouration, which is moulted annually between the end of the breeding season and the beginning of winter (Perrins 1979; Partali et al. 1987). Since carotenoids have limited environmental availability, physiological trade-offs exist in their allocation, and it is commonly hypothesised that carotenoid-based signals provide a reliable indicator of individual quality at the time of feather growth (Olson & Owens 1998; Møller et al. 2000). In **Chapter 3**, I used measures of carotenoid concentration in feathers to demonstrate that within the vitamin E treatment group, breeding populations consisted of individuals who were in significantly poorer condition before the onset of over-winter supplementation (**Figure 3.1**). An interesting and thought provoking result; which suggests that vitamin E supplementation has enabled relatively poorer quality individuals to survive winter and recruit, thereby perturbing natural selection and altering the phenotypic structure of populations.

Parental phenotypes were otherwise similar across all treatment groups during brood-rearing; showing little quantifiable variation in body mass and plasma antioxidant (α-tocopherol and total carotenoid) concentrations (**Table 3.2**). But, interestingly whilst susceptibility to oxidative stress appeared similar between females, males from vitamin E supplemented woodlands showed a reduction in plasma malondialdehyde concentrations (MDA; a biomarker for lipid peroxidation) (**Figure 3.2**). It is predicted that this result may be a reflection on the different reproductive roles females and males assume. By this stage of the breeding season, vitamin E supplemented females had already placed a proportional larger investment into relative yolk mass (**Chapter 2**), which may have been at the expense of their condition at this later stage. Males, however, do not make a substantial direct investment into offspring quality (c.f. egg production) until brood provisioning. Therefore the benefits of vitamin E

supplementation for condition may still prevail at this later stage, or alternatively improved oxidative status could be a reflection of males' increased capacity to combat ROS via the antioxidant system, having mobilised vitamin E from reserves acquired at feeders in the winter (see below). The impacts of this visible improvement is oxidative status were investigated in **Chapter 4**, however no effects on nestling phenotypes or fledging success were apparent. Furthermore, although parental provisioning was also studied in **Chapter 3**, this was only during 2008, and comparisons between male and female provisioning efforts were unable to be made. As such it was difficult to assess the impact of this result within this body of research. However, this is certainly worthy of further investigation and a good place to start would be in a study of male brood provisioning behaviour responses to over-winter supplementary feeding.

The results of **Chapters 2 and 3** have provided an indication of the mechanisms by which reproductive success may be influenced by over-winter feeding, and in **Chapter 4** I went on investigate how all of these factors came together to affect reproductive success. It was apparent that annual variation played a primary role in dictating productivity, with measures of nestling phenotype (body mass, plasma antioxidant and MDA concentration) predominantly influenced by annual variation (**Table 4.4**); and hatching success increased in response to vitamin E supplementation, but only in one year of study (**Figure 4.2**). However, despite this the effect of over-winter supplementary feeding on fledging success held true across all three years; demonstrating that 7.1% fewer chicks were fledged from unfed sites on average (**Figure 4.5**). It appears that winter provisioning may give birds false cues as to natural food availability and encourage them to make an unsustainable investment in nestling numbers, thereby acting as an ecological trap with detrimental consequences for reproductive success.

In **Chapter 5** the two candidate mechanisms were investigated in closer detail by accounting for individual variation in the uptake of winter supplements; and it was revealed that, as predicted, a large amount of variation exists in the use of supplementary food. In particular, it was noted that individuals of lowest expected over-winter survival took greatest advance of this additional resource, such as juveniles and females with lower concentrations of feather carotenoids (**Table 5.2, Figure 5.2**).

This suggests a lack of competitive exclusion at feeders, and therefore indicates that natural food availability may not be limiting for all individuals. Furthermore, pairs who utilised supplementary food more extensively during winter were shown to start laying earlier. This result had been masked at a population level in **Chapter 4**, and therefore it is highlighted again the importance of being able to account for individual variation in resource use in order make inferences about affects at a population level (Catoni et al. 2008).

6.2 AN APPRAISAL OF CARRY-OVER EFFECT MECHANISMS

It has been suggested throughout this thesis that the observed effects of over-winter supplementary feeding were operated via the indirect effects of energy and/or vitamin E on parental condition. But in addition to this, the possibility that micronutrients acquired at winter feeders were stored and used as a direct capital investment during the breeding season has also been alluded to. Although the capacity for antioxidant storage in lipid-rich tissues is well described (Negro et al. 2001; Surai 2007), the potential for these resources be to used for capital gains is a relatively novel and interesting possibility (McGraw & Toomey 2010; Metzger & Bairlein 2011). It has similarly been proposed that classically defined capital versus income breeding strategies are not dichotomous, but rather a continuum; and that micronutrient resources may be of benefit both indirectly (income) though improved condition, or directly (capital) via the allocation of stored reserved during the breeding season (Stephens et al. 2009; Harrison et al. 2011).

The results presented within this thesis do not provide definitive evidence to support this micronutrient storage hypothesis. In **Chapter 2**, maternal investment of α-tocopherol into egg yolk was not influenced by α-tocopherol supplementation during winter. Similarly plasma concentrations of α-tocopherol in parent birds during brood-rearing did not differ according to winter feeding treatment in **Chapter 3**. However, there are certain clues to suggest the utility of stored antioxidant reserves might be taking place; for instance, improvements in male oxidant status and in nestling plasma carotenoid concentrations may reflect mobilisation of stored antioxidants to protect against ROS build up during the metabolically stressful brood-rearing period (**Chapter**

3 and 4). Correlations between supplement uptake and deposition of carotenoids in egg yolk may also reflect a capacity to cache antioxidants in the winter (**Chapter 5**). This should certainly be a point of inquiry for future research.

6.3 THE ECOLOGICAL IMPLICATIONS OF GARDEN BIRD FEEDING

Until recently little has been know about the ecological impacts of garden bird feeding on wild bird populations. Undoubtedly supplementary feeding has a valuable role in conservation practices but an understanding of its consequences is imperative (Jones & Reynolds 2008).

The possibility that supplementary feeding might perturb natural selection and alter the phenotypic structure of wild bird populations has been hypothesised (Robb et al. 2008a), but this is the first study to produce empirical evidence which lends support to this hypothesis (**Chapter 3**). Furthermore the results of **Chapter 4** suggest concerns that supplementary feeding might cause feeder-dependency could be valid (Brittingham & Temple 1992). It would appear that a cessation of provisioning in spring could generate an ecological trap where birds receive inaccurate cues about natural food availability and consequently suffer reduced reproductive success. These findings suggest some clear negative implications of garden bird feeding, however there is certainly much more to do done before conclusions can be drawn. For instance benefits of provisioning for egg composition (**Chapter 2**) and male oxidative status (**Chapter 3**) could have positive repercussions outside of the context of this thesis, and in particular a better understanding of male responses to supplementary feeding could prove informative.

Furthermore, no attempts have been made to follow the survival and dispersal of fledglings after leaving the nest in this study. This would also be a valuable next step to improve understanding of the consequences of these results for wild bird population sizes. Supplementary feeding has the potential to influence many more target and non-target species than just the blue tit. Whilst this thesis has presented novel information to help interpret some the impacts wild bird feeding is having, an understanding of how

supplementary feeding might influence garden bird species assemblages and predator behaviour would provide new insight into the much bigger picture.

Finally, it is hoped that this body of work will encourage other researchers to draw focus away from the breeding season and start accounting for effects occurring during other periods of the annual cycle. Winter is a critical period, effectively separating 'the weak from the strong', and as seen here resource availability at this time can have importance consequences for health and productivity during the breeding season. These novel indications that antioxidants may act as carry-over effect mediators should be of great interest for behavioural ecologists and conservationists alike.

APPENDIX 1

A1. DESCRIPTION OF WOODLAND FIELD SITES

A1.1.1 Grouping of field sites into triplets

The nine woodland sites included within the study were grouped into three triplets, allowing for the three feeding treatments to be implemented simultaneously within similar sites in each year (**Table A1.1, Figure A1.1 (a)**). Grouping was primarily made according to similarities in vegetative structure, this included tree composition, and type and prominence of understorey and ground species (**Table A1.1**). Woodland size was also taken into account, and additional common features were scaled (low, medium, high) for consideration. These included amount of periphery woodland (low: isolated woodland; medium: additional woodland on ≤ 2 sides; high: additional woodland on 3 sides;), proximity to human settlement (within a 2 mile radius, low: <10 isolated households; medium: >10 isolated households; high: human settlement;), and level of public usage (low: private land; medium: public access to some parts of site; high: site regularly used members of the public) (**Table A1.1**).

A1.1.2 Nestbox and feeder distribution within field sites

Feeders and nest boxes were placed at an equal density and distribution across woodland sites, using parallel transects positioned within 50m of the woodland perimeter and at 100m intervals. Each site had an average of 38 boxes (total: 346; range: 32 – 44) and 9 feeders (range: 7 – 10). Feeders were only present in years when a named site was allocated to a fed treatment. Further description of the within-woodland experimental design is given in **Chapter 2 §2.3.1**, and see **Figure A1.1 (b)** for an example map detailing organisation of nest boxes and feeders within field sites.

A1.1.3 Uptake of over-winter supplementary food

Supplementary food uptake varied considerably between both sites and years. For a clearer understanding of between site, year and treatment differences see **Table A1.2**.

Table A1.1. Description of woodland field sites. Included is the pattern of winter feeding treatments by triplet; treatments include control (×), fat (fat) and fat-plus-vitamin E (VE). 'Key features' classified low (*) to high (***), see text for details. For vegetative structure, species are listed in order of prevalence.

	Winter feeding treatment			Key features				Vegetative structure		
Site	2007	2008	2009	Size (hec)	Periphery woods	Settlement proximity	Public access	Tree composition [a]	Understorey [b]	Ground cover [c]
Triplet (1)										
Kennall Vale	×	fat	VE	16.7	**	***	**	Beech, oak, sweet chestnut, sycamore	Holly, hazel	Ivy, bryophytes, ferns, brambles, bluebells
Devichoys	fat	VE	×	14.2	***	**	**	Oak, sweet chestnut	Holly, hazel	Brambles, honeysuckle, bryophytes, ivy
Tremayne	VE	×	fat	10.0	*	*	***	Oak, beech, ash, sweet chestnut	Holly, hazel	Brambles, ferns, ivy, bryophytes, honeysuckle
Triplet (2)										
Nansavallan	×	fat	VE	13.1	**	***	*	Oak (coppice)	Hazel, holly	Brambles, sedges, honeysuckle
Bonallack	fat	VE	×	6.6	**	***	*	Oak (coppice), beech, sycamore	Hazel, holly	Brambles, ivy, bluebells
Unity	VE	×	fat	9.5	*	**	***	Oak (coppice), beech, silver birch	Hazel, holly	Honeysuckle, brambles, bluebells, bryophytes
Triplet (3)										
Trevarno	×	fat	VE	9.3	**	*	*	Beech, ash, sycamore, oak	Laurel, holly	Ivy, bryophytes, ferns, bluebells
Trelissick	fat	VE	×	7.6	***	*	***	Ash, sycamore, beech, oak	Holly, hazel	Ivy, brambles, bryophytes
Carclew	VE	×	fat	9.4	**	*	*	Oak, beech, sycamore	Rhododendron	Bryophytes, brambles

[a] Tree species: ash, *Fraxinus excelsior*; beech, *Fagus sylvatica*, oak, *Quercus sp.*; silver birch, *Betula pendula*; sweet chestnut, *Castanea sativa*; sycamore, *Acer pseudoplatanus*.

[b] Understorey species: hazel, *Corylus avellana*; holly, *Ilex acquifolium*; laurel, *Prunus laurocerasus sp.*; rhododendron, *Rhododendron sp.*.

[c] Ground cover species: bluebells, *Hyacinthoides non-scripta*; brambles, *Rubus fruticosus*; honeysuckle, *Lonicera sp.*; ivy, *Hedera helix*; sedges, *Cyperaceae sp.*.

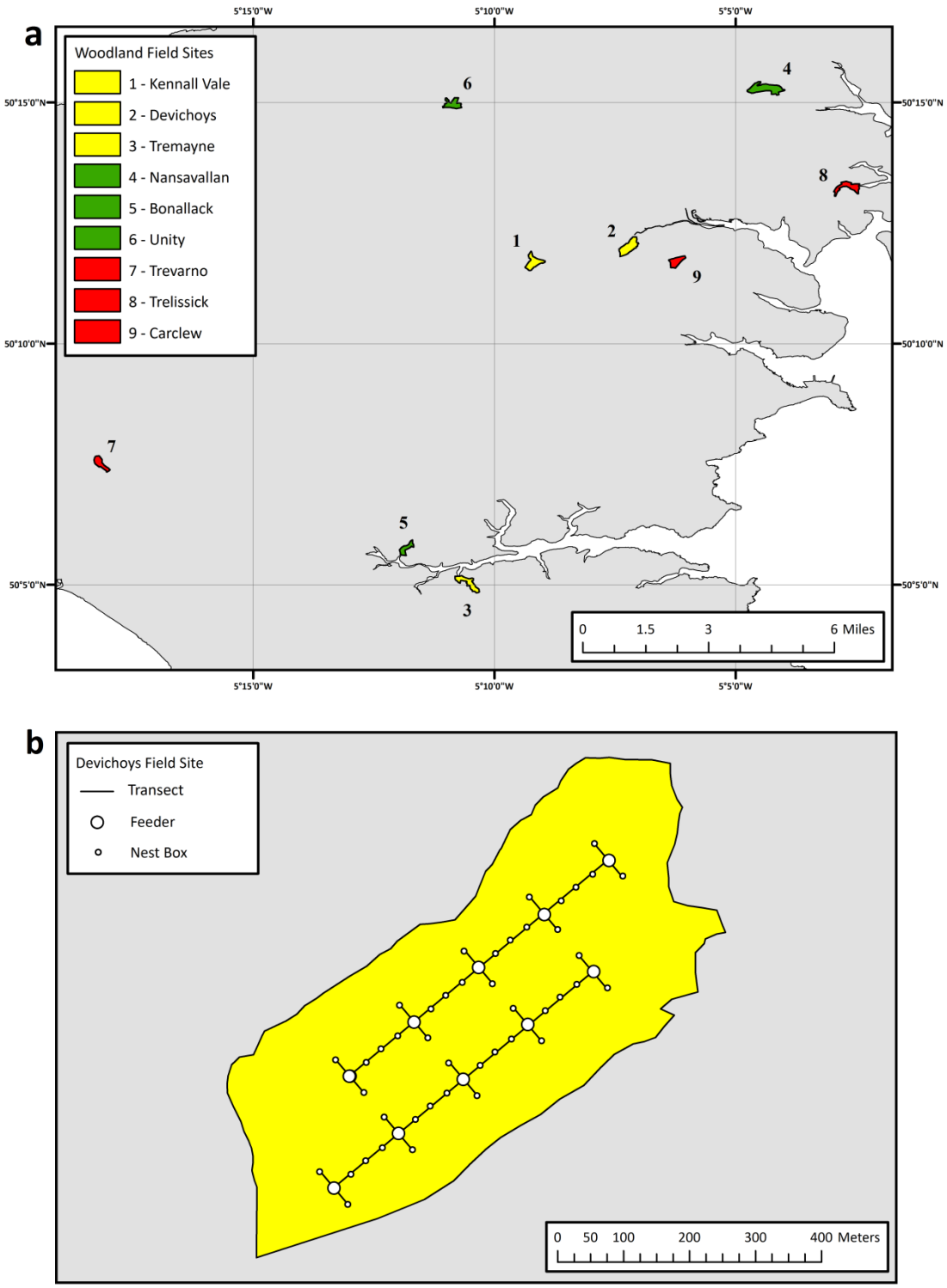

Figure A1.1. Maps detailing (a) The distribution of woodland field sites in west Cornwall coloured by triplet groups (as shown in Chapter 1, Figure 1.1); and (b) The within site layout of nest boxes and feeders, using Devichoys woodland as an example.

Table A1.2. Total mass of food consumed per site during the three winter feeding seasons. Sites are grouped according to feeding treatment, where dark-shading = fat fed sites and light-shading = fat+VE fed sites. Means per treatment group included for each year.

		Total mass of food consumed (g)		
Triplet	Site	2007/08	2008/09	2009/10
1	Kennall Vale		2400.1	8532.7
2	Nansavallan		538.0	3136.1
3	Trevarno		2361.9	7320.3
1	Devichoys	1400.0	3201.6	
2	Bonallack	291.1	703.4	
3	Trelissick	1987.0	4360.4	
1	Tremayne	457.4		8588.3
2	Unity	3436.4		6189.7
3	Carclew	109.8		4834.5
Mean for fat-fed sites		1226.0	1766.7	6537.5
Mean for fat+VE-fed sites		1334.6	2755.1	6329.7

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